NON UNIFORMITIES IN THE SIZE DISTRIBUTION OF THE NASCENT CHAINS OF GLOBIN FROM RABBIT RETICULOCYTES

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ABSTRACT

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By

Alberto Protzel

Evidence is presented to show that the nascent chains of rabbit globin do not have a uniform distribution of sizes.

Data are presented to show that rabbit reticulocyte ribosomes contain a significant component of completed α globin which is still attached to tRNA (α globyl tRNA). Additional data are presented to show that contamination by labeled supernatant hemoglobin or labeled α globin from the free globin pool present in reticulocytes is not a significant factor in these results.

Some 4.6% of the nascent α globin chains are present as α globyl tRNA, instead of 0.71% as predicted on the basis of the assumption that the size distribution of nascent globin chains is uniform. On the other hand β globyl tRNA comprises 0.69% of the nascent β globin chains. This value coincides closely with the predicted value for nascent β globin chains uniformly distributed in size along the polysome. Further evidence is presented to show that both α globyl tRNA and β globyl tRNA exhibit the kinetic properties expected for normal intermediates of soluble

hemoglobin biosynthesis following inhibition of the initiation of protein synthesis by pactamycin.

Radioactively labeled nascent chains of rabbit globin were also analyzed as a function of molecular weight by gel filtration on Bio Gel A-0.5M. The gel filtration analysis showed peaks of radioactivity for peptides in the molecular weight ranges of 10482-8854, 5891-6707, 4573-4994 and 3068-4088.

Gel filtration was done with Bio Gel A-0.5M, 10% agarose, mesh 200-400. The gel was equilibrated in 6M guanidine HCl-0.1 β mercaptoethanol. All elutions were done with this same solvent at a pressure differential of 57-60 cm of solvent. Calibration was done with peptide markers covering the molecular weight range from 356 to 16000.

The accumulation of nascent peptides of globin at discrete ranges of molecular weight indicates that the rate of movement of ribosomes along the mRNA of rabbit globin is not uniform.

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LIST OF ABBREVIATIONS

CM-cellulose	carboxy methyl cellulose
DEAE-Cellulose	diethylaminoethyl cellulose
β-ME	2-mercaptoethanol
mRNA	messenger ribonucleic acid
poly A	polyadenylic acid
poly T	polythymidilic acid
POPOP	1,4-bis 2-(4-methyl-5-phenyloxazolyl) -benzene
PPO	2,5- diphenyloxazole
SDS	sodium dodecyl sulphate
tRNA	transfer ribonucleic acid

INTRODUCTORY STATEMENT

It has been shown by Dintzis (1961) that the assembly of polypeptide chains of hemoglobin takes place by the sequential addition of amino acids, starting at the N-terminal end and continuing towards the C-terminal end of the polypeptide chain. A physical basis for this assembly process is provided by the polyribosome, a multiple ribosome structure (Warner et al., 1963, Rich et al., 1963).

Translation of genetic information into proteins (Nirenberg and Matthaei, 1961), is accomplished in the polyribosome (Rich et al., 1963), by stepwise (Erbe, Nau and Leder, 1969) movement of the ribosome along the mRNA (Lengyel et al., 1973) while carrying one nascent polypeptide chain (Warner and Rich, 1964).

Measurements of the rate of movement of the ribosome along the mRNA have been made by various authors. Thus, translation of the tryptophan operon of Escherichia coli takes place at the rate of approximately 1000 nucleotides per minute at 30° (Morse et al., 1969). These authors compared the kinetics of appearance of both mRNA and of enzyme activity to obtain these results. The rates of elongation of egg white proteins have been determined by Palmiter (1972) by measuring the time required for

radioactivity first observed as nascent peptides to appear as supernatant protein. This author estimates a translation rate of 900 nucleotides per minute for ovalbumin at 41°. A similar technique has been applied by Lodish and Jacobsen (1972) to the measurement of the rate of elongation of the α and β chains of hemoglobin. These authors observed a translation rate of 131 nucleotides per minute at 25° for both chains of hemoglobin. All these methods provide average values for the rate of ribosomal movement leaving unanswered the question about the relative rates of translation of specific portions of the mRNA.

The problem of relative rates of movement along the mRNA can be also studied by analyzing the size distribution of nascent peptides in polyribosomes that have achieved a steady state of synthesis. The distribution of sizes will be uniform if ribosomes go past every codon at the same rate. While if a ribosome spends a great deal of time at a given codon the corresponding nascent peptides will be present in an increased amount. There will be more polyribosomes with a ribosome present at that particular codon.

Several authors have studied the size distribution of nascent peptides of globin by means of Naughton - Dintzis plots (Naughton and Dintzis, 1962). According to this method, a population of nascent chains that is labeled with a certain radioactive amino acid is prepared. The specific activity of the amino acid at each one of its position of occurrence is determined. The specific activity data

are then plotted against the number that corresponds to the position along the chain at which the measurement was made. A straight line plot indicates a uniform size distribution. This method was applied by Hunt et al. (1968a) and by Luppis et al. (1970) to populations of nascent chains from reticulocyte polysomes. These authors concluded that the nascent chains of hemoglobin are uniformly distributed in size and that therefore the rate of ribosome movement along the mRNA of globin is constant. This thesis presents evidence that there are deviations from a uniform distribution in size for the nascent chains of hemoglobin. Hence, the rate of ribosome movement along the mRNA is not uniform.

Those nascent chains still attached to tRNA are referred to in this thesis as peptidyl tRNA, while those nascent peptides attached to tRNA whose primary amino acid sequences are those of the completed globin chains are referred to as globyl tRNA.

A procedure for the purification of the peptidyl tRNA component from rabbit reticulocyte ribosomes has been described by Slabaugh and Morris (1970). This method is particularly effective in removing soluble hemoglobin contamination from the peptidyl tRNA preparation.

The availability of this methodology has made feasible studies of the size distribution of the nascent chains of hemoglobin. A uniform distribution in size for the chains obained from this peptidyl tRNA implies that ribosomes move at a constant rate along the mRNA. As discussed above, this

thesis asks the question: Do the ribosomes move at a constant rate along the mRNA for globin?

Plan of the thesis.

After confirming that the method of Slabaugh and Morris (1970) does indeed give preparations of peptidyl-tRNA essentially free of soluble hemoglobin, the fraction corresponding to α globyl-tRNA and β globyl-tRNA was measured in that preparation.

The α and β globin chains of rabbit hemoglobin each contain 3 tyrosine residues in their amino acid sequence (Dayhoff and Eck, 1968). The C-terminal ends of α and β globin molecules consist of the amino acid sequences -Lys-Tyr-Arg and -Lys-Tyr-His respectively. Since the biosynthesis of hemoglobin is known to proceed from the N-terminal end toward the C-terminal end (Dintzis, 1961) an analysis of the purified peptidyl-tRNA fraction for the presence of the C-terminal dipeptides tyrosyl-arginine and tyrosyl-histidine, following tryptic digestion, has permitted a determination of the amounts of α globyl-tRNA and β globyl-tRNA in that fraction.

While β globyl-tRNA exists to the extent predicted by a uniform distribution of peptides the α globyl-tRNA was found to be present in an amount 6 times greater than the theoretical value predicted on the basis of the assumption of a uniform distribution of sizes of nascent α globin peptides. The size distribution of the remainder of the

population of nascent chains has been studied by means of a column chromatographic procedure. A Bio-Gel A0.5M agarose gel column has been calibrated for molecular weight determination of peptides ranging in molecular weight from 316 to 16000 daltons. This column procedure permits the display of the nascent peptides of hemoglobin as a function of molecular weight, thus allowing one to single out particular segments of the mRNA where ribosome movement might be slower or faster than others.

LITERATURE REVIEW

Translational Models of Control

Inherent to the differentiated state is the production of cell specific proteins. The biosynthesis of these cell specific proteins is usually associated with long lived messages. The mRNA for cocoonase has a half life of 100 hours (Kafatos, 1972), ovalbumin, 18 hours (Palmiter *et al.*, 1973). In the loach *Misgurnus fossilis* information issued by embryo nuclei in the middle blastula stage is realized only in the course of gastrulation (Spirin, 1969). Reticulocytes can synthesize hemoglobin for at least 48 hours after extrusion of the nucleus (Rifkind *et al.*, 1964).

Various models have been presented to account for the levels of proteins in eucaryote tissues. Kafatos (1972) attributes a key role to differential mRNA stability to account for specific protein levels that are high, in the presence of little or no gene amplification. Schimke (1970), emphasizes rates of degradation of enzymes. In more recent work, Palmiter and Schimke (1973), present the concept that more efficient translation of a long lived mRNA would lead to an increased rate of production of its corresponding polipeptide. This increase in efficiency would be due to more favorable competition for some rate limiting factor

once the more labile messages start to disappear. Any factor that would decrease the level of short lived messages would lead to superinduction of proteins with long lived mRNA. Sussman (1970), presents a "ticketing" theory to account for both qualitative and quantitative levels of proteins given the existence of long lived messages. Quantitative control would be achieved by allowing the ribosome to clip-off a particular "ticket codon" after a round of translation. After a certain number of clippings the message would be susceptible to RNAse attack. Qualitative control would be achieved by having at a given time a ribosome population capable of reading only certain messages. The ticketing theory might have some basis. Measurements of the length of poly-adenylic acid, poly (A), segments in mRNA of HeLa cells have been performed by Sheiness and Darnell (1973). These authors labeled the cells briefly with ^{3}H-adenosine, and transferred them to fresh nonradioactive medium. They observed a maximum shortening from 200 to 100 nucleotides in the poly (A) of the mRNA between 3 and 6 hours after transfer to fresh medium. In long term experiments, they observed pieces only 50 adenylate residues long in the poly (A). Similar ticketing phenomena might be operating in the case of hemoglobin mRNA. Duck erythrocytes labeled for 4 hours with [^{3}H] adenosine have poly (A) segments of at least 150 nucleotides in length (Pemberton and Baglioni, 1972). Messenger RNA from rabbit reticulocytes labeled for 18

hours before removal from the animal had poly (A) sequences 50-70 nucleotides in length (Lim and Canellakis, 1970). Furthermore mRNA from circulating reticulocytes, older than 18 hours, was found to have poly (A) sequences of 8 residues (Burr and Lingrel, 1971).

Tomkins et al. (1969) have presented a model based on inducers and repressors to explain their results of the steroid mediated induction of liver tyrosine amino transferase. They suggest that the sole role of the steroid is to antagonize a post transcriptional repressor which both inhibits messenger translation and promotes messenger degradation. The reticulocyte presents a convenient system for the study of models of translational control, as shall be discussed below.

The Reticulocyte

Over 90% of the protein synthesized by the reticulocyte is hemoglobin. In addition to hemoglobin, reticulocytes synthesize six other proteins, two of which are membrane proteins (Lodish, 1973 a, b). The degree of compartmentation of protein synthesis in the reticulocyte is presumably very limited. Disk gel electrophoresis patterns of proteins synthesized by whole reticulocytes and by membrane free lysates are identical Lodish (1973a). These observations reflect the progressive specialization of the reticulocyte as it becomes an erythrocyte.

The reticulocyte is the last step of erythroid cell

differentiation that is morphologically distinct from the erythrocyte (Ham, 1969). Erythropoiesis starts with the multipotential stem cell, capable of becoming committed to differentiation into erythrocytes, leucocytes or megacaryocytes which give rise to the platelets (Lajtha, et al., 1971). Further differentiation leads to a succession of morphological states, designated according to their varying staining capacities. These states are in succession, the proerythroblast, basophilic erythroblast, polychromatophilic erythroblast and the orthochromatic erythroblast. Extrusion of the nucleus by the orthochromatic erythroblast leads to the reticulocyte (Ham, 1969; Tarbutt and Blackett, 1968). The rate of synthesis of hemoglobin is maximum at the polychromatophilic stage of development. These results were obtained by measuring authoradiographically the uptake of ^{59}Fe by cells from the hepatic erythroid population of the mouse (Djaldetti et al., 1970). Reticulocytes are cells in the process of degeneration. The number of ribosomes in marrow erythroid cells is 5.4 times that in reticulocytes (Lingrel and Borsook, 1963).

Control of Hemoglobin Biosynthesis

Reticulocyte mRNA

The mRNA for globin has been widely sought for. Labrie (1969) reported the finding of a 10S RNA species. Its specific activity was five times higher than that of 18S, 29S and 5S RNA 17 hours after injection of $^{32}PO_4$ into the

rabbit. Its T1 RNAse digest did not correspond to that of 18S or 29S RNA. Lockard and Lingrel (1972) reported the preparation of mouse 9S RNA capable of synthesizing the α and β globin chains of the mouse in a duck reticulocyte lysate system. Williamson et al. (1971) reported the preparation of mRNA from mouse reticulocytes having a molecular weight of 170,000, which would correspond to 65 nucleotides in excess of the number necessary to code for a polypeptide the size of globin. This mRNA from mouse has been translated in a mouse liver S-30 cell free system giving α and β chains in a ratio of 1.5 to 1 (Sampson et al., 1972). Poly (A) sequences will bind to millipore filters and to poly [T] sequences. Brawerman et al. (1972) have obtained preparations enriched in 10S RNA by passing crude preparations of polysomal RNA through millipore filters. Similar preparations have been passed through oligothymydilic acid-cellulose columns to obtain a 9S RNA capable of synthesizing rabbit globin in a Krebs II ascites tumor cell free system (Aviv and Leder, 1972). Gianni et al. (1972) have prepared 10S RNA from the post ribosomal supernatant of rabbit reticulocytes which will only direct synthesis of α globin chains in a 30S supernatant of rat liver. A similar protein synthesizing system programmed with 10S RNA obtained from the polysomes by these authors, yielded both α and β chains in a ratio of 1.5 to 1 respectively. Jacobs-Lorena and Baglioni (1972) have isolated a 20S ribonucleoprotein from reticulocyte post ribosomal supernatant that gives 10S RNA. A Krebs II

ascites cell-free system programmed with this mRNA gives only α chains while the same system using reticulocyte polysomal mRNA gives a ratio of α to β chains of 0.48. Similar results have been obtained by Housman et al. (1971) using the same incubation system. The widely varying efficiency of heterologous cell-free systems for synthesizing the α and β chains of hemoglobin can be compared to the reticulocyte itself. Reticulocytes produce about equal ratios of the α and β chains of hemoglobin (Baglioni and Colombo, 1964). These results obtained with heterologous systems programmed with reticulocyte mRNA might reflect the effect of supernatant cofactors on the rate of mRNA translation.

Rates of growth of the α and β Globin Chains

The direction of elongation of proteins is from the N-terminal end to the C-terminal end, Dintzis (1961). This means that the last amino acid added to a protein before release would be expected to be the C-terminal amino acid. One would thus expect that shortly after the addition of a radioactive amino acid to reticulocytes synthesizing hemoglobin, the amino acids towards the carboxy end of the supernatant hemoglobin would have more radioactivity (Dintzis, 1961).

The time lag between the first appearance of radioactivity at the carboxy end and the first appearance of radioactivity at the N-terminal end represents the time it

took to assemble and release the protein (Knopf and Lamfrom, 1965). In practice, the method of Knopf and Lamfrom (1965) measures the time it takes for tryptic peptides labeled with the same amino acid and situated at opposite extremes of the protein to reach the same specific activity. Using this method, Hunt et al. (1969) have found that the α chain of globin is translated on the average 70% faster than the β chain of globin.

Lodish and Jacobsen (1971) have criticized these results on the grounds that the label was not being incorporated at the same rate into all of the peptides studied. If label were being inserted by a degenerate pair of tRNA's (Soll et al., 1966) each charged with the same amino acid but at different specific activities, mistakenly high or low rates of translation would be obtained. High specific activity for the aminoacyl-tRNA at the amino end would produce a rate of elongation shorter than the actual rate and vice versa. To avoid these artifacts Lodish and Jacobsen (1971) concentrated on a given peptide and measured the time lag between the first time it was observed to incorporate label and the time it appeared as part of a completed soluble protein. By using this approach an elongation time of 200 seconds per chain and a rate of release of 15 seconds per chain was found for both chains, at 25°.

Control of Release of α and β Chains

The striking equality of the amounts of α and β chains

synthesized by the reticulocyte led to the suggestion that there was an interrelationship between the synthesis of the α and β chains. Balanced synthesis has been observed for example for the A and B subunites of tryptophan synthetase in E. Coli but these are produced by a polycistronic messenger (Morse et al., 1969). In the case of the α and β globin chains, the corresponding genes are not linked (Itano, 1960). The molecular weight of the 10S RNA that codes for globin is around 170,000 - 190,000 (Williams et al., 1971; Labrie, 1969). This range of molecular weights corresponds to a size of an average of 520 nucleotides, which would code for a protein of around 170 amino acid residues, assuming three nucleotides per codon (Nirenberg et al., 1965). The α and β chains of globin have 141 and 146 amino acid residues, respectively. Thus, the mRNA for globin, as isolated, cannot contain coding information for both globin chains in the same polynucleotide backbone.

To account for the balanced synthesis of the two chains of hemoglobin Colombo and Baglioni (1966) proposed that completed α chains aided the release of β chains. Many studies have presented evidence against this view.

In the genetic disease α-thalassemia, characterized by decreased synthesis and the α chain of hemoglobin H(B_4) can be detected (Motulsky, 1964). Hemoglobin H accumulates and appears as inclusion bodies in older erythrocytes, showing that β chain synthesis can proceed in the absence of α chain synthesis (Motulsky, 1964). By the same token, studies

in patients with β thalassemia show that α-chains will accumulate in the absence of β chain synthesis (Fessas, 1966). Honig et al. (1969) have selectively blocked synthesis of the human α chain from fetal hemoglobin leaving the synthesis of α chain unaffected as compared to controls. These authors used the O-methyl threonine analog of isoleucine which prevents its incorporation in hemoglobin (Hori and Rabinovitz, 1968). Since the α chain of human fetal hemoglobin has isoleucine (Dayhoff and Eck, 1968), while the α chain does not, it is possible to selectively inhibit the growth of one chain. Rabinovitz et al. (1969) have performed a similar inhibition experiment using rabbit reticulocytes. They have used a heterozygous rabbit in which one half of the β chains have no isoleucine. The α chains contain the three isoleucines found in normal rabbits while the remainder of the β chains have the normal presence of 1 isoleucine. When the synthesis of α chains was retarded to 10% of controls the formation of the isoleucine-less β chain was stimulated by at least 30%. Ascribing this stimulation of the variant β chains to increased availability of limiting factors these authors conclude that each globin subunit is synthesized independently of the other one.

Seemingly contradictory results concerning the independence of the rates of synthesis of the chains of globin have been obtained by Schaeffer et al. (1969). These authors added human β chains to a hemoglobin

synthesizing cell free system from rabbit reticulocytes. There was a 40-50% decrease in the amount of radioactivity of the rabbit β chain component in the supernatant fraction and an increase in the amount of completed or almost completed β chains in the ribosome fraction.

Role of Heme

As shown by Kruh and Borsook (1956), there is a parallelism in the rates of synthesis of heme and globin. Murine proerythroblastoid cells (T-3-Cl-2) transformed by Friend Leukemia virus show detectable amounts of globin mRNA 2 days after induction with dimethyl sulphoxide. Globin mRNA reaches a maximum value 4 days after induction with dimethyl sulphoxide (Ross et al., 1972). At this time a hemoglobin like color can be detected (Friend et al., 1971). The synthesis of globin is dependent on the presence of iron, a precursor of heme Borsook (1958). Removal of iron by chelation will lead to polysome disagregation and cessation of synthesis of hemoglobin (Rabinovitz and Waxman, 1965).

Heme has been shown to be implicated in hemoglobin biosynthesis both at the level of initiation of translation (Zucker and Schulman, 1968; Adamson et al., 1968) and at the level of the completed chains by regulating the level of the pool of free α chains (Tavill et al., 1972).

A role of heme in initiation was suggested by the observation that addition of hemin to an unfractionated reticulocyte lysate increased the rate of initiation of

globin chains (Zucker and Schulman, 1968) and helped prolongue the linear rate of synthesis (Howard et al., 1968). Hemin was found to prevent the formation of an inhibitor that would form during incubation at 37°. Addition of an aliquot from a lysate incubated without hemin was able to inhibit protein synthesis in a fresh, unincubated lysate (Maxwell and Rabinovitz, 1969; Howard et al., 1970). This effect was shown to be temperature dependent (Hunt et al., 1972). At temperatures over 28° the rate of hemoglobin synthesis has declined markedly by 10 minutes in a lysate incubated without hemin. At 23° the rate does not decline until after 30 minutes. Gross and Rabinovitz (1972) have presented evidence that this inhibitor might exist in two states, as a reversible inhibitor and as an irreversible inhibitor. The reversible inhibitor would be in equilibrium with a proinhibitor that is stabilized by hemin. Absence of hemin would displace the equilibrium to reversible inhibitor which then would transform into the irreversible inhibitor. Legon et al. (1973) have shown with sucrose gradients that incubation of a lysate with ^{35}S methionine and hemin produces a complex between met-tRNA and the 40S ribosomal subunit. A similar incubation without hemin showed a rapid disappearance of the complex after two minutes of incubation, coupled with cessation of protein biosynthesis. This sparing effect of heme is not limited however to the initiation of globin chains. Lodish and Desalu (1973) have

observed that reticulocyte lysates incubated in the absence of hemin show a depression in the synthesis not only of globin but in the synthesis of the six other major proteins known to be produced in the reticulocyte and are unable to synthesize any of the 8 known reovirus-specific proteins. McDowell et al. (1972) have shown that the complete lysate system will synthesize the 8 reovirus proteins. These results on the scope of the heme effect have been confirmed by Mathews et al. (1973) using mRNA for mouse globin, calf lens crystallins and the RNA from EMC virus. In all these cases heme stimulates the synthesis of the corresponding proteins in the rabbit reticulocyte lysate.

Effect of tRNA

Changing patterns of isoaccepting tRNA species or of levels in a given species of tRNA have been associated with changes in differentiation. Benzoylated DEAE cellulose column chromatography has shown the presence of the $tRNA^{lys}$ isoaccepting species ($tRNA_1^{lys}$, $tRNA_2^{lys}$) in vegetative or sporulating Bacillus subtilis, while in spores $tRNA_2^{lys}$ is missing or found in very low concentrations (Chuang and Doi, 1972). Methylated Albumin Kieselguhr (MAK) chromatographic profiles of $tRNA^{met}$ and $tRNA^{arg}$ in erythrocytes of larval bull frog (Rana catesbeiana) differ from that found in the adult erythrocytes (DeWitt, 1971).

In cells committed to synthesis of specific proteins, the tRNA population tends to correlate with the amino acid

composition of the proteins being synthesized. Sheep reticulocytes contain different levels of $tRNA^{ile}$ and $tRNA^{met}$ in accordance with the particular allele of the β chain of hemoglobin that is being synthesized (Litt and Kabat, 1972). Transfer RNA from rabbit reticulocytes contains a high ratio of acceptance activity for histidine as compared to isoleucine. Histidine is very frequent in hemoglobin and isoleucine is very frequent in hemoglobin and isoleucine is very infrequent (Smith and McNamara, 1972). These authors find, however, that leucine acceptance activity is unusually low relative to the number of leucine residues present in hemoglobin. The role of modulator tRNA (Ames and Hartman, 1963) in rabbit reticulocytes awaits further studies.

MATERIALS AND METHODS

1. Reagents

Cycloheximide, bovine hemin (2x crystallized) and ribonuclease A (5x crystallized, protease free) from bovine pancreas were purchased from Sigma Chemical Company, St. Louis, Middouri. Sparsomycin was generously donated by Drug Research and Development, Division of Cancer Treatment, National Cancer Institute, Bethesda, Maryland. Trypsin treated with L-(1-Tosylamido-2-phenyl) ethyl chloromethyl ketone was obtained from Worthington Biochemical Corporation, Freehold, New Jersey. Pactamycin was donated by the Upjohn Company, Kalamazoo, Michigan. Penicillin G was purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio. Streptomycin Sulfate, U.S.P., was acquired from General Biochemicals, Chagrin Falls, Ohio. Diethylaminoethyl cellulose (DE-52) and carboxymethyl cellulose (CM-32) were purchased from H. Reeve Angel and Company, Clifton, New Jersey, and Bio Gel P-10 was from Bio Rad Laboratories, Richmond, Ca. Aquasol and Liquifluor were obtained from New England Nuclear, Boston, L-[^{35}S] Methionine and L-[3,5-^{3}H] Tyrosine were purchased from Amersham/Searle Corporation, Arlington Heights, Illinois. Specific activities ranged from 33 to 40 Ci per mole for tyrosine and from 40

to 133 Ci per mole for methionine, respectively. L-[^{3}H] Tryptophan (7.1C) mole was purchased from Schwarz/Mann, Orangeburg, New York. L-[^{14}C] Tyrosine, 455 Ci per mole, was ordered from Schwarz/Mann, Orangeburg, New York. Nitrocellulose filters (0.4 μ pore size) were from Schleicher and Schuell Company, Keene, New Hampshire. The synthetic dipeptides, L-tyrosyl-L-arginine and L-tyrosyl-L-histidine, were prepared by Cyclo Chemical, Los Angeles, California. Bio Gel -A 0.5M, 200-400 mesh, 10% agarose, was purchased from Bio-Rad Laboratories, Richmond, California. Blue Dextran 2000 was purchased from Pharmacia. DNP-alanine was kindly furnished by Dr. R. J. Evans of Michigan State University. Guanidine HCl was purchased from Sigma, grade I. All other reagents used were reagent grade.

2. Pretreatment of Reticulocytes for Labeling

Male New Zealand rabbits were made reticulocytic by four daily subcutaneous injections of 2.5% phenylhydrazine. The rabbits received no injections on days 5 and 6. The phenylhydrazine was dissolved in an isotonic solution containing 0.13 M NaCl, 5.2 mM KCl and 7.5 mM $MgCl_2$ (NKM) (Allen and Schweet, 1962). Following the addition of glutathione to a final concentration of 10^{-3} M, the pH was adjusted to about 7.3. The resulting solution was filtered and frozen until used. On day 7 of the injection sequence the animals were given a light ether anesthesia followed by an injection of 100 mg of Nembutal and 2000 I.U.

of heparin via the marginal ear vein. Blood was obtained by heart puncture and the collected blood cooled immediately to 4°. Hematocrits ranged from 12 to 16. All subsequent steps were carried at 4°. The blood was passed through glass wool and the cells were separated from the plasma by centrifugation for 20 minutes at 4000 X g in a Sorvall refrigerated centrifuge. The plasma was decanted and the volume measured. The packed cells were then washed with a volume of the "reticulocte saline," RS, described by Lingrel and Borsook (1963), equal to the plasma volume. The RS contained 0.13 M NaCl, 5 mM KCl and 7.4 mM $MgCl_2.6\ H_2O$. The cells were resuspended in a small volume of RS, the remainder of the RS was added, the suspension stirred and the cells recovered by centrifugation for 20 minutes at 4000 X g. The washing procedure was identically repeated once more and the cells recovered by centrifugation as before.

3. Labeling of Reticulocytes

A suspension of reticulocytes was incubated in a modified medium of Lingrel and Borsook (1963), Table I. Plasma from the same rabbit was dialyzed 1 hour against 35 volumes of cold RS prior to use in the incubation medium. The amino acid mixture of Lingrel and Borsook (1963) was used, except that hydroxyproline was omitted and L-asparagine and L-leucine were added to a final concentration of 0.51 mM and 2.58 mM in the incubation medium

(Hunt, 1968). Final concentrations of all the amino acids in full medium appear in Table II. Modifications were made as indicated in the text and in the legends to figures. These modifications were made depending on the particular radioactive amino acid used in the labeling experiment. When radioactive tyrosine was used, it was absent from the medium until the radioisotope was added. Nonradioactive L-tyrosine was added to the isotopically labeled tyrosine to give a final concentration of 0.021 mM in the incubation medium. This concentration of tyrosine was used for all incubation unless otherwise indicated. When [^{35}S] methionine was used as a label, nonradioactive methionine was omitted entirely from the medium. L-[^{35}S] methionine was added undiluted to the reaction mixture in amounts of 1 or 2 Mci and specific radioactivities averaging 150 Ci/mole. Particular values used are indicated for specific experiments in the legends to the figures. When tryptophan was used no amino acids were omitted from the incubation mixture. All incubations were performed at 37°. After an initial 2 minute warm-up period the radioactive amino acid was added to the reaction mixture. This addition of radioactivity defined zero time of incubation. The incubation was terminated by pouring the entire incubation mixture or suitable aliquots thereof into a 12 fold volume of ice cold RS containing cycloheximide at a concentration of 16.5µg per ml, (0.059 mM). The cells were then collected by centrifugation and washed once with fresh RS containing cycloheximide.

4. Preparation of Ribosomal Pellets

The washed reticulocytes were lysed for 10 minutes with 4 volumes of 2.5 mM $MgCl_2$ containing 0.09 mM cycloheximide and 0.21 mM sparsomycin. The solution was then made isotonic by the addition of one volume of 1.5 M sucrose-0.15 M KCl. Cell debris was removed by centrifugation at 2000 X g for 20 minutes. The supernatant solution was then centrifuged at 64000 X g for 3 1/2 hours to obtain the radioactive ribosomal pellets (1X). Where indicated the ribosomal pellets were resuspended in medium B (Allen and Scweet, 1962), and reisolated by sedimentation as before to yield washed (2X) ribosomes. Medium B contains 0.25 M sucrose, 17.5 mM $KHCO_3$ and 2 mM $MgCl_2$. The concentration of ribonucleoprotein was determined by measuring the absorbance at 260 nm using an absorption coefficient of 11.3 for a concentration of 1 mg per ml (Ts'O et al., 1961).

5. Preparation of Peptidyl-tRNA

Ribosomal pellets were resuspended in a small volume (approximately 1 ml) of 0.25 M sucrose containing 0.059 mM cycloheximide and 0.14 mM sparsomycin. The ribosomal suspension was then used to prepare peptidyl-tRNA according to the method of Slabaugh and Morris (1970). It was found that reduction of the urea concentration of buffers I and II from 8.0 to 7.6 avoids the occasional problem of crystallization of the urea solutions at 4°. This modification

Table I. Incubation of Reticulocytes according to Lingrel and Borsook (1963).

I. Reagent Mixture

In order of addition:

Component	mls
Amino acid Mix.* In RS. ph 7.75	54.00
$MgCl_2$ (0.25 M) plus Glucose 10%	2.70
TRIS.HCl (0.164M) pH 7.75	27.00
Sodium Citrate (10^{-3}M) in plasma	21.60
Sodium Bicarbonate (10^{-3}M) in plasma	32.40
	137.70

II. Reaction Mixture

In order of addition:

Component	mls
Reticulocytes (Packed cell volume)	10.00
Reagent Mixture	26.40
$KFe(NH_4)_2(SO_4)_2.6H_2O$ (10.5 mg/10ml) in RS	1.50
Radioactive amino acid in RS	2.40
	40.30

*Table II gives the concentrations of the amino acids in the reaction mixture (final concentration). Amino acids are 3.893 times as concentrated in the stock solution referred to as Amino Acid Mix.

Table II. Final Concentration of Amino Acids in the Modified Reaction Mixture of Lingrel and Borsook (1963)*

Amino Acid	Concentration (mM)
Alanine	0.514
Arginine	0.128
Asparagine	0.514
Aspartic acid	0.732
Glycine	1.361
Histidine	0.617
Isoleucine	0.077
Leucine	2.569
Lysine	0.462
Methionine	0.077
Phenylalanine	0.411
Proline	0.360
Serine	0.424
Threonine	0.437
Tryptophan	0.077
Tyrosine	0.206
Valine	0.822
Cysteine	0.103
Glutamine	2.055

*Any deviations from these final concentrations are indicated in the legends to the figures or in Methods.

together with the utilization of 360 ml of Buffer I during the "Buffer I wash" during DEAE-cellulose chromatography have been employed through out this thesis, Figure 1. The pooled fraction containing the purified peptidyl-tRNA was reduced to a volume of approximately 1.8 ml by ultrafiltration in an Amicon cell with a UM-2 Diaflo membrane. The concentrated sample was then dialyzed against 3 - 1500 ml portions of deionized water, lyophilized and stored at -21°.

Preparation of Urea Stock Solutions

Urea solutions (8.54 M) were prepared at room temperature and deionized by stirring with Amberlite MB-3 for approximately 1 1/2 hours. The ion exchange resin was removed by filtration and the resulting solution used as a stock urea solution for the preparation of the other urea containing solutions. A solution containing 6M LiCl and 7.6 M urea was prepared from this stock solution. Solid LiCl was added slowly to an 8.54 M stock urea kept in an ice bath. This solution was then kept at 4° until used.

Preparation of Urea Buffers

Buffer I contained 7.6 M urea, 0.1 M sodium acetate pH 5.6 and 0.05 M 2-mercaptoethanol (2-ME). Buffer I was prepared from stock solutions of 8.54 M urea, 5 M 2-ME and glacial acetic acid. Buffer I was titrated to pH 5.6 at room temperature using 6N NaOH. Buffer II was identical except it contained 0.75 M sodium acetate and was titrated with saturated NaOH.

Figure 1. DEAE-cellulose step during preparation of peptidyl-tRNA. A sample of [^{3}H] tyrosine-labeled ribosomes was treated with LiCl/urea and the soluble fraction desalted on Bio-Gel P-10 as described in the text. The desalted material (35 ml, 10.76×10^6 dpm) from the Bio-Gel P10 column was applied to a DEAE-cellulose column, as described in Methods. The sample was washed with 350 ml of buffer I. Buffer II was then applied. Aliquots of each fraction were analyzed as described in Methods.

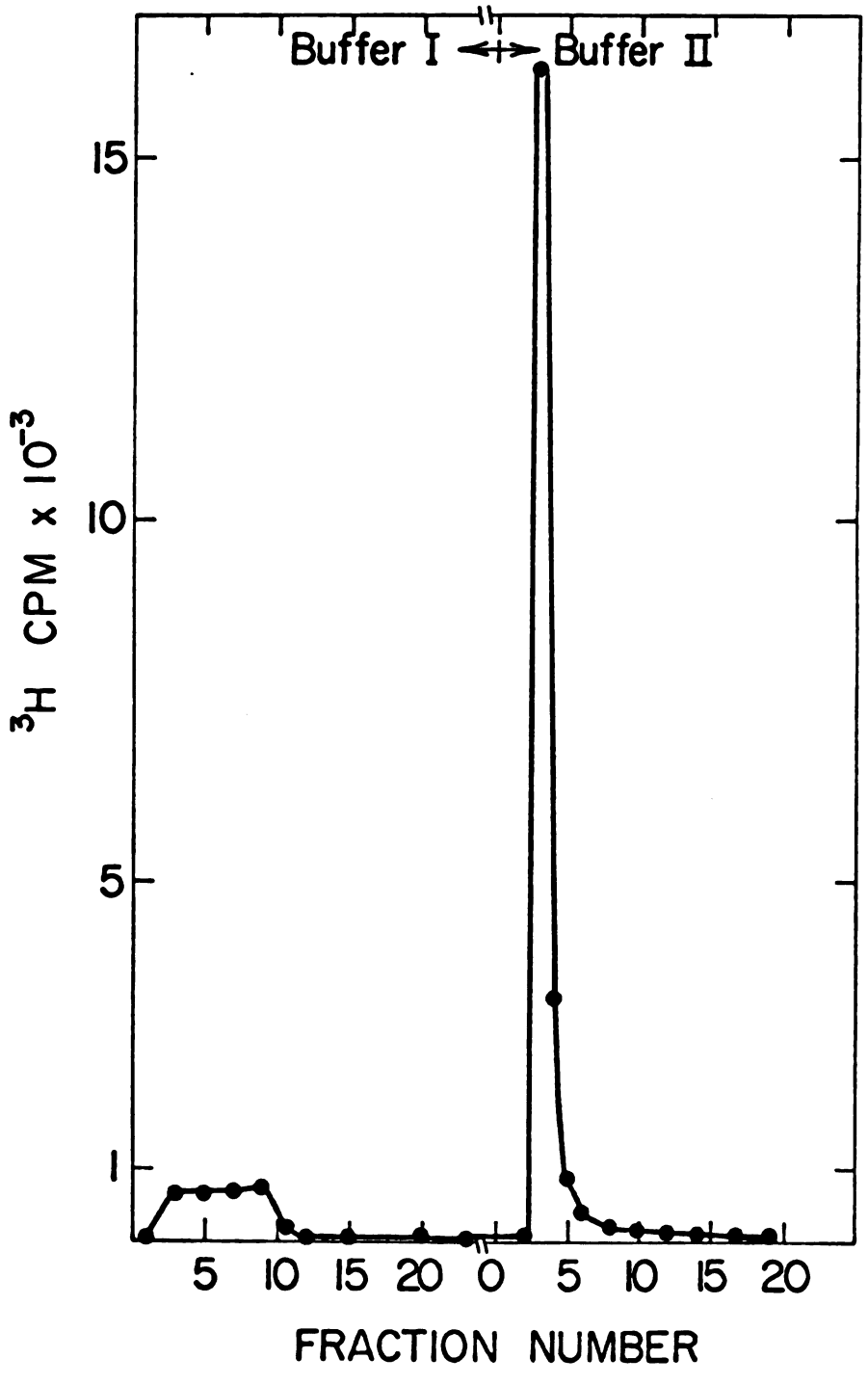
Buffer I
Buffer II
15
10
5
1
^{3}H CPM x 10^{-3}
5 10 15 20 0 5 10 15 20
FRACTION NUMBER

Figure 1

Bio-Gel P-10 Column Chromatography

Bio-Gel P-10, 50-100 Mesh, column chromatography was used to remove LiCl from the solution containing peptidyl-tRNA. Bio-Gel P-10, 9.5g was soaked overnight in approximately 250 ml of Buffer I. The slurry was allowed to settle for 15 minutes and the supernatant was removed by aspiration. The total remaining P-10 was poured into a column 1.9 cm in diameter to a bed height of 33 cm. Prior to use the column was washed with 50 ml of buffer I and run at a flow rate of about 0.37 ml/min. Three ml fractions were collected.

DEAE-Cellulose Chromatography (Figure 1)

Whatman De-52 microgranular cellulose was used. Seven grams of the cellulose exchanger was suspended in 60 ml of 0.5 N acetic acid and aspirated with agitation to remove CO_2. The slurry was titrated to pH 5.6 using 6 N NaOH, and the "fines" removed by allowing the cellulose to settle for a number of minutes equal to 2.5 times the height of the slurry in cm. The "fines" were then removed by aspiration of the supernatant solution. Approximately 60 ml of buffer I was added and the removal of fines repeated. This was followed by two subsequent removals of fines with buffer I. The total remaining DEAE-cellulose was poured into a 2 cm diameter column to give a bed height of approximately 6 cm. Prior to use the column was washed with about 50 ml of buffer I. The sample was applied to the column and the absorbed

peptidyl-tRNA was washed with at least 360 ml of buffer I at a flow rate of about 12 ml per 30 min.

6. Preparation of Globin Uniformly Labeled with [^{14}C] or [^{3}H] Tyrosine

Washed reticulocytes were incubated in the presence of tyrosine labeled with the appropriate isotope as described above. The tyrosine concentration in the medium was 0.1 mM. Penicillin and streptomycin were added to the reaction mixture to a final concentration on 0.11 mg per ml of each. Incubations were allowed to proceed at 37° for 3 1/2 to 4 hours. The cells were washed, lysed and the post ribosomal supernatant used to prepare hemoglobin according to the method of Winterhalter and Huehns (1964). The post ribosomal supernatant was dialyzed at 4° against 2-1 liter portions of 0.01 M sodium phosphate buffer, pH 6.8. The dialyzed solution was applied to a CM-Sephadex column (1.8 x 20 cm), equilibrated with 0.01 m sodium phosphate pH 6.8. This was followed by a wash with 100 ml of the same buffer. Elution was done with a convex gradient formed by placing 250 ml sodium phosphate pH 6.8 in a constant volume chamber and adding 0.02 M Na_2HPO_4 to the chamber while stirring. Fractions containing hemoglobin were combined and dialyzed for 48 hours against 3 portions (1 lt each) of deionized water at 4°. Hemoglobin was determined by the method of Austin and Drabkin (1936). Globin was prepared by the cold acid acetone method of Rossi Fanelli *et al*. (1958). The dialyzed hemoglobin (15 mg per ml) was added dropwise and

with magnetic stirring to 30 volumes of cold acetone containing 6 mM HCL. The acetone HCL solution was kept cold in dry ice - acetone bath, -86°. The precipitated globin was collected by centrifugation at 1020 X g for 15 minutes at -20°. The supernatant was decanted and the globin dissolved in the minimal volume of deionized water. The globin was dialyzed against 3-1 liter portions of deionized water at 4°. Recovery of radioactivity is approximately 79%. The globin was stored at -20° as a lyophilized powder.

7. Separation of Uniformly Labeled Alpha- and Beta-Globin Chains

The α and β globin chains of [^{14}C] labeled rabbit globin were separated on carboxymethyl cellulose (CM-32) columns (1 x 22 cm) with a nonlinear gradient modified from the procedure of Rabinovitz et al. (1964). The gradient was generated by placing concentration multiples of 1,3,5,7,1,7 and 9 fold of the starting buffer (0.2 M formic acid - 0.02 M pyridine) in successive chambers of a 10 chamber rectangular Varigrad (Buchler Instruments Inc., Fort Lee, New Jersey). The contents of each chamber (50 ml) were 0.05 M in β-mercaptoethanol (Lodish, 1971). Prior to chromatographic separation the globin samples were dialyzed overnight against 0.05 M β-mercaptoethanol and then adjusted to 0.2 M formic acid, 0.02 M pyridine. Globin (45 mg or less) was applied to the column and eluted at a flow rate of 14-16 ml per hour. The separated α and β globin chains were then lyophilized and each was rechromatographed on a CM-32 column

by the same procedure in order to obtain further purification, Figure 2. Lyophilized samples of separated globin chains were stored at -20°. The purity of the separated α and β globin chains obtained in this manner was established by the addition of nonradioactive carrier globin and digestion of the mixture with trypsin at 37° for 4 hours as described below. The six tyrosine containing peptides (αT4, αT6, αT15, βT4, βT14, βT16) were separated according to the method of Hunt et al. (1969) and analyzed for radioactivity. By this means it could be shown that the α chain preparation contained approximately 0.82% β chain while the β chain preparation contained approximately 1% contamination by α globin.

8. Analysis of Nascent Globin Chains

a. Analysis of α and β Globyl-tRNA

Pretreatment of Peptidyl-tRNA for Tryptic Digestion

The lyophilized sample of labeled peptidyl-tRNA was resuspended in 1.0 ml of water containing 0.1 mg of pancreatic RNase incubated at 37° for 25 minutes and lyophilized. After redissolving in 0.15 ml of 0.1 N NaOH the material was incubated for 3 1/2 hours at 37° in order to cleave the peptidyl-tRNA ester bond. The solution was then neutralized with 1 N HCL to a pH of 5.4-5.6 as determined with pH indicator paper. Purified α and β rabbit globin chains of known radioactivity content (uniformly labeled with [^{14}C]-tyrosine) were then added as an internal standard. Nonradioactive globin was added, if necessary, to give a mass of 3-4 mg of

Figure 2. Separation of the α and β chains of rabbit globin.

A) Rabbit globin (45 mg, 7.5 x 10^4 cpm per mg) labeled uniformly with [^{14}C] tyrosine was analyzed by CM-cellulose chromatography as described in Methods.

B) Rabbit globin α chain obtained from A plus α chain obtained from a similar experiment were pooled and rechromatographed as in A.

C) Same as in B except that rabbit globin β chain was used.

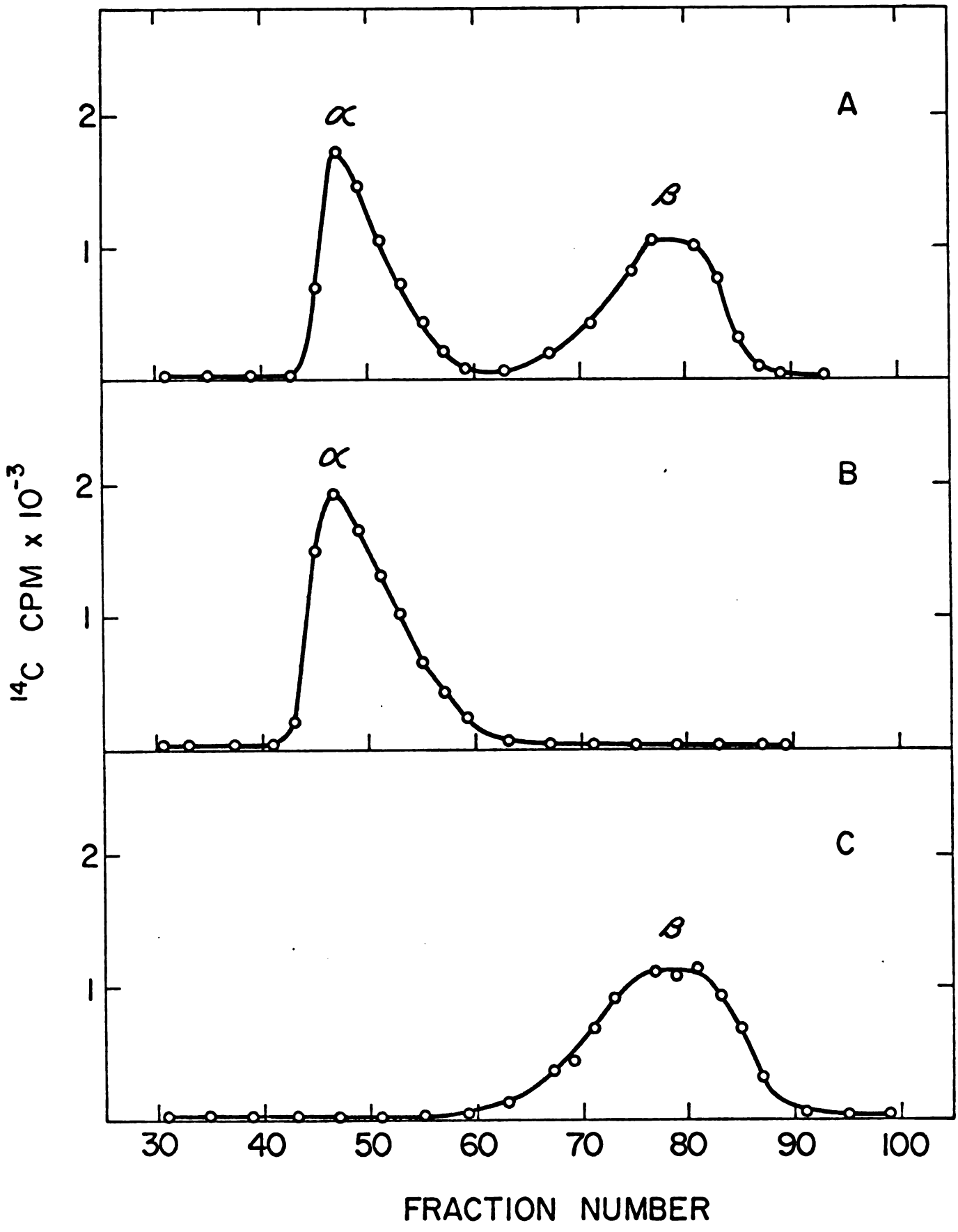
A
B
C
α
β
α
β
2
1
2
1
2
1
^{14}C CPM x 10^{-3}
30
40
50
60
70
80
90
100
FRACTION NUMBER

Figure 2

protein in the sample. The synthetic dipeptides, L-tyrosyl-L-arginine (αT15) and L-tyrosyl-L-histidine (βT16), were added as carrier peptides (50 nmoles each) prior to tryptic digestion.

Tryptic Digestion

Tryptic digestion was carried out in 0.1% $NaHCO_3$, Schapira et al. (1968), at a final globin concentration of 3-4 mg per ml. Trypsin was added in an amount equal to 2% (w/w) of the total globin present. After 2 hours incubation at 37°, 1% (w/w) trypsin was again added and the incubation continued for an additional 2 hours. Samples were then frozen and lyophilized. To prepare tyrosine labeled tryptic peptides for agarose gel filtration chromatography, tryptic digestion was done in 0.2 M ammonium bicarbonate (ABC) at a final globin concentration of 1.3 mg per ml. Trypsin was added in an amount equal to 1.3% (w/w) of the total purified β chain (16 mg) from rabbit globin uniformly labeled with ^{14}C -tyrosine. Incubation was continued for an additional 8 hours and the sample lyophilized.

Removal of trypsin seemed necessary to prevent any possible hydrolysis of the larger peptide markers during the pretreatment of the sample for gel filtration as described further ahead. Besides it seemed desirable to remove any large products of partial digestion which might interfere with the identification of the larger peptide markers. The tryptic digest was therefore purified by

passing through a Bio-Gel P-10 column. Results of one such removal of trypsin are shown in Figure 3. Trypsin was assumed to elute with the void volume of the column. Figure 4 shows a similar experiment in which the tryptic peptide markers for the experiment shown in Figure 11 were purified.

Separation of Tryptic Peptides

Separation of tyrosine containing tryptic peptides from rabbit globin was performed by the two dimensional method of Hunt et al. (1969), which combines high voltage electrophoresis and paper chromatography, Figure 5. Tryptic peptides are numbered according to their position of occurrence relative to the N-terminal end of the α and β globin chains of rabbit hemoglobin (Gerald and Ingram, 1961).

The lyophilized sample containing the tryptic peptides was dissolved in 0.1 ml of 10% formic acid v/v and was applied to a 6 x 22 inch sheet of Whatman 3-MM Chromatography paper in two 50 µl aliquots. The sample was applied as a 1 inch long streak along a line 5 cm away from the anode (-) edge of the paper. The paper was wetted with the pH 4.7 electrophoresis buffer (Kitchen et al., 1968), (1.25% pyridine, 1.25% acetic acid) before electrophoresis for 3.25 hours at 2000 volts. After drying the paper, a 5.0 cm wide lane was cut containing the sample and scanned for peaks of radioactivity by means of a Packard Radiochromatogram paper strip scanner. The three areas of radioactivity were detected. These areas are referred to as areas 1, 2 and 3,

Figure 3. Calibration of Bio-Gel P-10 column for removal of trypsin from a tryptic digest of radioactive globin. Rabbit globin (10 mg) uniformly labeled with [^{3}H] tyrosine was hydrolyzed in 0.2 M ammonium bicarbonate with trypsin as described in methods. The lyophilized tryptic digest was dissolved in 1 ml of 0.2 M ammonium bicarbonate. To this solution 0.2 ml of blue dextran (18 mg/ml in 0.2 M ABC) and 1 drop of 0.1% (w/w) phenol red in water was added. The sample was then applied to a Bio-Gel P-10 column for analysis. Bio-Gel P-10 was soaked overnight in 0.2 M ammonium bicarbonate (0.2 M ABC). The Bio-Gel P-10 was then poured into a 1 cm diameter column to give a bed height of 27 cms. Three milliliter fractions were collected and assayed for radioactivity and for absorbance at 630 nm (blue dextran) and 540 nm (phenol red). Aliquots, 0.5 ml, were drawn from the odd numbered fractions and counted in 5 ml of Bray's solution, see Methods.

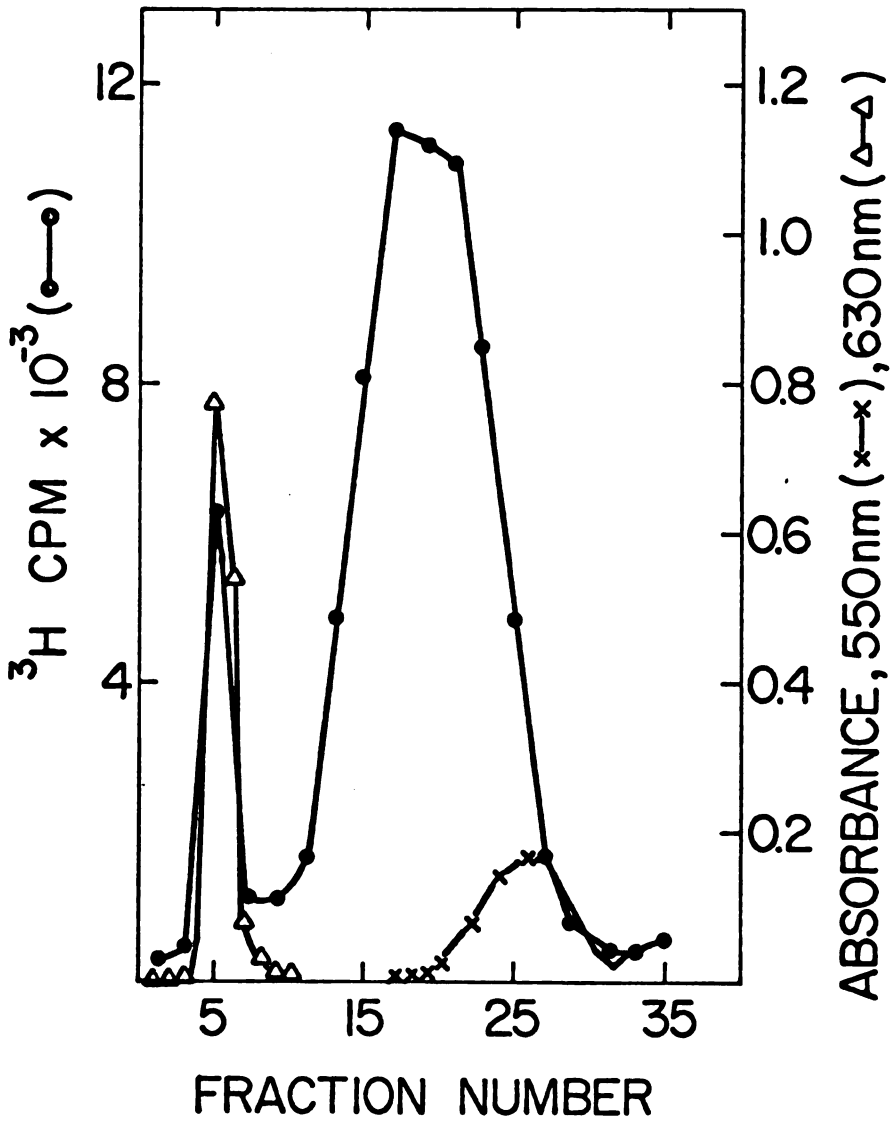
12
8
4
^{3}H CPM x 10^{-3} (●—●)
1.2
1.0
0.8
0.6
0.4
0.2
ABSORBANCE, 550nm (×—×), 630nm (△—△)
5
15
25
35
FRACTION NUMBER

Figure 3

Figure 4. Removal of trypsin from a tryptic digest of [^{3}H]-globin. The β-chain (16 mg) of rabbit globin uniformly labeled with [^{3}H] tyrosine was hydrolyzed in 0.2 M ammonium bicarbonate containing 2% (w/w) trypsin as described in Methods. This sample was processed as in Figure 8 except that the blue dextran and phenol red were omitted and the bed height of the P-10 column was 40 cms. Odd numbered fractions were assayed for radioactivity by diluting aliquots (50 µl) with 0.5 ml of water and counting in Bray's solution. Fractions were pooled as indicated by the horizontal line.

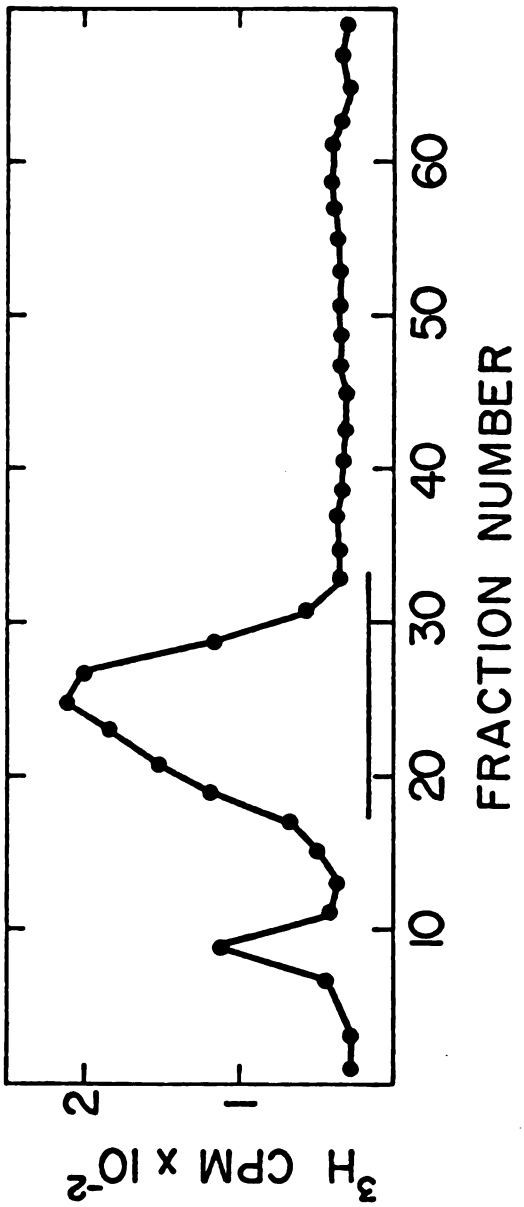

Figure 4

Figure 5. Separation of tyrosine-containing tryptic peptides from rabbit globin by two-dimensional high voltage electrophoresis and paper chromatography. Experimental procedures appear in Methods. Non-radioactive globin and the β-chain of rabbit globin uniformly labeled with [^{14}C] tyrosine (40,000 cpm) were mixed and subjected to trypsin digestion as described in Methods. Tyrosine containing peptides appear cross hatched.

A. Strip scanner recording of the radioactivity present in β following high voltage electrophoresis at pH 4.7.

B. Paper strip containing tryptic peptides from rabbit globin. The position of the origin and the direction of movement of the tryptic peptides during electrophoresis at pH 4.7 are indicated by an O and an arrow, respectively, between panels A and B. The vertical wavy lines indicate where segments containing radioactivity were cut for further analysis in a second direction. From left to right these segments are referred as areas 1, 2 and 3 as indicated by the numbers underneath.

C. Separation of the peptides in Area 1 by electrophoresis at pH 2 in the direction shown by the

vertical arrow. The broken horizontal lines indicate where the segment containing area was sewed for separation in a second dimension. All spots were first visualized with ninhydrin, see Methods. Cross hatched spots were visualized by further staining with a tyrosine specific stain.

D. Separation of the peptides in area 2 by descending paper chromatography. All other details same as in panel C.

E. Separation of the peptides in area 3 by electrophoresis at pH 8.9. All other details same as in panel D.

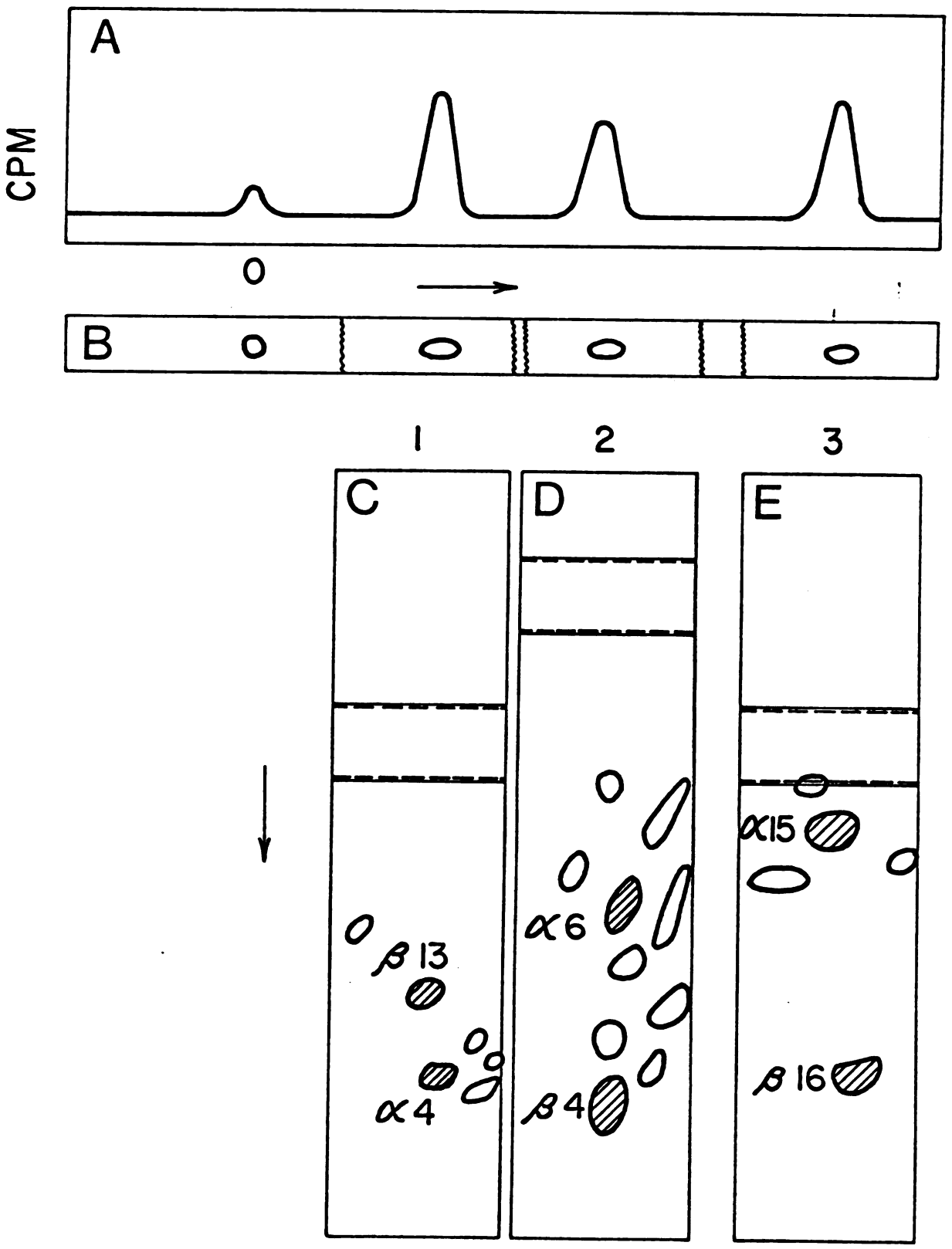

Figure 5

from anode to cathode. The boundaries were marked and cut to give 2 x 4 in rectangles. These rectangles were sewn to 4 x 22 inch sheets of Whatman 3 MM chromatography paper. The papers were then trimmed to preserve the overall length of 22 inches. The sheet containing area 1 tryptic peptides was wetted with pH 2 electrophoresis buffer (8% acetic acid, 2% formic acid) and run in the same buffer for 1 hour at 3000 volts. The sheet containing area 2 tryptic peptides was placed in a cylindrical chromatography jar and the paper equilibrated for 2 to 3 hours with the solvent system of Waley and Watson (1953), (90:60:18:72::n-butanol:pyridine: acetic acid:water, v/v). The chromatogram was developed with this same solvent for 15 hours. The sheet containing area 3 tryptic peptides was wetted with pH 8.9 electrophoresis buffer, (1% ammonium carbonate) and run in the same buffer for 1.75 hours at 2000 volts. Peptides were visualized by dipping through buffered ninhydrin containing 0.3% (w/v) ninhydrin in acetone which was 1% (v/v) in both glacial acetic acid and pyridine (Easley, 1965). Tyrosine-containing spots in parallel control runs were identified using a 1-nitroso-2-naphtol stain. The paper was dipped in a 0.1% (w/V) solution of this compound in acetone, dried and dipped through acetone containing 10 ml concentrated HNO_3 per 100 ml. The appropriate areas containing the radioactive tyrosine peptides were removed from the sheets, remaining solvents removed <u>in vacuo</u> and the paper was then

cut into small sectors and placed in scintillation vials for the elution procedure. Three extractions with 2 ml of 0.01 N HCl were performed at 80°. The eluates were pooled into fresh scintillation vials and lyophilized for counting of radioactivity.

Counting of Radioactivity

The tryptic peptides were dissolved in 0.01 N HCl and combined with 10 ml of Aquasol and counted in a Packard liquid scintillation spectrometer model 3310. Counting efficiencies were determined by the channels ratio method for doubly labeled samples. Counting efficiencies of samples containing a single-radioisotope were sometimes established by internal standardization with [^{3}H] or [^{14}C] labeled toluene of known radioactivity content (New England Nuclear, Boston, Mass.). Data expressed as decompositions per minute (DPM) were determined from the observed cpm and the counting efficiency.

The elution of radioactive materials during column chromatography was monitored by placing 25-50 ml aliquots of the eluate fractions in 0.5 ml of H_2O, 5 ml of Aquasol and counting in a Nuclear Chicago, Unilux, liquid scintillation counter.

Counting procedures for Agarose gel chromatography are explained in the legends to the figures. Bray's solution (Bray, 1960) contained per liter: 60g naphtalene, 4g PPO, 200 mg POPOP, 100 ml absolute methanol, 20 ml ethylene

glycol and p-dioxane to volume.

b. Analysis of Nascent Globin Chains by Gel Filtration Recrystallization of Guanidine Hydrochloride

Guanidine hydrochloride was recrystallized according to the method of Nozaki and Tanford (1967). Guanidine hydrocholride, 500 g was dissolved with stirring in 2 liters of absolute ethanol, kept close to its boiling point. Norite, 2 g, was added to the solution and stirred for 2 minutes. Norite was removed by gravity filtration of the warm solution through two sheets of fluted filter paper. To the clear solution 1.1 liters of benzene was added to precipitate the guanidine hydrochloride. The precipitate was allowed to stand overnight at 4°. The crystals were harvested in a Buchner funnel. The harvested crystals were transferred to a container which was placed in a vacuum desicator. Benzene was removed by connecting the dessicator to a water aspirator pump. The dry crystals were ground in a mortar and placed under high vacuum. The dry guanidine hydrochloride crystals were further recrystallized from absolute methanol. To 67 ml of absolute methanol near its boiling point 100 g of guanidine hydrochloride was slowly added with stirring. Any guanidine hydrochloride remaining undissolved was brought into solution by adding small aliquots of methanol. The warm methanolic solution was allowed to stand overnight at 4°. The crystals were harvested in a Buchner funnel. A further crop of crystals was obtained by

cooling the mother liquor in a dry-ice acetone bath. Excess methanol was removed from the damp crystals by placing them in a vacuum dessicator connected to a water aspirator pump. The dry crystals were ground in a mortar and evaporated under high vacuum in a lyophilizer to remove the last traces of organic solvents.

Bio-Gel A-0.5M Gel Filtration Chromatography

Bio-Gel A-0.5M, (lots 104754 and 11607), mesh 200-400, with a nominal agarose content of 10% was suspended in water and allowed to settle several times to remove TRIS and NaN_3 added as a preservative. Water was decanted each time. The agarose gel was then equilibrated with the eluting solvent (6M guanidine HCl-0.1M β-ME, pH 6.5). Appropriate amounts of dry guanidine HCl and β-ME were added to the settled agarose gel. Following gentle swirling, enough water was added to dissolve the guanidine HCl. The slurry was degassed and litrated to pH 6.5 with 0.1N NaOH. The agarose suspension was then allowed to equilibrate overnight at 4°. All other operations were performed at 4°. Gel beds ranging in height from 78 to 83 cm were formed in Pharmacia K 15/90 columns. The columns were packed and run under a pressure differential of 57-60 cm of solvent. The pressure differential was maintained during the runs with the use of a 500 ml Marriot flask. Flow rate was 3.0 ml per hour (1.73 ml per hour per cm^2). Prior to use the column was allowed to flow for at least 36 hours. To apply

the sample, the column flow was momentarily stopped and the sample, 0.2 ml, was layered under the solvent onto the top of the gel with a Sage pump. Column flow was started immediately after application of the sample. Fractions were collected with a Gilson fraction collector. The fraction size ranged from 11 drops (0.3 ml) to 26 drops depending on the experiment, as indicated in the legends to the figures. In other experiments 25 drops were collected with an ISCO Golden Retriever fraction collector. Fractions were either collected into test tubes and aliquots removed for counting of radioactivity or directly into scintillation vials and counted. In the latter case the scintillation vials were fastened to the rotary table of an ISCO fraction collector. In this latter case 11 drops were collected per fraction (0.3 ml). Positions of elution of the blue dextran and DNP alanine markers were detected by measuring their absorbance at 630 nm and 360 nm respectively (Fish et al., 1969).

Treatment of the Sample for Bio-Gel A-0.5M Gel Filtration

A lyophilized preparation of peptidyl-tRNA was dissolved in 0.5 ml of 0.1 N NaOH and was incubated for 3 1/2 hours at 37° in order to cleve the peptidyl-tRNA ester bond. The solution was then neutralized with 1N HCl to a pH of 8.0-8.4 as determined with pH indicator paper. The sample was then lyophilized. In experiments where peptidyl-tRNA was not used this step was omitted.

A lyophilized sample containing free peptides was dissolved in 0.5 ml of column solvent, the pH was raised to 8.6 by adding 1N NaOH and the sample was allowed to stand at room temperature for 24 hours to reduce disulphide bonds. The pH of the solution was then lowered to pH 6.5 as determined with pH indicator paper. At this time 70 µl of 3.6% blue dextran in column solvent was added followed by 80 µl of 0.1% DNP-alanine in 60% sucrose prepared in column solvent. The clear solution was then centrifuged at 12000 x g to remove dust particles and 0.21 ml were loaded onto the Bio-Gel A-0.5 m column.

Cyanogen Bromide Cleaveage of Globin Chains

Globin or separated globin chains were dissolved in 70% formic acid (v/v) at a concentration of 5 mg per ml (Schroeder et al., 1968). Depending on the experiment, CNBr was present in amounts ranging from 90 to 500 moles of CNBr per mole of methionine present. Reaction times ranged from 18 hours to 72 hours. Reactions were carried in the dark. At the end of the reaction, the reaction mixture was diluted with 10 times its volume of water and lyophilized.

Removal of Guanidine and β-ME from Peptides

The guanidine HCL - 0.1 M β-ME solvent used for Bio-Gel A-0.5M chromatography was removed by chromatography in columns packed with Bio-Gel P-2. Bio-Gel P-2 was swolen overnight in 0.2M Ammonium Bicarbonate. Gel beds 1.2 x 40 cms were formed in glass columns. The fractions to be

analyzed were applied directly to the top of the gel bed and eluted with 0.2 M ammonium bicarbonate. Three ml fractions were collected. Peptides were detected by counting of radioactivity and guanidine HCl by measurement of conductivity in a Radiometer conductivity apparatus. The fractions containing the radioactive peptides were pooled and lyophilized.

Treatment of Data from Bio-Gel A-0.5 M Gel Filtration Chromatography

Plotting of Data

Elution data from Bio-Gel A-0.5 gel filtration chromatography was presented according to Fish et al. (1969). Elution volumes corresponding to different experiments were normalized by using the distribution coefficient (K_d), corresponding to each fraction as a representation of the position of elution. The distribution coefficient (K_d) as defined by Fish et al. (1969) is

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

Where V_e is the weight of solvent used to elute a given compound.

V_i is the weight of solvent contained within and without the gel.

V_o is the void volume, i.e. the weight of solvent in the column, external to the gel matrix.

Blue dextran 2000 was used to determine the exclusion volume, by monitoring its absorbance at 630 nm. In this

thesis the fraction number containing the maximum absorbance at 630 nm is defined as the void volume. V_i was determined by monitoring the absorbance of DNP-alanine at 360 nm. The fraction with an absorbance maximum at 360 nm was defined as V_i. V_e is any fraction between V_o and V_i.

The cube root of the distribution coefficient, $(K_d)^{1/3}$, of known peptide markers have been plotted against their molecular weight raised to the 0.555 power (Fish et al., 1969). Straight lines have been obtained from these plots. The slopes and intercepts of these straight lines have been calculated by the least squares method.

Using distribution coefficients determined in two separate agarose gel filtration analyses (Table VI) and the molecular weights of the peptides as determined by their amino acid sequence (Dayhoff and Eck, 1968) the following linear relationship has been obtained:

$$K_d^{1/3} = 1.0568 - 0.002101 \times M^{0.555} \quad [1]$$

This relationship has been used to relate molecular weight to distribution coefficient for each fraction.

Construction of Theoretical Curves

To interpret the observed elution pattern of nascent globin chains, hypothetical elution curves have been computed. These curves are based on the following assumptions.

a. The distribution of sizes of nascent peptides is uniform. This assumption defines a base line to compare

the experimental values.

b. The ratio of nascent α chains to nascent β chains is 1. Hunt et al. (1968a,b) find an average ratio of about 1.1. This thesis find a ratio of 1.04.

c. The relationship between the distribution coefficient of each nascent peptide and its molecular weight is given by [1], as determined by calibration of the column.

d. The elution pattern of any single peptide will be a Gaussian curve. This gaussian curve will be centered at the K_d value of its corresponding peptide. The contribution from each peptide is independent of the contribution of the other peptides.

e. For a given labeled amino acid, the area under the elution pattern of a peptide carrying this amino acid will be proportional to the number of residues of this amino acid present in the peptide.

f. Nascent α chain peptides and β chain peptides with the same number of residues will have the same distribution coefficient. This is a simplifying assumption used to permit calculation of the K_d values in steps.

g. Molecular weight increase in steps of 110 the average weight per residue for the β chain of hemoglobin. Essentially the method used is equivalent to drawing a gaussian curve with a maximum at the K_d value corresponding to each nascent chain and adding the ordinates of the resulting curves for each value of K_d. This gives the composite elution pattern as a function of

K_d. A computer program implementing this idea is shown in Appendix I.

Smoothing of Bio-Gel Filtration Elution Data

In one instance, figure 21, excessive fluctuation of elution data was removed by the data smoothing procedure of Savitzky and Golay (1964). A point was chosen along the elution pattern. Four successive points were taken immediately to the right and to the left of the chosen point. A quadratic polynomial was then fitted by least squares to the nine points. From the known abscissa of the chosen point and the computed quadratic a new ordinate was computed for the chosen point. The coefficients for a nine point fitting listed by Savitzky and Golay (1964) were used. This fitting procedure was repeated for all points of the elution pattern except for the last four at either extreme of the elution pattern.

RESULTS

1. Purified Peptidyl-tRNA is Free of Contamination with Soluble Hemoglobin

The analyses conducted in this thesis require that purified peptidyl-tRNA be free of significant amounts of contamination by soluble (labeled) hemoglobin. The two analyses described below were performed to assess this degree of contamination.

A mixture of nonradioactive reticulocyte ribosomes and purified [^{3}H] labeled hemoglobin was prepared (See Legend of Table 3). This mixture was then subjected to the procedure for preparation of peptidyl-tRNA, Slabaugh and Morris (1970). Radioactivity present in the purified peptidyl-tRNA fraction thus represents the extent of contamination by hemoglobin in that fraction. Results from the two separate analyses appear in Table 3. These results indicate that not more than 0.030% of the labeled hemoglobin originally added remains in the purified peptidyl-tRNA fraction.

2. Accumulation of the Completed α Chain on the Polyribosome Labeling of the Ribosomes in the Whole Reticuloyte

Figure 6 shows the time course of incorporation of [^{3}H] tyrosine into the ribosomes and into soluble hemoglobin

Legend

(Table III)

Rabbit reticulocytes (0.5 ml packed cell volume) were incubated as described in Methods. The tyrosine concentration in the incubation medium was 0.1 mM. Labeled alanine, valine and leucine (0.5 m Ci each) were added and the incubation was allowed to proceed for 45 minutes at 37°. The post ribosomal supernatant was dialyzed against 0.1 M sodium acetate (pH 5.6) and passed through a DEAE-cellulose column (1.5 x 5 cm) which had been equilibrated with the same buffer. The labeled hemoglobin was then further purified by CM-cellulose chromatography (see Methods). Unlabeled 2x ribosomes in 0.25 M sucrose (21.4 mg/ml) were then combined with the purified [^{3}H] hemoglobin (7.4 x 10^6 DPM/mg) and purified peptidyl-tRNA was prepared. Samples were counted by liquid scintillation using Bray's solution.

Table III Added ^{3}H Hemoglobin Found in the Purified Peptidyl-tRNA Fraction

Experiment	[^{3}H] Hemoglobin added	Unlabeled ribosomes added	[^{3}H] Hemoglobin recovered in the purified peptidyl-tRNA fraction	
	DPM x 10^{-6}	mg	DPM	%
I	25.8	40.0	7,850	0.030
II	18.1	48.4	4,450	0.025

Figure 6. Time course of incorporation of [^{3}H] tyrosine into soluble hemoglobin of rabbit reticulocytes. Rabbit reticulocytes (5 ml packed cell volume) were incubated as described in Methods. At zero time 0.1 m Ci of ^{3}H tyrosine (240 μ Ci per mole) was added to a final concentration of 0.021 mM. At the time points indicated 3 ml aliquots were withdrawn from the reaction mixture. The specific activity of the soluble hemoglobin and of the ribosomes was measured in the post ribosomal supernatant fraction and in the twice washed (2X) ribosomes obtained from each aliquot. Solutions containing 0.5 mg of hemoglobin in 1 ml of water or 0.090 mg of ribonucleoprotein in 1 ml of 0.25 M sucrose were precipitated with an equal volume of 20% trichloroacetic acid. The precipitates were collected on nitrocellulose membranes and counted in a toluene Liquifluor mixture.

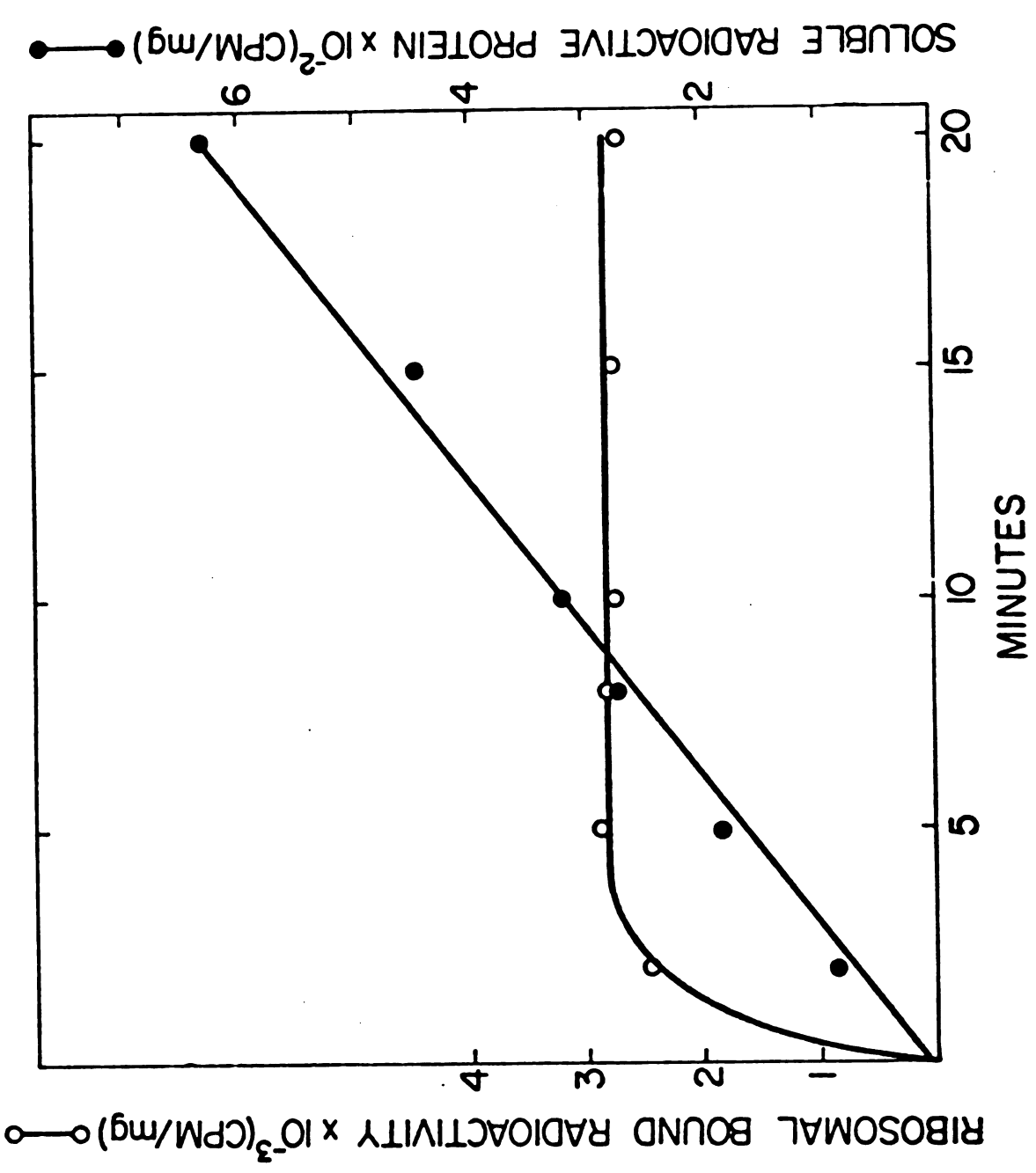
RIBOSOMAL BOUND RADIOACTIVITY x 10^{-3}(CPM/mg)
SOLUBLE RADIOACTIVE PROTEIN x 10^{-2}(CPM/mg)
MINUTES
1
2
3
4
2
4
6
5
10
15
20

Figure 6

of intact reticulocytes. The incorporation of radioactivity into ribosomes reached a constant value by 4 minutes after addition of [^{3}H] tyrosine to the incubation medium. The specific radioactivity of the ribosomes remained constant for at least the next 16 minutes. The incorporation of radioactivity into soluble hemoglobin was linear for at least the first 20 minutes of incorporation. The constant level of radioactivity found in the ribosomal fraction after 4 minutes of incubation assures that a steady state of labeling of precursor pools and nascent protein has taken place. Nascent globin chains prepared from cells collected at 10 minutes of incubation thus possess uniform specific activity of the 6 tyrosine residues present in the nascent globin chains.

Determination of the Amount of α and β Globyl-tRNA

Rabbit reticulocytes were incubated in a medium containing [^{3}H] tyrosine for 10 minutes at 37°. The ribosomal pellets obtained from the labeled reticulocytes were used to prepare the purified peptidyl-tRNA fraction. Following the addition of [^{14}C] labeled α and β globin chains to the peptidyl-tRNA as internal standards, the mixture was digested with trypsin and the tyrosine containing tryptic peptides were isolated and analyzed as described in Methods.

The relative specific activities ([^{3}H]/[^{14}C] ratio) of the tryptic peptides are shown in Figure 7. The [^{3}H]/[^{14}C] intercepts were calculated by the method of least squares

Figure 7. Relative specific activities of the nascent globin peptides from purified peptidyl-tRNA. The ordinate represents the [^{3}H]/[^{14}C] ratios obtained in experiment I of Table IV. Each tryptic peptide is positioned on the abscissa according to the position of the C-terminal amino acid of that tyrosine-containing tryptic peptide in the sequence of rabbit hemoglobin. Tryptic peptides have been numbered according to their position of occurrence relative to the N-terminal end of the corresponding rabbit globin chains. Lines drawn through each set of points thus represent the relative specific activities to be expected for each amino acid present in a uniform distribution of nascent chains on the polysome. The [^{3}H]/[^{14}C] intercept has been used as a measure of the total nascent chains present and the ordinate value corresponding to α T15 or β T16 has been used as a measure of α globyl-tRNA or β globyl-tRNA present, respectively.

A
B
RELATIVE SPECIFIC ACTIVITY (^3H)/(^{14}C)
100
50
α4
α6
α15
β4
β14,β16
POSITION OF TRYPTIC PEPTIDE

Figure 7

in order to obtain a relative measurement of the number of α and β nascent chains present, see Discussion. The [^{3}H]/[^{14}C] ratios observed in the C-terminal tryptic peptides (α T15 and β T16) were used as a relative measurement of the number of completed α and β globin chains in the peptidyl-tRNA fractions since only the completed globin chains in the population of nascent chains can yield those tryptic peptides upon hydrolysis, Dintzis (1961).

The results of three independent experiments are shown in Table IV. Each set of experimental data was analyzed as shown in Figure 7 for experiment I. It is apparent from these data that rabbit reticulocyte ribosomes contain a significant component of completed α globin which is still attached to tRNA (α globyl-tRNA). Some 4.6% of nascent α globin chains are present as α globyl-tRNA. On the other hand, completed β chains attached to tRNA (β globyl-tRNA) constitute only 0.70% of the nascent β globin chains.

Effect of Hemin

The presence of a pool of free soluble globin chains has been shown to be present in the reticulocyte (Tavill et al., 1972; Baglioni and Campana, 1967). It has also been reported that this pool is decreased in size if the reticulocytes are incubated with hemin (Tavill et al., 1972). In order to examine the possible effects of hemin on the accumulation of α globyl-tRNA on the ribosomes two paralled incubations of rabbit reticulocytes were performed. One

LEGEND

(Table IV)

Rabbit reticulocytes (10 ml packed cell volume) were incubated for 10 minutes at 37°. The reaction mixture contained 2 m Ci of [^{3}H] tyrosine (2421 μ Ci/μ mole). The incubation conditions, preparation of peptidyl-tRNA, trypsin digestion and analysis of labeled tryptic peptides are described in detail in Methods. For each of the analyses 47,400 DPM of [^{14}C] tyrosine labeled α-globin and 50,100 DPM of [^{14}C] tyrosine labeled β-globin were added as a uniformly labeled internal standard.

TABLE IV Analysis of ^{3}H Tryosine Labeled Tryptic Peptides from Purified Peptidyl tRNA

	Globin Peptides					β Globin Peptides					
	tryptic peptide			N-terminal intercept+	α globyl-tRNA**	tryptic peptide			N-terminal intercept+	β globyl-tRNA**	α globyl tRNA / β globyl tRNA
Experiment	αT4	αT6	αT15			βT4	βT14	βT16		%	
I [^{3}H]/[^{14}C]	94.4	71.7	5.38	118	4.56	89.1	14.7	0.83	122	0.72	6.1
I [^{3}H]*	1490	1130	84.9	-	-	1410	232	13.9	-	-	-
II [^{3}H]/[^{14}C]	127	97.8	6.93	160	4.33	112	22.6	1.04	154	0.63	6.7
II [^{3}H]*	2010	1530	109	-	-	1770	357	16.6	-	-	-
III [^{3}H]/[^{14}C]	86.4	64.6	5.31	107	4.96	86.3	15.6	0.82	118	0.69	6.5
III [^{3}H]*	1360	1020	83.9	-	-	1360	246	12.9	-	-	-
Average					4.62					0.70	6.4

* Tritium content of the tryptic peptides is expressed as total DPM x 10^{-3} by equating [^{3}H] recoveries to observed [^{14}C] recoveries.

\+ The [^{3}H]/[^{14}C] intercepts are calculated by the mothod of least squares as described in Methods.

** $\frac{\text{Observed } [^{3}H]/[^{14}C] \text{ (of } \alpha T15 \text{ of } \beta T16 \text{ resp.)} \times 100}{\text{Calculated } [^{3}H]/[^{14}C] \text{ intercept (of } \alpha \text{ peptides or } \beta \text{ peptides resp.)}}$

incubation mixture was conducted in the usual manner (see legend of Table IV) while to the other was added hemin to a concentration of 1 x 10^{-4} M. Analysis of the six tryosine containing peptides from each preparation, conducted as before revealed that hemin addition to the incubation medium did not alter the proportion of nascent α or β globin chains present as α globyl-tRNA or β globyl-tRNA, respectively, in the purified peptidyl-tRNA fraction.

Pactamycin Induced Decay of Radioactivity in the Nascent Globin Chains

The antibiotic pactamycin, at a concentration of 10^{-6} M, has been shown to inhibit preferentially the initiation of protein synthesis in the reticulocyte (Stewart Blair et al., 1971; Kappen et al., 1973). Since elongation and release of nascent globin chains are not inhibited, preparations of purified peptidyl-tRNA obtained from reticulocytes that have been exposed to pactamycin should show a progressive decrease of radioactivity in the peptidyl-tRNA fraction with an increased time of incubation.

The effects of pactamycin addition to rabbit reticulocytes whose ribosomes were in a steady state of labeling (10 minutes at 37°) is shown in Figure 8. The specific activity of hemoglobin in the soluble phase of the reticulocyte was found to increase very little following pactamycin addition to the incubation medium, hence the amount of contamination of the ribosomal pellets with [^{3}H] labeled hemoglobin should be similar in each of the preparations

Figure 8. Effect of pactamycin addition to reticulocytes labeled in the steady state. Rabbit reticulocytes (10 ml packed cell volume) were incubated as described in Methods. At zero time 5 m Ci of [^{3}H] tyrosine (6053 Ci per mole) were added. After 10 minutes of incubation the first aliquot (10 ml) was withdrawn to serve as a control prior to pactamycin addition. After an additional 15 seconds, as indicated by the arrow, the incubation mixture was made 1 x 10^{-6} M in pactamycin by the addition of 0.6 ml of 0.5 x 10^{-4} M pactamycin in saline (RS). Three further 10 ml aliquots were withdrawn at the time intervals indicated in the figure. Peptidyl-tRNA was prepared from each aliquot and the total radioactivity content was determined as described in Methods. Hemoglobin specific activities were measured in the post ribosomal supernatant fractions.

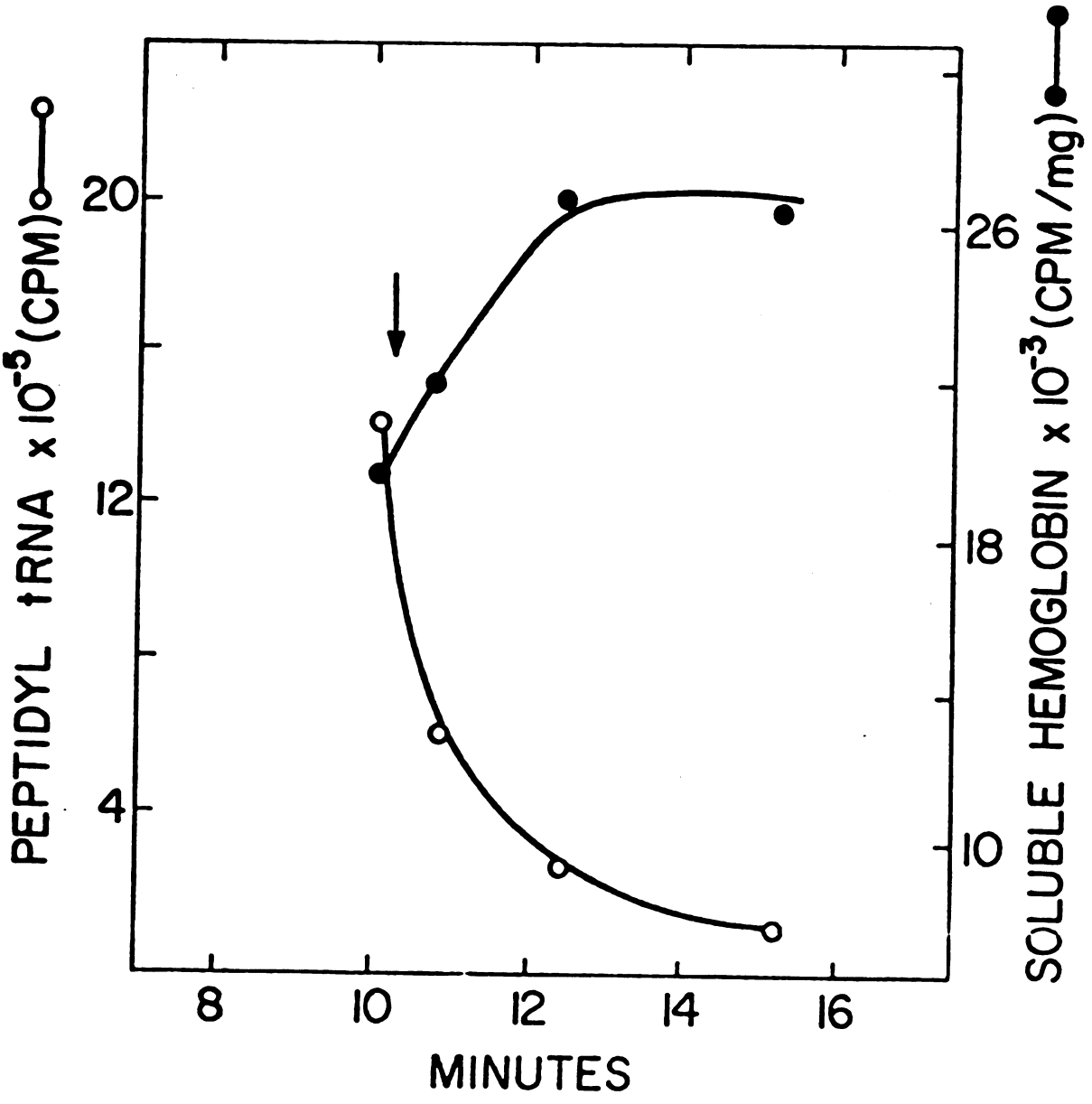
PEPTIDYL tRNA x 10⁻⁵(CPM)
SOLUBLE HEMOGLOBIN x 10⁻³(CPM/mg)
20
12
4
26
18
10
8
10
12
14
16
MINUTES

Figure 8

obtained at the respective time periods after pactamycin addition. However, the radioactivity found in the purified peptidyl-tRNA fraction prepared from samples withdrawn following incubation in the presence of pactamycin decrease rapidly with time. After 5 minutes of incubation in the presence of pactamycin only 8.9% of the original radioactivity remained associated with peptidyl-tRNA.

The radioactivity content of each of the six tyrosine containing tryptic peptides in each of the four samples was analyzed. These data presented in Figure 9. The radioactivity present in all tyrosine containing peptides (including α T15 and β T16) declines precipitously after pactamycin addition. Peptides α T4 and β T4, closest to the N-terminal portion of the respective globin chains, decline most rapidly following the addition of pactamycin. These results are consistent with completion and release of nascent peptides without initiation of new peptide chains.

Figure 10 presents the relative radioactivity content of tryptic peptides near the N-terminal portion (α T4, β T4) of the nascent peptides as compared to the radioactivity present in the C-terminal tryptic peptides (α T15, β T16). The proportions of α globyl-tRNA and β globyl-tRNA in the nascent protein fractions were markedly increased with time after pactamycin addition. Since α T15 is derived only from globyl-tRNA while α T4 is derived from both globyl-tRNA and the other tyrosine containing α globyl nascent peptides as well, one can determine that by 5

Figure 9. Pactamycin induced decay of radioactivity in the 6 tyrosine-containing tryptic peptides of rabbit globin. The tryptic peptides were prepared from each of the four peptidyl-tRNA preparations shown in Figure 8 (See Methods for preparation and analysis of labeled tryptic peptides). To each of the peptidyl-tRNA preparations was added [^{14}C] tyrosine labeled α globin (33,800 DPM) and [^{14}C]-tyrosine labeled β globin (34,600 DPM) as a uniformly labeled internal standard prior to tryptic digestion. Figures 9A through 9F present the tritium radioactivity found associated with each of the six tyrosine containing tryptic peptides as a function of time following pactamycin addition. Values are expressed as per cent of those found in the control sample removed prior to pactamycin addition.

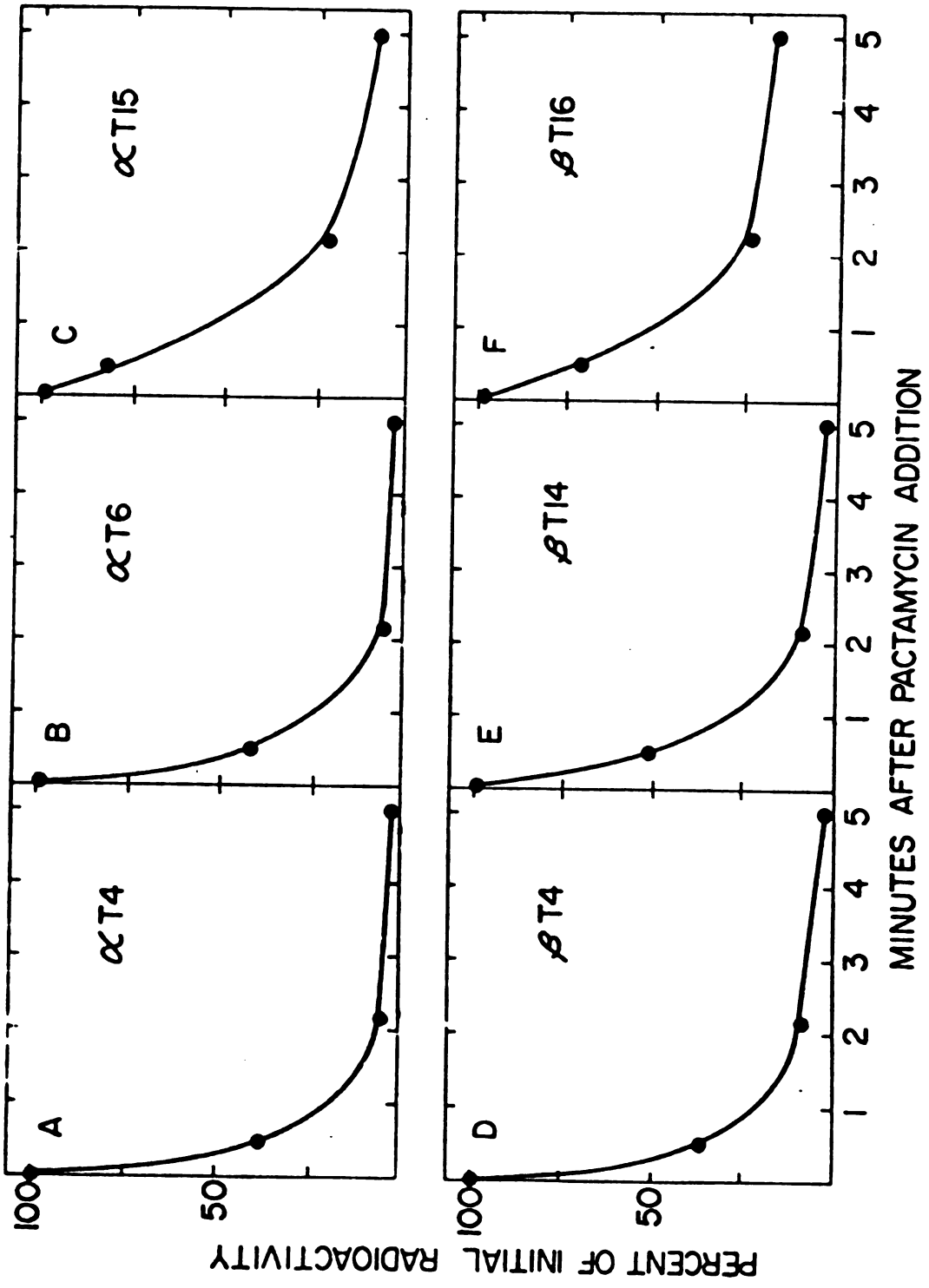
A
αT4
B
αT6
C
αT15
D
βT4
E
βT14
F
βT16
100
50
PERCENT OF INITIAL RADIOACTIVITY
1
2
3
4
5
MINUTES AFTER PACTAMYCIN ADDITION

Figure 10. Ratios of radioactivity found in tryptic peptides from the N-terminal and C-terminal portions of the nascent protein fraction following pactamycin addition. Each ratio of radioactivity was calculated from the total tritium content (DPM) of the tryptic peptides indicated. The four peptidyl-tRNA samples analyzed are those described in Figure 8.

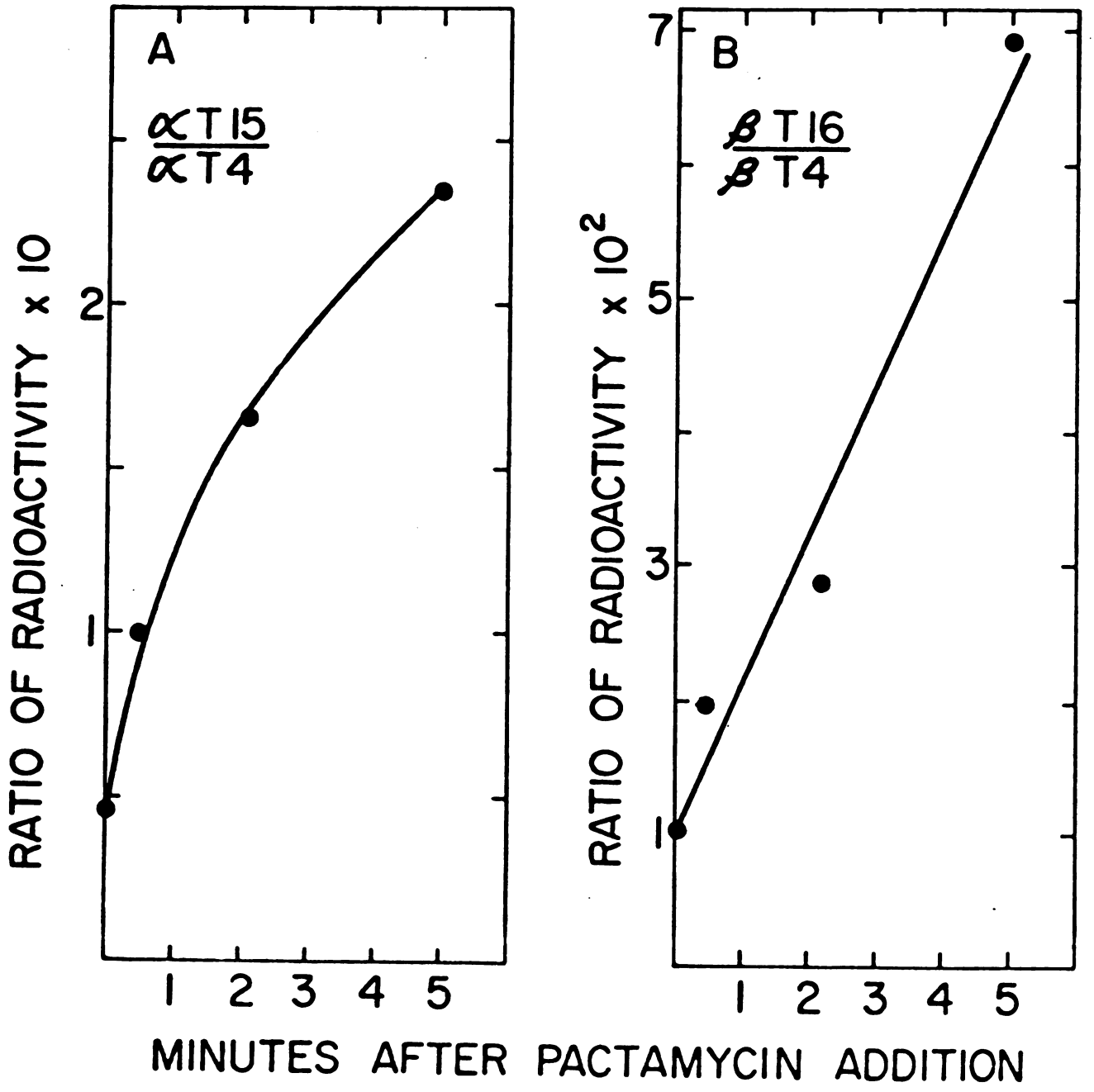
A
αT15/αT4
RATIO OF RADIOACTIVITY x 10
2
1
1 2 3 4 5
B
βT16/βT4
RATIO OF RADIOACTIVITY x 10²
7
5
3
1
1 2 3 4 5
MINUTES AFTER PACTAMYCIN ADDITION

Figure 10

minutes after pactamycin addition approximately 24 percent of the nascent α globin peptides are α globyl-tRNA. A similar calculation indicates that β globyl-tRNA reaches approximately 7 percent of the nascent β globin peptides by 5 minutes after pactamycin addition. The total radioactivity content of all components decreased rapidly following pactamycin addition, however, collectively these data are consistent with the concept that the globyl-tRNA molecules are normal intermediates of soluble hemoglobin biosynthesis.

3. Accumulation of Growing Globin Chains on the Polyribosome

The experiments that have just been described gave evidence for the presence of at least one species of peptidyl-tRNA (i.e. α-globyl-tRNA) in an amount larger than would have been expected if the size distribution of nascent peptides were uniform. To explore the possibility that peptides shorter than α-globin might also accumulate during their assembly a series of column chromatographic experiments was undertaken. A gel filtration procedure that would separate peptides according to size was developed. This procedure was then used to separate the nascent peptides of rabbit globin obtained from preparations of radioactively labeled peptidyl-tRNA.

a. Calibration of the Bio-Gel A0.5M Gel Filtration Column Peptide Markers

The set of nascent peptides of globin range in length from 2 amino acid residues to 146 amino acid residues. This is deduced from the mechanism of protein biosynthesis (Dintzis, 1961) and from the amino acid sequence of hemoglobin (Dayhoff and Eck, 1968). Since nascent globin chains were going to be analyzed the calibration of the gel filtration column was undertaken using peptides derived from globin itself. To obtain markers in the molecular weight range of 3400 to 12000 advantage was taken of the presence of only one methionine residue per globin chain. The α globin chain has methionine at position 32 and the β chain has methionine at position 55 (Dayhoff and Eck, 1968). Cyanogen Bromide cleaveage (see Methods) of the α chain of globin yields 2 peptides with molecular weights of 3413 and 11996 respectively. The β chain of globin gives CNBr fragments of molecular weights of 5980 and 10000 respectively. To obtain a set of short peptide markers advantage was taken of the presence of tyrosine in tryptic peptides β T16, β T4 and β T14, which are 2, 10 and 12 amino acid residues long, respectively (Dayhoff and Eck, 1968). Tryptic peptides were prepared from the β chain of rabbit globin as described in Methods.

Calibration of the Column

The completed β chain and its 3 tryptic peptides plus

the four CNBr peptides obtained from globin were pretreated for Bio-Gel A-0.5M gel filtration as described in Methods. Blue Dextran and DNP-alanine were added to define the void volume and internal volume respectively. These peptides were then analyzed by Bio-Gel A-0.5M gel filtration chromatography as described in Methods. The results of this experiment appear in figure 11. A set of 7 major radioactive peaks is apparent. A wide peak is centered around fraction 108. The leading and trailing portions of this peak contain two different radioactive components, as shown in figure 12 by means of a double label experiment. Column fractions containing radioactive components D through J in figure were pooled as indicated by the horizontal bars.

Identification of the Column Markers

The longer peptides (D-G) were identified by their amino acid composition. The shorter peptides (H-J) were identified from the known electrophoretic mobilities of the tyrosine containing peptides, as described in Methods.

Analysis of Peptides D, E, F and G

Peptides D-G were hydrolyzed in vacuo with 6N HCL at 110° for 22 hours. Table V shows the results of automated amino acid analysis of the hydrolysates. Peptide G matches very closely the amino acid composition of the 32 amino acid long CNBr fragment of the β chain. Particularly noticeable is the absence of phenylalanine and the low content of leucine. The presence of homoserine, product

Figure 11. Bio-Gel A-0.5M agarose gel filtration analysis of peptides for column calibration. Rabbit globin (25 mg) uniformly labeled with [^{14}C] tyrosine was reacted with a 390 fold molar excess of CNBr for 48 hours as described in Methods to obtain markers D, E, F and G. Tritium labeled small peptides were obtained by tryptic digestion of the β chain of rabbit globin as described in Methods. Purified β chain of rabbit globin uniformly labeled with [^{3}H] tyrosine was added to obtain peak C. Twenty six drops per fractions were collected. Odd numbered fractions were assayed for radioactivity. Aliquots (150 µl) were diluted with 0.5 ml of water and counted with 5 ml of Aquasol in a Nuclear Chicago Liquid Scintillation counter. Details of the gel filtration analysis are described in Methods. Fractions were pooled as indicated by the horizontal bars. Peaks A and K indicate the exclusion volume and the internal volume respectively.

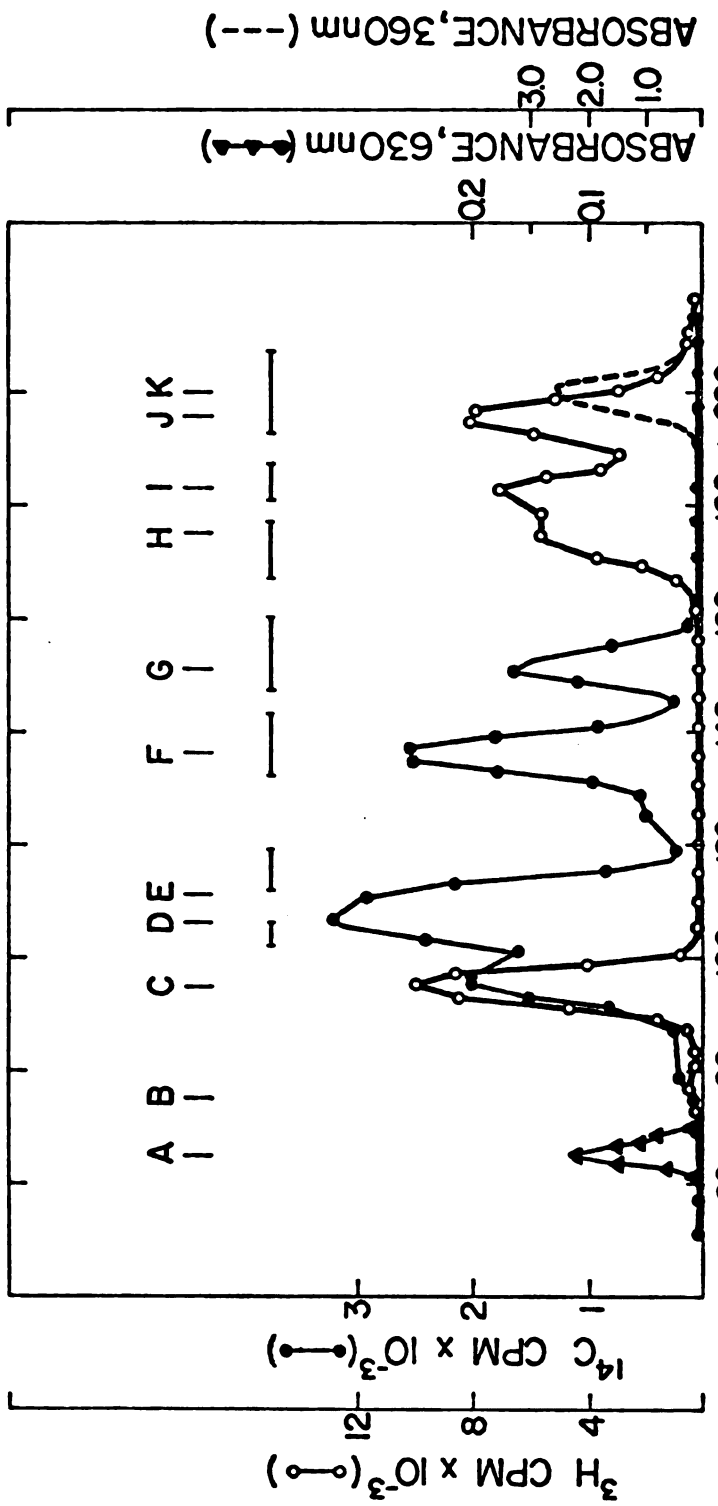
ABSORBANCE, 360nm (---)
ABSORBANCE, 630nm (•—•)
^{3}H CPM x 10^{-3} (o—o)
^{14}C CPM x 10^{-3} (•—•)
FRACTION NUMBER
A B C DE F G H I JK

Figure 11

Table V. Amino Acid Analysis of Peptides D, E, F and G from Figure 11

Amino Acid	Peptide		Peptide		Peptide		Peptide	
	G	αCB-1	F	βCB-1	E	βCB-2	D	αCB-2
Aspartic	2.20	2	3.41	3	9.36	9	9.03	9
Threonine	2.08	2	2.12	2	4.13	2	7.49	9
Serine	2.16	2	5.09	6	5.56	4	7.29	9
Glutamic	4.02	4	5.25	7	8.04	7	5.91	4
Proline	0.92	1	-	1	4.30	3	-	6
Glycine	4*	4	3.66	5	8.14	6	6.23	5
Alanine	3.90	4	5*	5	10.68	10	9.0*	9
Valine	1.54	2	4.93	8	9.40	10	7.91	9
Cystine	-	-	-	-	-	-	-	-
Methionine	-	-	-	-	-	-	-	-
Isoleucine	1.68	2	0	0	1*	1	1.01	1
Leucine	1.59	1	5.82	6	13.28	12	14.36	16
Tyrosine	0.84	1	1.05	1	1.92	2	1.88	2
Phenyalanine	0.0	0	2.97	3	5.69	5	6.58	8
Lysine	4.28	4	2.82	3	9.07	10	8.01	9
Histidine	0.98	1	1.53	1	7.54	8	8.54	9
Arginine	1.13	1	1.86	2	1.38	1	2.32	2

*By definition, for normalization of data.

of the CNBr reaction is suggested by the apparent presence of 4 lysine residues contrasted to the theoretical value of 3 lysines. Under the conditions of amino acid separation, lysine is not separated from homoserine. Peptide F matches the composition of the 55 amino acid long CNBr fragment from the β chain. Particularly noticeable is the absence of isoleucine. Peptides D and E are incompletely separated from each other, therefore the amino acid composition of the corresponding peptide samples should show only a tendency to reflect the composition of the pure peptide. This is illustrated in the case of serine and threonine and to a lesser degree in the case of glutamic acid and leucine. This conclusion is further substantiated by Figure 12 where peak E is obtained from a [^{14}C]-labeled β chain while peak D is obtained from a tritium labeled α chain.

Significance of Peak X in Figure 11

Figure shows the results of agarose gel filtration of a reaction in which rabbit globin labeled uniformly with [^{3}H] tyrosine was incubated for 18 hours with a 90-fold molar excess of CNBr. Four major radioactive peaks are observed between fractions 105 and 200. Figure 13 shows the results of agarose gel filtration of a reaction in which ^{3}rabbit globin labeled with ^{3}H-tyrosine was incubated for 18 hours with a 90-fold molar excess of CNBr. Four major radioactive peaks are observed between fractions 105 and 200. Figure 14 shows a similar experiment in which

Figure 12. Bio-Gel A-0.5M agarose gel filtration analysis of peptides for column calibration in the presence of peptidyl-tRNA. Separated α chain (9.8 mg) and β chain (7.8 mg) from rabbit globin uniformly labeled with [^{3}H]-tyrosine and [^{14}C]-tyrosine respectively were incubated for 72 hours with a 500 molar excess of CNBr as described in Methods. Short peptides labeled with [^{14}C] were obtained from the appropriately labeled β-chain of rabbit globin as described in Methods. Non-radioactive peptidyl-tRNA was prepared from one rabbit, as described in Methods. The peptidyl-tRNA ester bond was cleaved with base as described in Methods. This preparation was then combined with the radioactive peptides. The mixture was than analyzed by Bio-Gel A-0.5M agarose gel filtration. Odd numbered fractions were assayed for radioactivity. Twenty one drops per fraction were collected. Peaks A and K correspond to those in Figure 11.

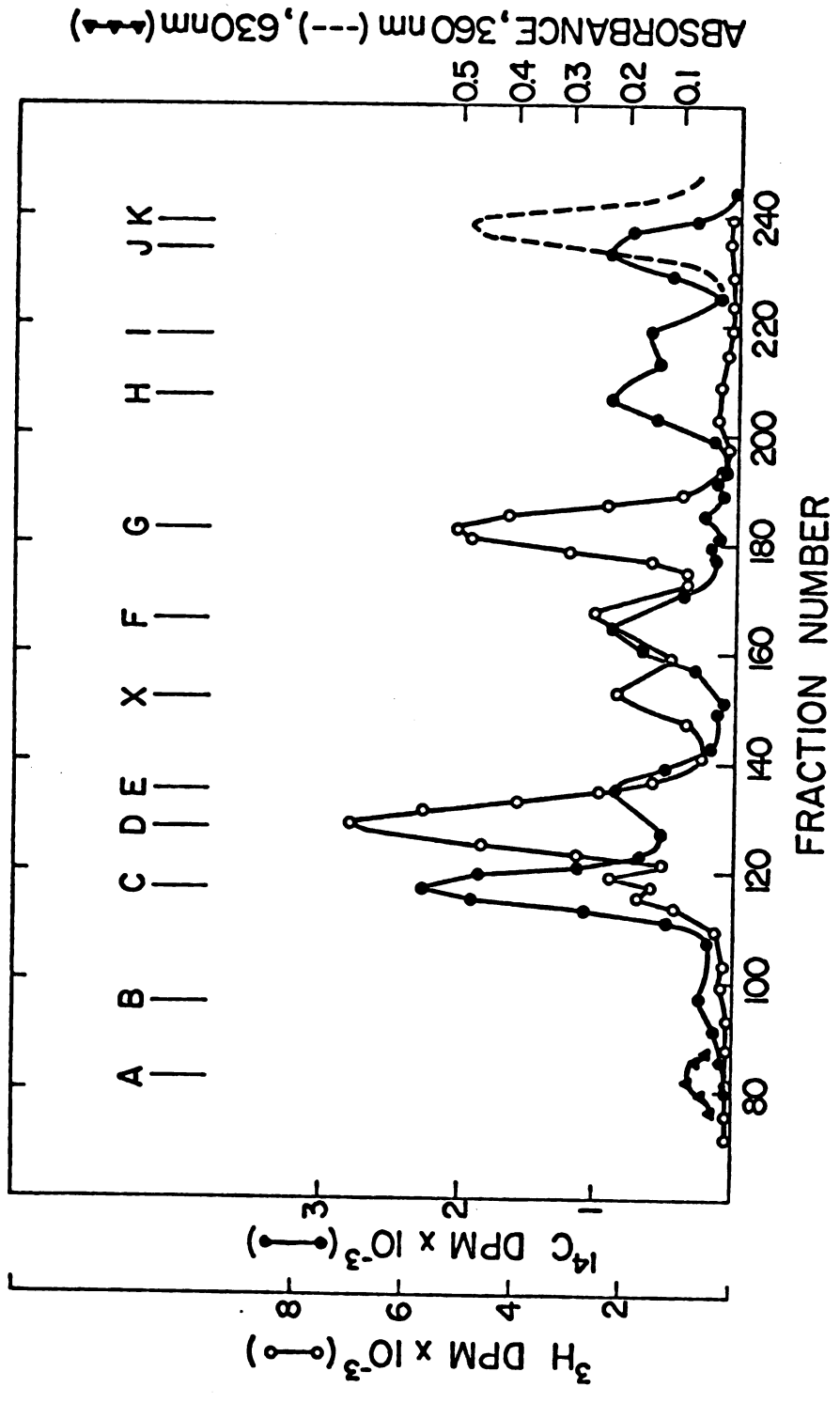
ABSORBANCE, 360nm (---), 630nm (▲—▲)
0.5
0.4
0.3
0.2
0.1
A
B
C
D
E
X
F
G
H
I
J
K
80
100
120
140
160
180
200
220
240
FRACTION NUMBER
14C DPM x 10-3 (●—●)
3
2
1
3H DPM x 10-3 (○—○)
8
6
4
2

Figure 12

Figure 13. Bio-Gel A-0.5M gel filtration analysis of the peptides obtained from rabbit globin by cleaveage with cyanogen bromide. Rabbit globin, 15 mg uniformly labeled with [^{3}H] tyrosine was reacted for 18 hours with a 90 fold molar excess of CNBr as described in Methods. The preparation of radioactive globin, and the details of the Bio-Gel A-0.5M gel filtration analysis are given in Methods. The small radioactive peak at fraction 244 is due to [^{3}H] lysine to indicate the inclusion volume of the column. Twenty five drop fractions were collected. The profile of radioactivity was determined by counting the whole fraction plus 0.5 ml of water with 5 ml of Bray's solution, see Methods.

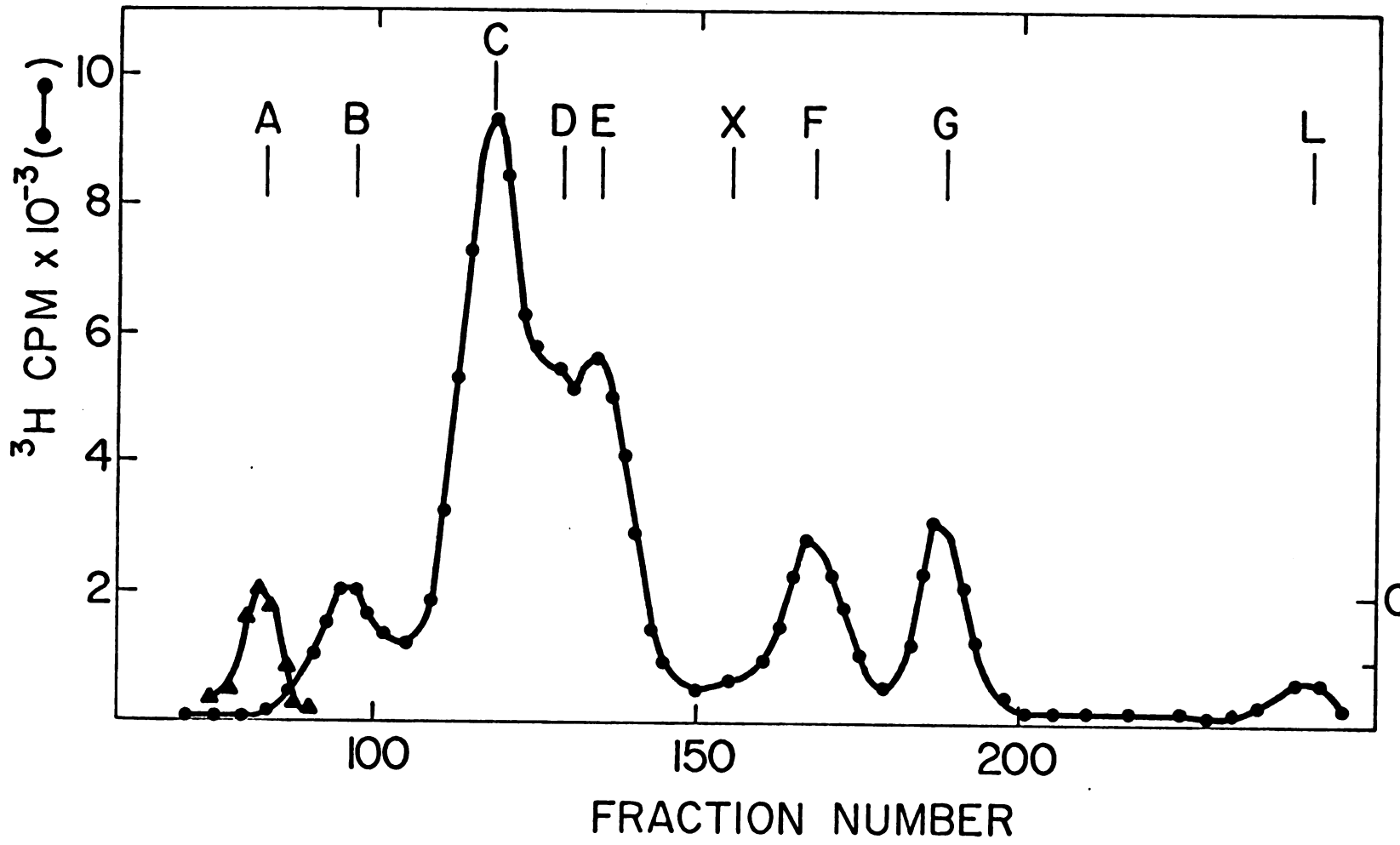
C
A
B
D
E
X
F
G
L
10
8
6
4
2
^{3}H CPM x 10^{-3}(●—●)
0.1
ABSORBANCE 630nm (●—●)
100
150
200
FRACTION NUMBER

Figure 13

Figure 14. Bio-Gel A-0.5M gel filtration analysis of the peptides obtained from rabbit globin by cleaveage with cyanogen bromide. Purified rabbit α chain (15 mg) and β chain (17 mg) uniformly labeled with [^{3}H] tyrosine were incubated for 48 hours with a 390 fold molar excess of CNBr as described in Methods. Twenty one drops were collected and odd numbered fractions were assayed for radioactivity. The preparation of radioactive α and β globin and the details of the Bio-Gel A-0.5 filtration analysis are described in Methods.

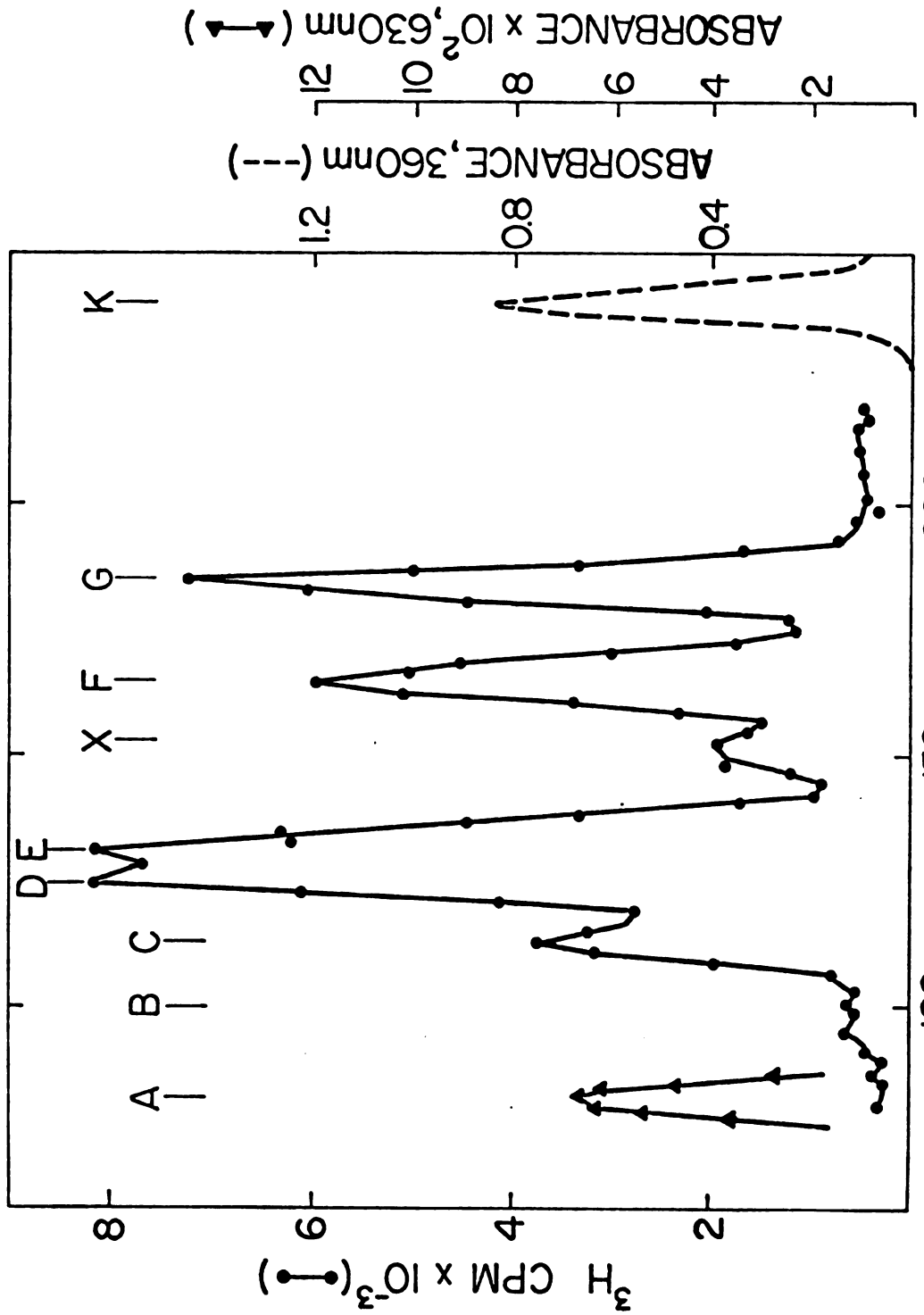
ABSORBANCE x 10^2,630nm (▲—▲)
ABSORBANCE,360nm (---)
^{3}H CPM x 10^{-3}(●—●)
FRACTION NUMBER
A
B
C
D
E
X
F
G
K

Figure 14

rabbit globin labeled with [^{3}H] tyrosine was reacted with a 390 fold molar excess of CNBr for 48 hours. A similar pattern is observed but now a small peak, marked X, has appeared. This same small peak is observed in figure 11 in which markers D, E, F, and G were obtained under the same conditions as those used in figure 14. Figure 12 shows a similar experiment in which markers D-G were obtained by reacting a mixture of α chains labeled with [^{3}H] tyrosine and β chains labeled with [^{14}C] tyrosine with a 500 fold molar excess of CNBr for 72 hours. This time peak X is very prominent relative to peak G, which carries the same isotope. These observations suggest that peak X is a side product which appears under drastic conditions. Peak B was not characterized.

Analysis of Peptides H, I and J

Peptides H, I and J were characterized by high voltage paper electrophoresis at pH 4.7. Tryptic peptides β T4, β T14 and β T16 are completed separated under these conditions as described in Methods. Peptides H and I were applied onto Whatmann 3MM chromatographic paper to migrate in separate lanes during high voltage electrophoresis. The lane containing the tryptic digest from globin was stained for identification of tyrosine containing peptides. The other two lanes were analyzed for radioactivity as described in Methods. Figure 15 shows the results of this analysis. Sample H shows two peaks of radioactivity in figure 15A.

Figure 15. Identification of Pooled Samples H and I from Figure 11.

A) High voltage electrophoresis of desalted sample H. Electrophoresis was done at pH 4.7 as described in Methods. The sample lane was cut into 1 cm strips and counted in toluene Liquifluor mixture.

B) High voltage electrophoresis of desalted sample I. Electrophoresis and counting of radioactivity same as in panel A.

C) High voltage electrophoresis of a tryptic digest of nonradioactive rabbit globin analyzed in a parallel lane as the samples shown in panels A and B. The spots indicated correspond to tyrosine containing peptides, visualized as described in Methods. From left to right, the tyrosine containing spots correspond to trypic peptides β T14, α T4, unresolved α T6 plus β T4, α T15 and β T16, respectively.

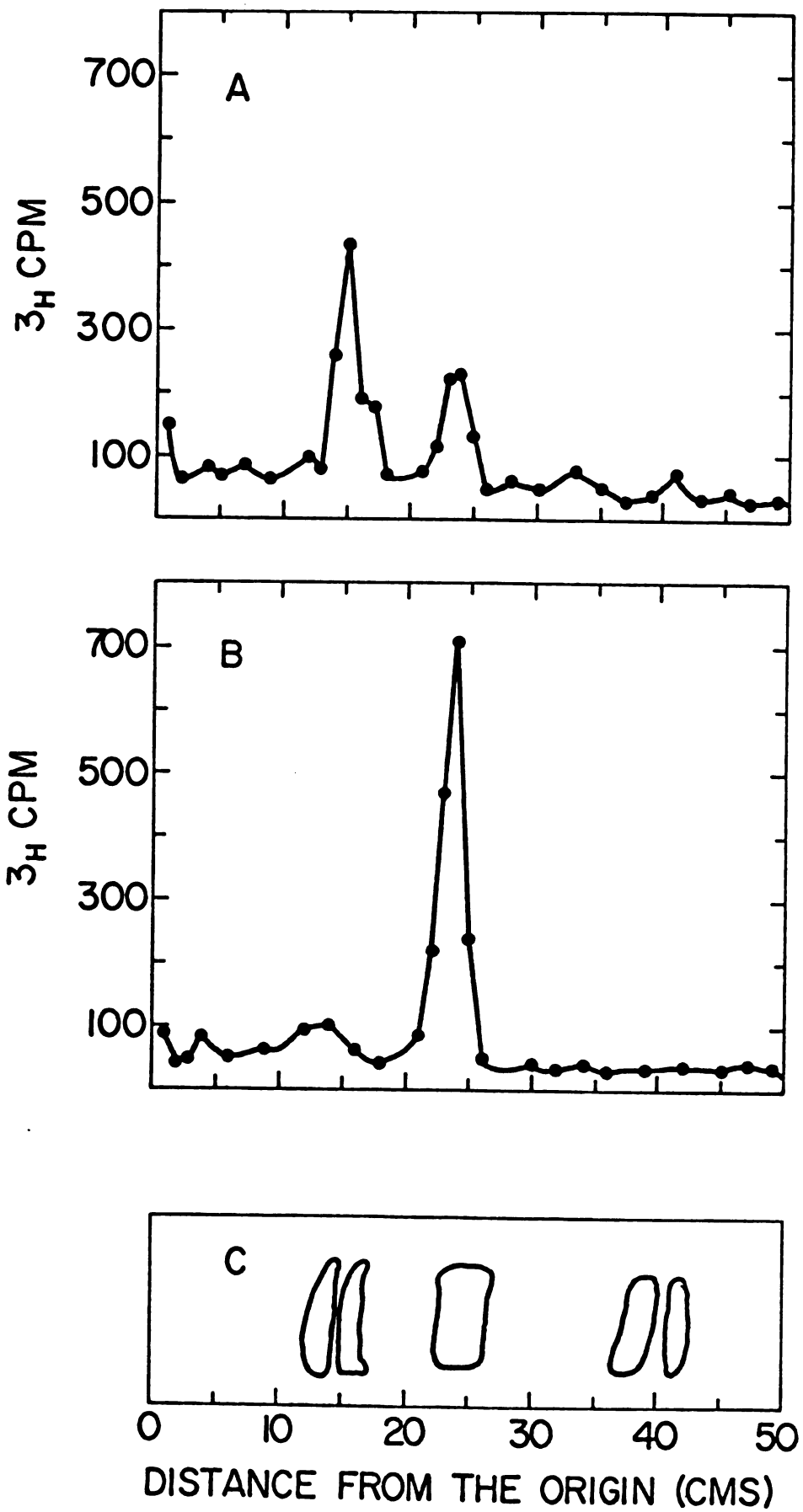
A
700
500
300
100
^{3}H CPM
B
700
500
300
100
^{3}H CPM
C
0
10
20
30
40
50
DISTANCE FROM THE ORIGIN (CMS)

Figure 15

A large peak at the position of β T14 and a smaller peak at the position of β T4 is observed. Sample I, figure 15B shows a single large peak in the position of β T4. The conclusion is that peptide H is β T14 and peptide I is β T4. A similar electrophoretogram shows that peptide J is β T16, figure 16.

The Calibration Curve

Using agarose gel filtration in 6M guanidine HCl (Fish et al., 1969) obtained useful molecular weight estimates between the extreme limits of 76000 and 1540. These authors obtained a straight line by plotting the cube root of the distribution coefficient ($K_d^{1/3}$) of polypeptide chain markers against their respective molecular weight raised to the 0.555 power. Figure 17 shows the result of a similar plot obtained from the observed distribution coefficient (K_d) and the known molecular weights of the peptides labeled C through J in figure 11. A similar plot obtained from the markers in figure 12 is shown in figure 18. Table VI shows the observed distribution coefficients and calculated molecular weights used to obtain figures 17 and 18.

b. Nonuniformity in Size Distribution in the Population of Nascent Globin Chains

Nascent Chains Labeled with Tyrosine

The mechanism of protein biosynthesis requires that a preparation of peptidyl-tRNA contain a population of nascent globin chains of varying lengths. In order to display the

Figure 16. Identification of Pooled Sample J from Figure 11

A) High voltage electrophoresis of desalted sample J. Electrophoresis and counting of radioactivity the same as in figure 15.

B) Tryptic digest of whole globin, analyzed in a parallel lane with sample J. Tyrosine containing peptides were stained as described in Methods. From left to right, the tyrosine containing spots correspond to tryptic peptides β T14, α T4, unresolved α T6 plus α T4 and unresolved α T15 plus β T16, respectively.

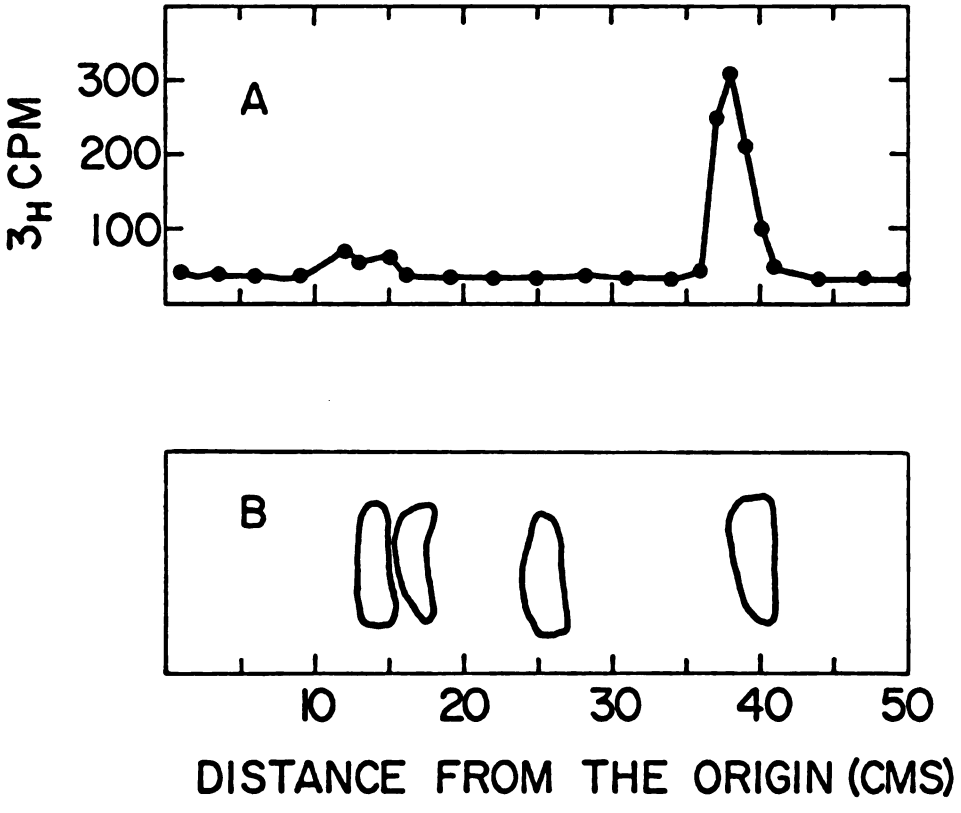
A
B
300
200
100
^{3}H CPM
10
20
30
40
50
DISTANCE FROM THE ORIGIN (CMS)

Figure 16

Figure 17. Calibration of the Bio-Gel A-0.5M column or gel filtration in the absence of peptidyl-tRNA. Plot of the experimentally determined distribution coefficient (K) according to the method of Tanford-Porath. $K^{1/3}$ is plotted as a function of the molecular weight to the 0.555 power. β T16, β T4 and β T14 (J, I and H of Figures 11 and 12) are tryptic peptides of rabbit globin defined in the Methods section. α CB-1 and α CB-2 (G and D respectively of Figure 11 and 12) are the two peptides obtained by CNBr cleaveage of the β chain of rabbit globin. β CB-1 and β CB-2 (F and E of Figures 11 and 12) are the corresponding peptides obtained from the β-chain of rabbit globin. The β-globin chain (C of Figures 11 and 12) was also used for calibration of the column. The numbers in parenthesis indicate the number of amino acid residues present in each peptide. The experimental values of the distribution coefficients were obtained from the marker peptides shown in Figure 11.

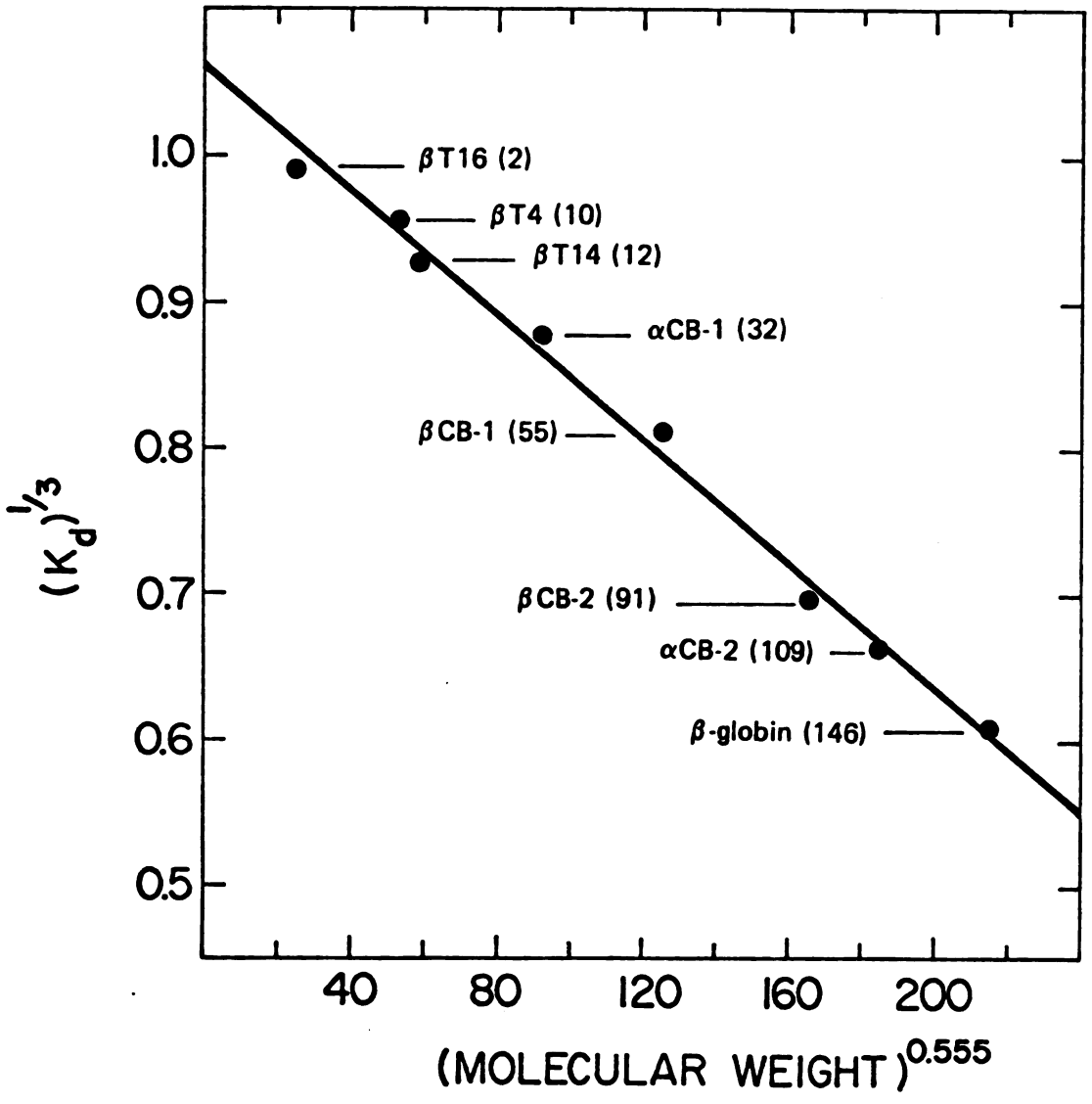
βT16 (2)
βT4 (10)
βT14 (12)
αCB-1 (32)
βCB-1 (55)
βCB-2 (91)
αCB-2 (109)
β-globin (146)
1.0
0.9
0.8
0.7
0.6
0.5
40
80
120
160
200
$(K_d)^{1/3}$
(MOLECULAR WEIGHT)$^{0.555}$

Figure 17

Figure 18. Calibration of Bio-Gel A-0.5M column for gel filtration in the presence of peptidyl-tRNA. The labels on the peptides and the coordinate axes are identical to those of figure 17. The experimental values for the distribution coefficients were obtained from the marker peptides shown in figure 12.

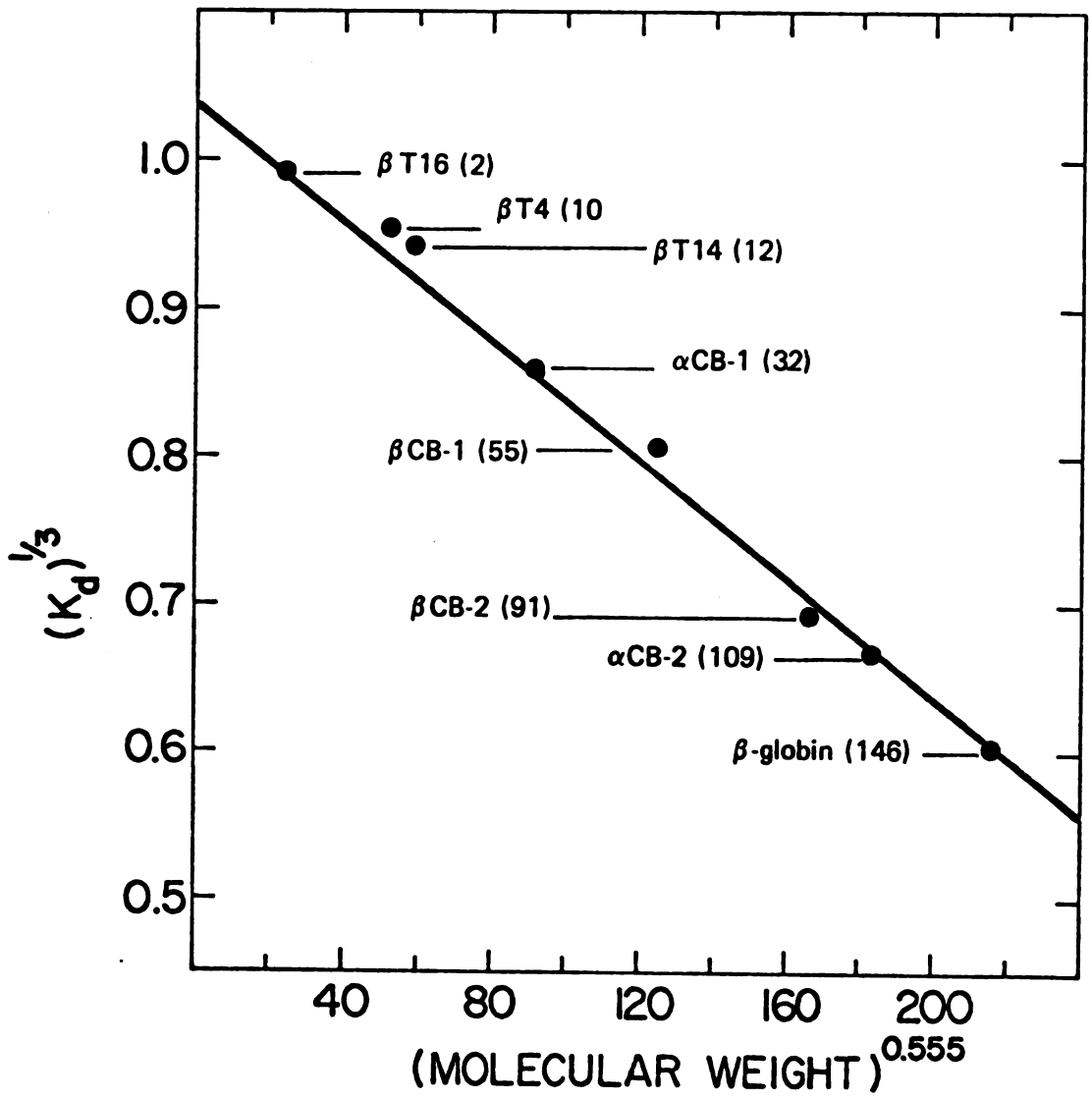
βT16 (2)
βT4 (10
βT14 (12)
αCB-1 (32)
βCB-1 (55)
βCB-2 (91)
αCB-2 (109)
β-globin (146)
1.0
0.9
0.8
0.7
0.6
0.5
40
80
120
160
200
(MOLECULAR WEIGHT)^0.555
(K_d)^1/3

Figure 18

Table VI. Distribution Coefficients (K_d) of the Marker Peptides Measured in Figures 11 and 12

Peptide*	Molecular Weight	K_d		$(K_d)^{1/3}$		(Molecular Weight)$^{.555}$
		Fig. 11	Fig. 12	Fig. 11	Fig. 12	
(C) β chain (146)	16000	0.2205	0.2243	0.6041	0.6075	215.44
(D) α CB-2 (109)	11996	0.3014	0.2949	0.6705	0.6656	183.60
(E) β CB-2 (91)	10000	0.3308	0.3397	0.6916	0.6977	166.03
(F) β CB-1 (55)	5980	0.5220	0.5385	0.8051	0.8135	124.76
(G) α CB-1 (32)	3413	0.6323	0.6795	0.8583	0.8792	91.38
(H) β T14 (12)	1526	0.8161	0.8013	0.9435	0.9288	58.46
(I) β T4 (10)	1274	0.8676	0.8719	0.9537	0.9553	52.88
(J) β T16 (2)	316	0.9705	0.9743	0.9901	0.9914	24.40

*The letter inside the parenthesis indicates the position of the peptide in figures 11 and 12. The parenthesis at the right indicates the number of amino acid residues present in the chain.

components of such a population of globin chains according to size, peptidyl-tRNA labeled with [^{3}H] tyrosine was prepared as described in Methods. The nascent globin chains were released from the tRNA by treatment with 0.1 N NaOH and then subjected to agarose gel filtration. Figure 19 presents the results of such an experiment. The eluted radioactivity was plotted as a function of the distribution coefficient corresponding to each eluted fraction. A pattern of peaks is visible at K_d values of 0.28, 0.36, and 0.44. Troughs are visible at K_d values of 0.32 and 0.4. To assess the significance of the experimental curve a theoretical elution curve for a population of nascent peptides uniformly distributed in size was plotted, figure 20. The construction of this curve has been discussed in the Methods section. Points of insertion of tyrosine during chain growth are indicated by arrows. Comparison of figures 19 and 20 shows that the overall range of K_d values displayed by both experimental and theoretical curves is very similar, as expected. These values range from approximately 0.2 to about 0.7. The theoretical curve has a peak at K_d 0.25, corresponding to the peak at K_d of 0.28 of the experimental curve. No troughs or peaks are observed in the theoretical curve. These results suggest that some members of the population of nascent chains displayed in figure 19 are either decreased or increased in amount relative to the other members of the population. Figure 21 shows a repeat analysis of the same sample shown in figure 19. The same

Figure 19. Bio-Gel A-0.5M gel filtration analysis of the [^{3}H] tyrosine-labeled nascent peptides of rabbit globin. Rabbit reticulocytes (10 ml packed cell volume) were incubated as described in Methods. The incubation conditions were identical as those described for labeling with [^{3}H] tyrosine, except that final concentration of leucine in the medium was 1.0mM. At zero time 2 m Ci of [^{3}H] tyrosine (2420 μCi per μ mole) was added. After 10 minutes of incubation incorporation of radioactivity was stopped as indicated in Methods and peptidyl-tRNA was then prepared. An aliquot of [^{3}H] tyrosine labeled peptidyl-tRNA was then analyzed by Bio-Gel A-0.5M gel filtration as described in Methods. The effluent from column chromatography was collected directly into scintillation vials fastened to an ISCO rotary fraction collector. The rotary fraction collector was actuated by a Gilson drop counter. Ten drop (approximately 0.3 ml) fractions were collected. Radioactivity was assayed by adding 0.2 ml of water to each scintillation vial and counting in 5 ml of Aquasol. Counting was done in a Packard liquid scintillation spectrometer model 3310. Counting efficiencies were determined by the channels ratio method. All data are presented as decompositions per

minute (DPM) as determined from the observed cpm and the counting efficiency. To normalize the data from different gel filtration analyses the fraction number has been expressed as the distribution coefficient (K_d) as described in Methods. By definition, K_d for blue dextran is 0 and K_d for DNP-alanine is 1.0.

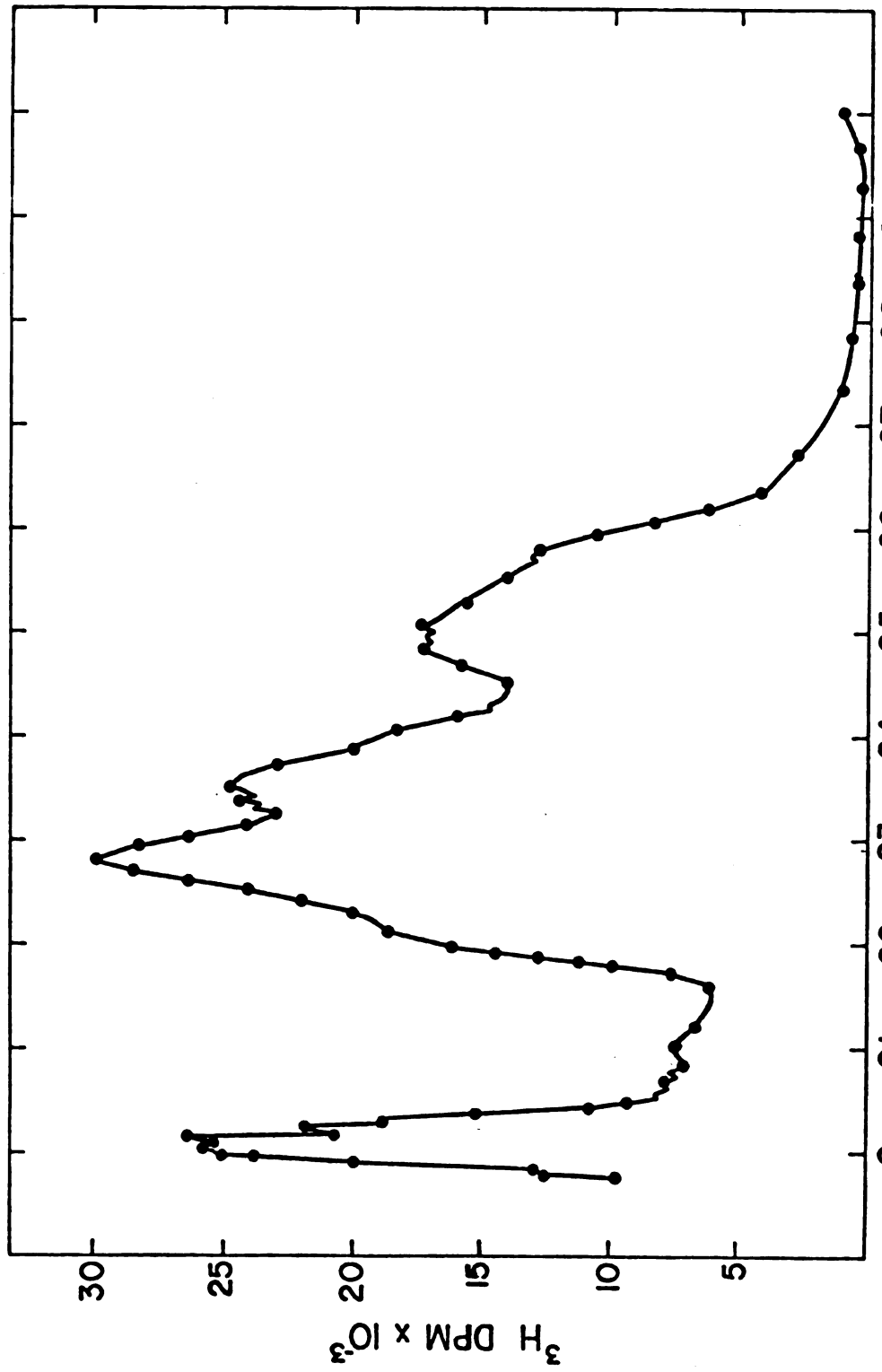

Figure 19

Figure 20. Theoretical elution pattern for a population of [^{3}H] tyrosine-labeled nascent peptides from globin analyzed by Bio-Gel A-0.5 gel filtration chromatography. A population of nascent globin chains with a uniform distribution in size has been assumed. It has also been assumed that there are equal numbers of nascent α-chains and nascent β-chains. See Methods for further assumptions. The theoretical curve has been plotted as a function of K_d to facilitate comparison with the experimental curves Figures 19 and 21. See Methods for explanation of arbitrary units. The arrows indicate the position of insertion of tyrosine residues along the nascent chains.

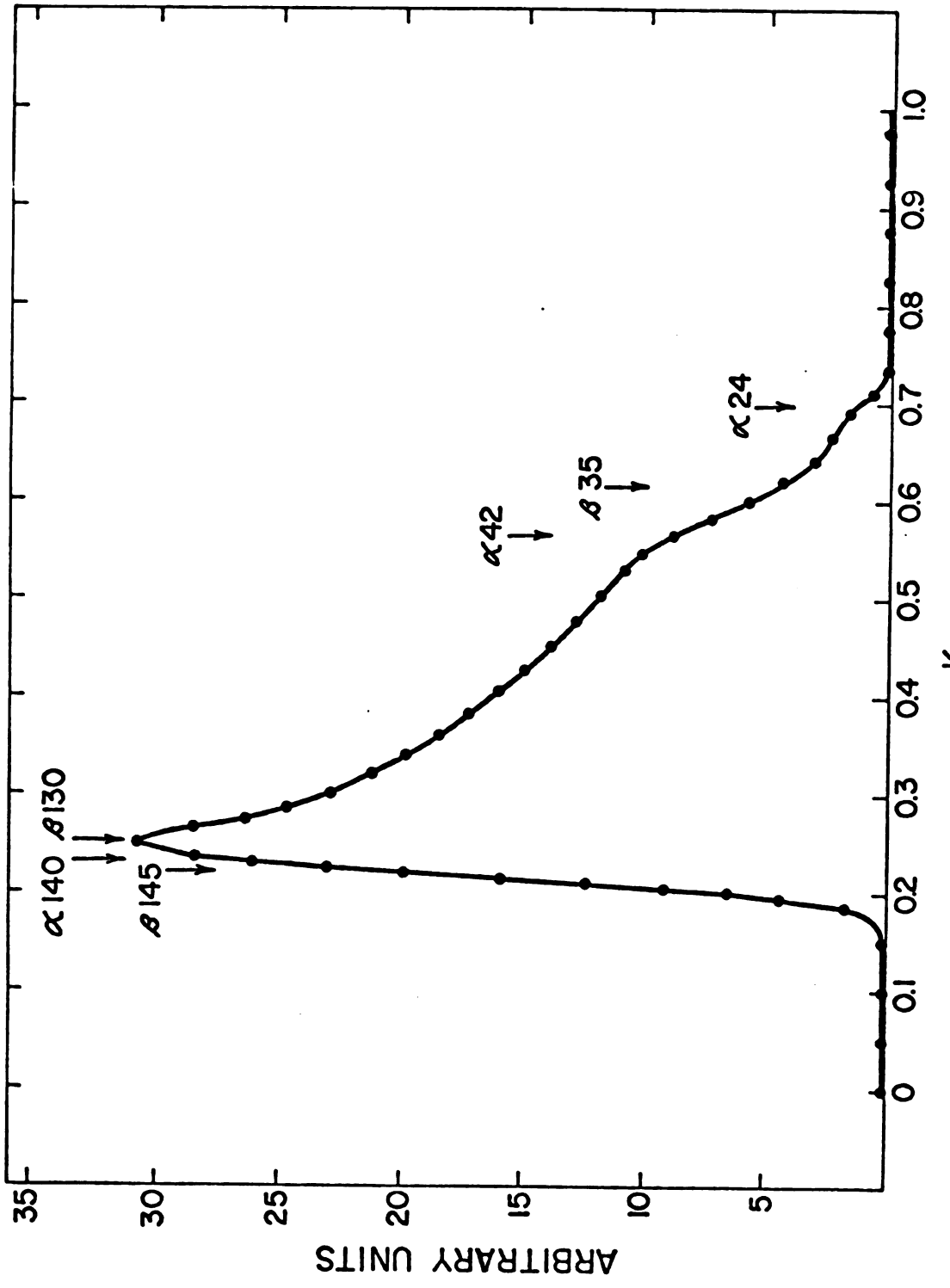
ARBITRARY UNITS
35
30
25
20
15
10
5
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
K_d
α140 β130
β145
α42
β35
α24

Figure 20

Figure 21. Bio-Gel A-0.5M gel filtration analysis of the [^{3}H] tyrosine labeled peptides of rabbit globin. An aliquot of the sample analyzed in Figure 20 was analyzed identically. A jagged curve was obtained. This was smoothed out by a least squares procedure (Savitzky and Golay, 1964) that filters out noise. This procedure is described in Methods. The smoothed data are presented as in figure 19.

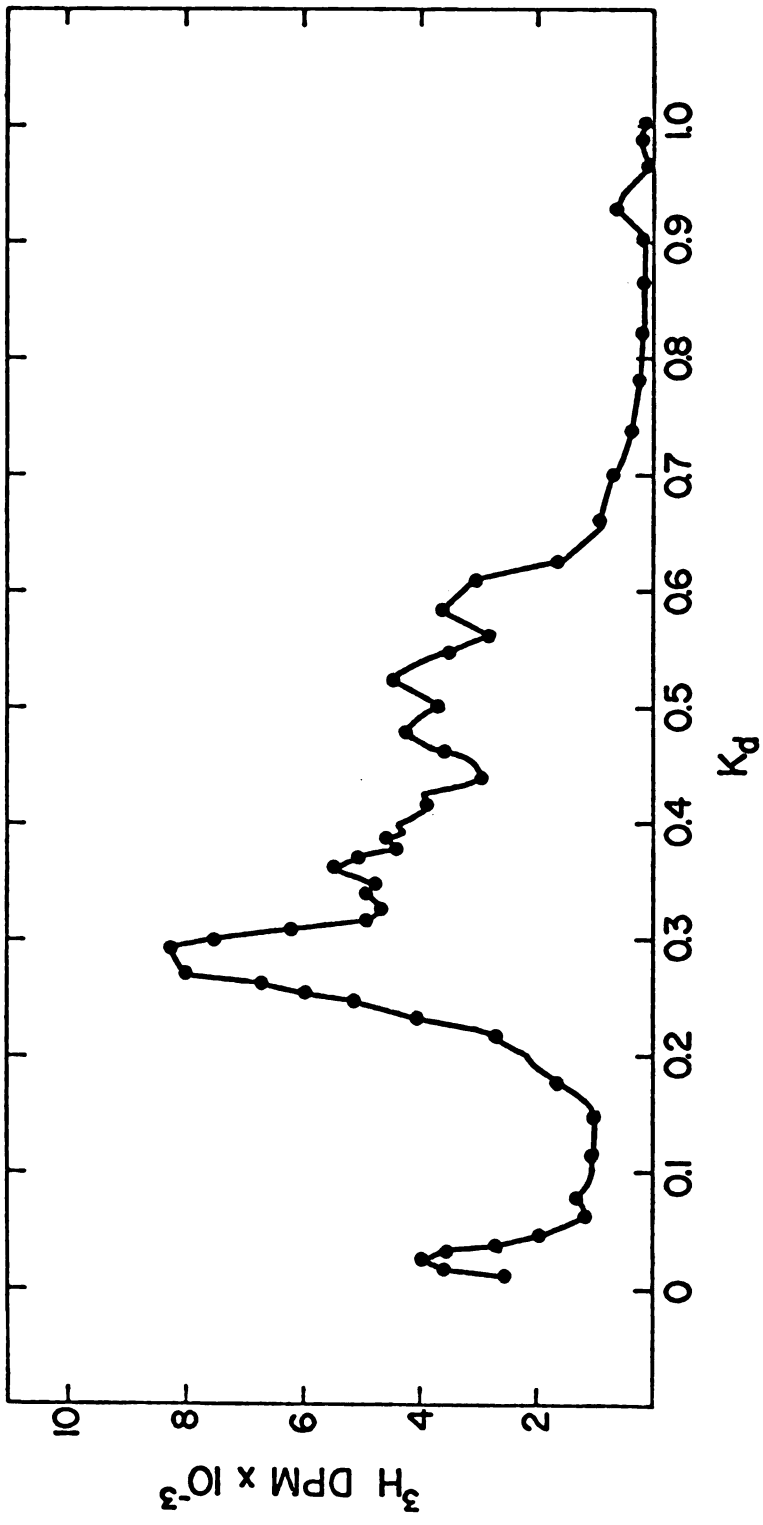

Figure 21

pattern of troughs at K_d values of 0.32 and 0.45 is observed. Comparison of these two figures is important in connection with the sharp peak of radioactivity present at a K_d value close to 0. This peak would contain peptides with molecular weight greater than 30,000. The patterns shown in figures 19 and 21 have the same general features in spite of the very different sizes of the leading peak.

Nascent Chains Labeled with Tryptophan, full Medium

Rabbit globin has two tryptophan residues at positions 14 and 15 of the α chain respectively, and one residue at position 37 of the β chain,(Dayhoff and Eck, 1968). These amino acid residues inserted early during chain growth, make tryptophan a convenient label for displaying a population of nascent peptides. Tryptophan is no longer inserted past residue 37 in the β chain. Thus, steep curves that might obscure some peaks are avoided. Peptidyl-tRNA was therefore prepared with reticulocytes incubated with a full complement of amino acids plus tritiated tryptophan. Figure 22 shows the population of nascent globin chains obtained in this experiment. Troughs at K_d values of 0.33 and 0.45 are again observed, together with peaks of radioactivity at K_d values of 0.29 and 0.37 plus a shoulder at K_d 0.62. Due to instrument failure between K_d values of 0.47 and 0.56 a dotted line has been drawn suggesting a peak around 0.55. This value was suggested by the slopes of the two limbs of the incomplete peak. A theoretical curve for tryptophan labeling

Figure 22. Bio-Gel A-0.5M gel filtration analysis of the [^{3}H] tryptophan labeled nascent peptides of rabbit globin. Rabbit reticulocytes (10 ml packed cell volume) were incubated as indicated in Methods for labeling with tryptophan. At zero time 2 mCi of [^{3}H] tryptophan (7100 µCi per µmole) was added. Following this addition the specific activity of the 40.3 ml reaction mixture was 2275 µCi per µmole. After 10 minutes of incubation, incorporation of radioactivity was stopped as indicated in Methods and peptidyl-tRNA was then prepared. An aliquot of [^{3}H] tryptophan-labeled peptidyl-tRNA was then analyzed by Bio-Gel A-0.5M gel filtration. Collection and analysis of fractions identical as in Figure 19. Fractions between K_d values of 0.47 and 0.56 were lost. These are indicated by broken lines.

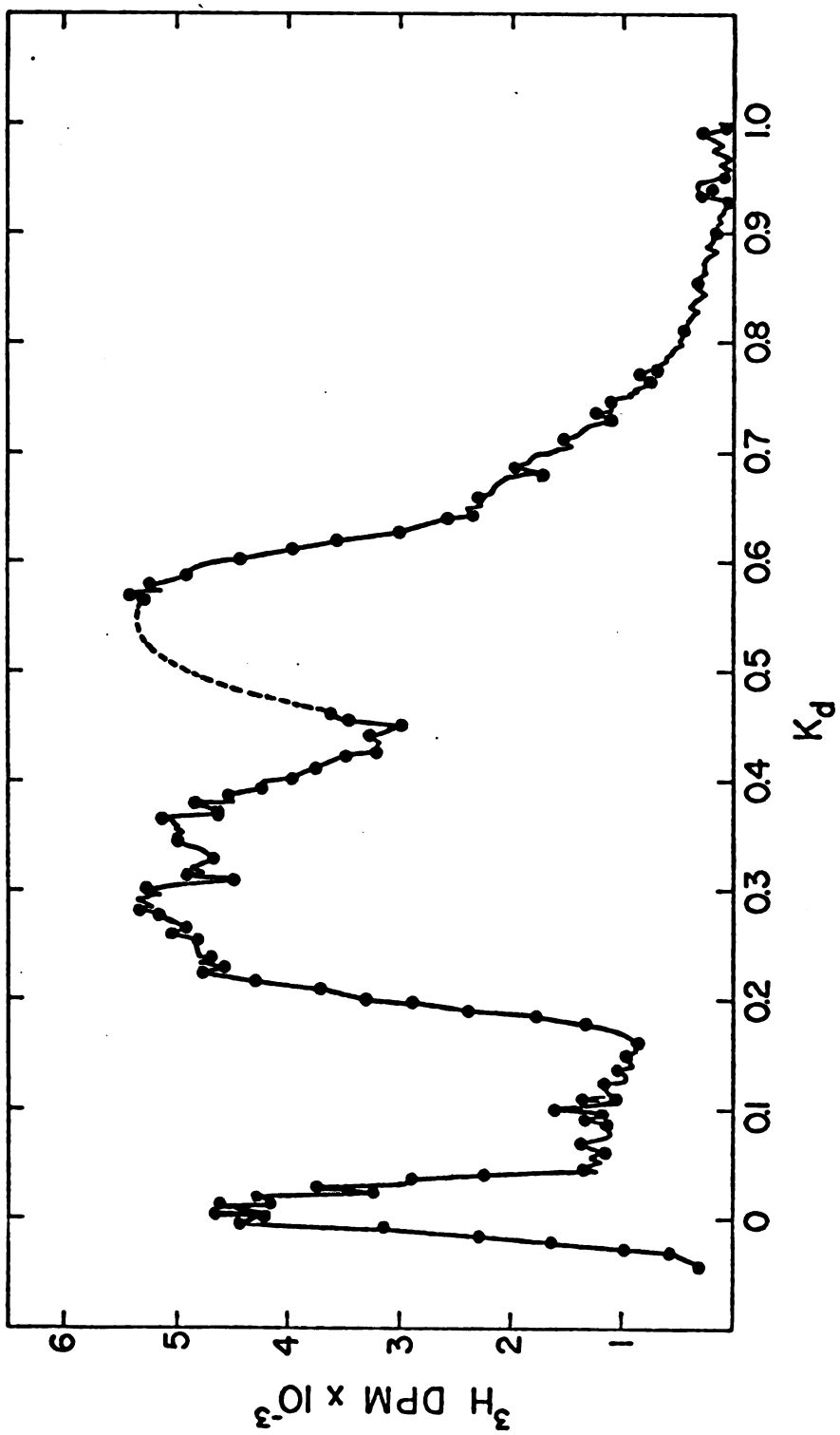

Figure 22

appears in figure 23. Again, as in the case of the theoretical curve for tyrosine, there are no troughs in the theoretical curve for tryptophan.

Effect of RNAse on the Elution Pattern of Nascent Chains

A peak of radioactivity was observed to elute close to the void volume in all Agarose gel filtration experiments. Figures 19 and 21 have shown that the pattern of troughs and peaks is independent of the size of this peak. The possibility that this peak might represent RNA-polypeptide complexes (Huang and Baltimore, 1970), was investigated. An aliquot of the same sample of [^{3}H] tryptophan peptidyl-tRNA that appears in Figure 22 was incubated for 25 minutes at 37° with protease-free pancreatic RNAse. After RNAse treatment this sample was analyzed by Bio-Gel A-0.5M gel filtration chromatography. The results are shown in Figure 24. This pattern is nearly identical to that of Figure 22.

Nascent Chains from Whole Blood

Rabbit reticulocytes kept at 0° for one hour show an almost complete disappearance of polysomes. Upon warming to 37° the polysome pattern is almost completely restored after one minute and completely restored after two minutes (Tepper and Wierenga, 1972). These same authors find an oscillatory rate of hemoglobin synthesis in the precooled reticulocytes. In reticulocytes kept at 37° prior to incubation the rate of protein synthesis is linear. This phenomenon was observed only with precooled reticulocytes.

Figure 23. Theoretical elution pattern for a population of [^{3}H] tryptophan labeled nascent peptides from globin analyzed by Bio-Gel A-0.5M gel filtration as described in Methods. The assumptions and presentation of data are identical as in Figure 20.

pulation of
ides from
filtra-
sumptions
l as in

Figure 23

Figure 24. Bio-Gel A-0.5M gel filtration analysis of the [^{3}H] tryptophan-labeled nascent peptides of rabbit globin after treatment with pancreatic RNAse. An aliquot of the sample shown in Figure 23 was incubated with 0.1 mg of pancreatic RNAse (protease free), for 25 minutes at 37° and analyzed as by Bio-Gel A-0.5M as in figure 19.

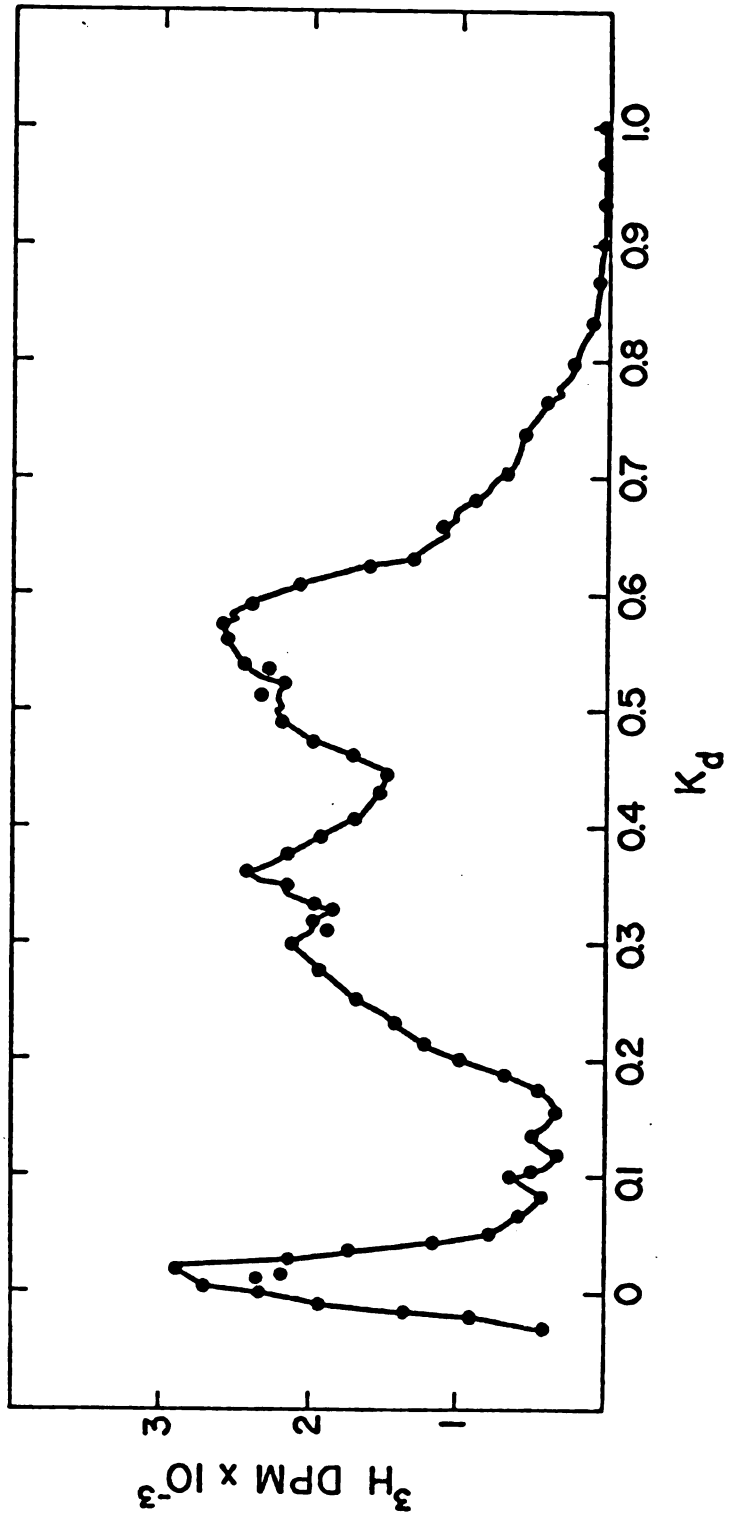

Figure 24

As reported by these authors a low degree of reticulocytosis (30%) and attainment of final temperature within 40 seconds are necessary to observe oscillations. In the cell incubations reported in this thesis, reticulocytes were kept under 4° for at least one hour and were brought up to 37° within two minutes, at the most. The possibility existed that the agarose gel filtration patterns were artifacts induced by cold synchronization. To explore this possibility peptidyl-tRNA was prepared from reticulocytes that had not been exposed to low temperatures prior to incubation. Blood (40 ml) was drawn from an anemic rabbit by heart puncture, filtered and while still warm added to an Erlenmeyer flask containing [^{3}H] tryptophan (3mc, 7.1 mCi/μmole) dissolved in 0.5 ml of RS. The cells were incubated for 12 minutes at 37°. Peptidyl-tRNA was prepared from this incubation mixture and analyzed by Bio-Gel A-0.5M gel filtration. The results of this experiment appear in figure 25. The pattern is essentially identical to that seen in figures 22 and 24. Therefore any results observed cannot be ascribed to synchronization of ribosomes following exposure to the cold.

Labeling with Methionine

To study patterns of nascent peptides, methionine has the advantage that it is inserted into globin during initiation of protein synthesis (Wilson and Dintzis, 1969; Hunter and Jackson, 1971; Koffer - Gutmann and Arnstein, 1973). N-terminal methionine is removed during elongation of both

Figure 25. Bio-Gel A-0.5M gel filtration analysis of the [^{3}H] tryptophan-labeled nascent peptides of rabbit globin synthesized in whole blood. Details of the experiment are discussed in the text. Bio-Gel filtration analysis as in figure 19.

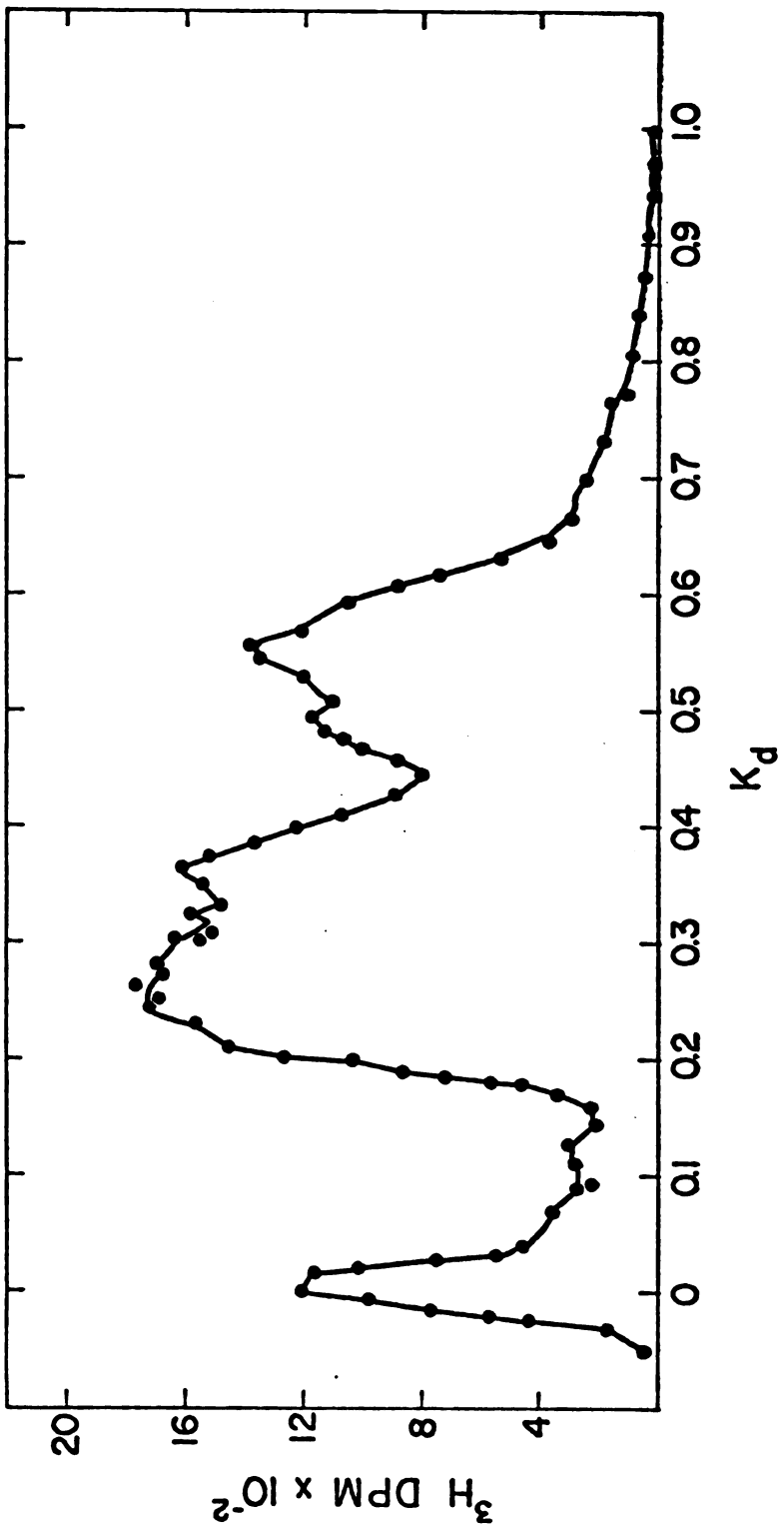

Figure 25

the α and the β chains of rabbit globin. The stage of protein synthesis at which the removal of N-terminal methionine takes place is not certain. Jackson and Hunter (1970) have estimated that peptides between 15 and 20 amino acids long loose their N-terminal methionine. Similar results were obtained by Yoshida et al. (1970) who found that peptides shorter than 16 amino acids had N-terminal methionine. Koffer-Gutman and Arnstein (1973) found N-terminal methionine in peptides up to 50 amino acids long. There is one methionine at residue 32 in the α chain and one at position 55 of the β chain of rabbit globin. Thus, assay for the presence of nonuniformity in size distribution in the population of nascent chains of globin can be done for all size ranges if radioactive methionine is used as a label.

The attainment of steady state labeling of the cells was verified, as shown in figure 26. As shown in this figure a steady state of labeling had been achieved after about eight minutes. The sample incubated without methionine had achieved a steady state that was lower by 7%, than the sample without methionine. Borsook (1957) has shown that methionine is not rate limiting. Eleven minutes seems to be an adequate point for collecting the cells. Figure 27 shows the effect of incubating reticulocytes in the presence of all amino acids except methionine. This figure shows the same peaks that were observed with the [^{3}H] tyrosine and [^{3}H] tryptophan labeled peptides. Peaks at K_d values of 0.289, 0.341, 0.470 and 0.602 are observed.

Figure 26. Time course of incorporation of [^{3}H] tyrosine into rabbit reticulocytes in the absence and in the presence of methionine. Rabbit reticulocytes (10 ml packed cell volume) were suspended in the incubation medium described in Methods, except that leucine was added to a final concentration of 1 mM in the incubation medium and methionine and tyrosine were omitted. The cell suspension was then divided in two aliquots. At zero time the following additions were made. To one aliquot [^{3}H] tyrosine (30 µC per µmole) and methionine was added to give final concentrations of 0.1 mM and 0.077 mM respectively. To the other aliquot only tyrosine was added to the same specific activity and concentration in the final reaction mixture. At the time points indicated 4 ml aliquots were withdrawn from the reaction mixture. The specific activity of the ribosomes was measured in the twice washed (2X) ribosomes obtained from each aliquot. Solutions containing 0.21-0.25 mg of ribonucleoprotein in 1 ml of 0.25 sucrose were precipitated with an equal volume of 20% tricholoacetic acid. The precipitates were collected on nitrocellulose membranes and counted in a Toluene Liquifluor mixture.

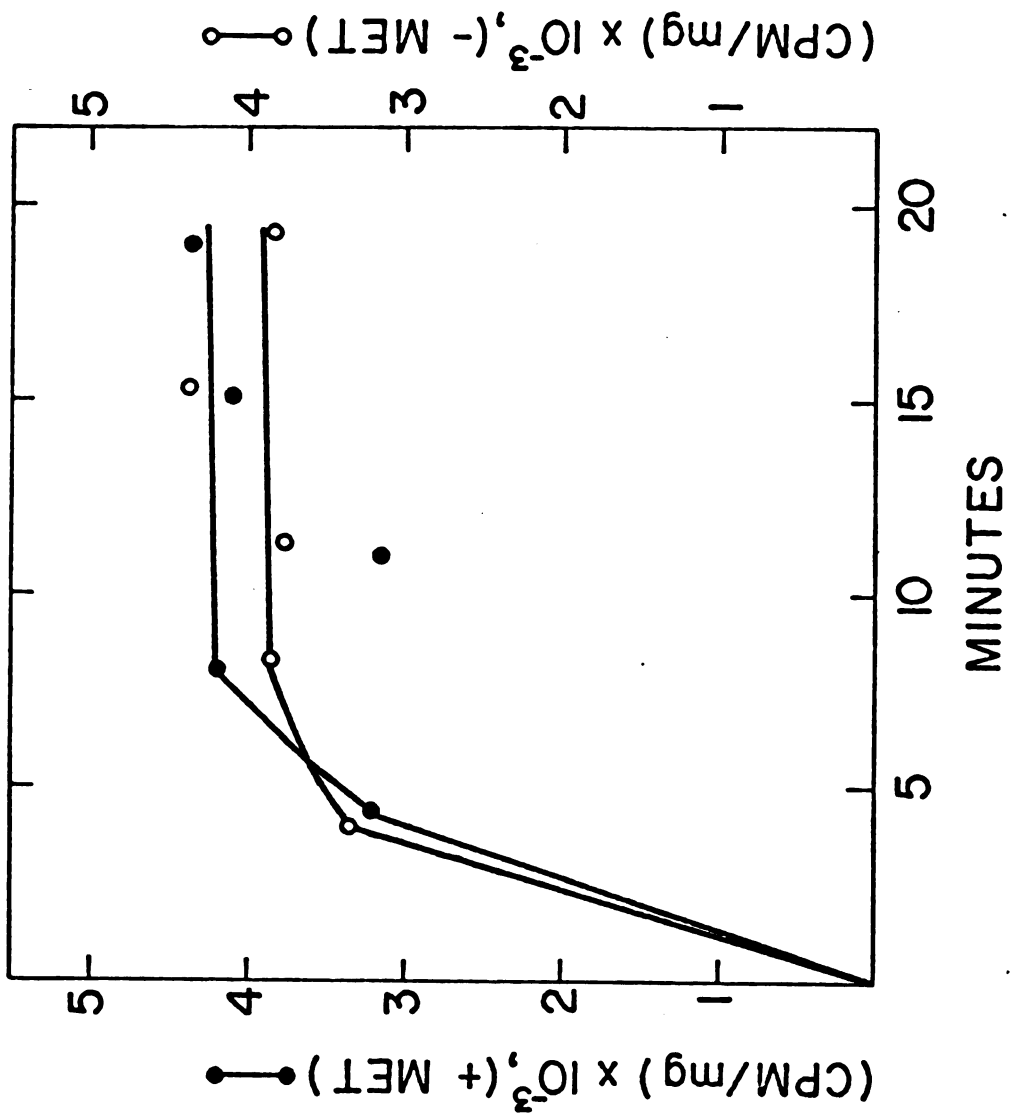

Figure 26

Figure 27. Bio-Gel A-0.5M gel filtration of [^{35}S] labeled nascent peptides of rabbit globin with standard leucine. Rabbit reticulocytes (10 ml packed cell volume) were incubated as described in Methods. At zero time 0.5 mCi of [^{35}S] methionine (3.0 x 10 mCi per μmole) was added, resulting in a final exogenous concentration methionine of 0.41 μM. After 11 minutes the reaction was stopped as described in Methods to prepare peptidyl-tRNA. Bio-Gel A-0.5M gel filtration was done as described in Methods. Collection of fractions, counting of radioactivity and presentation of data identical to Figure 19 except that the data are expressed as counts per minute.

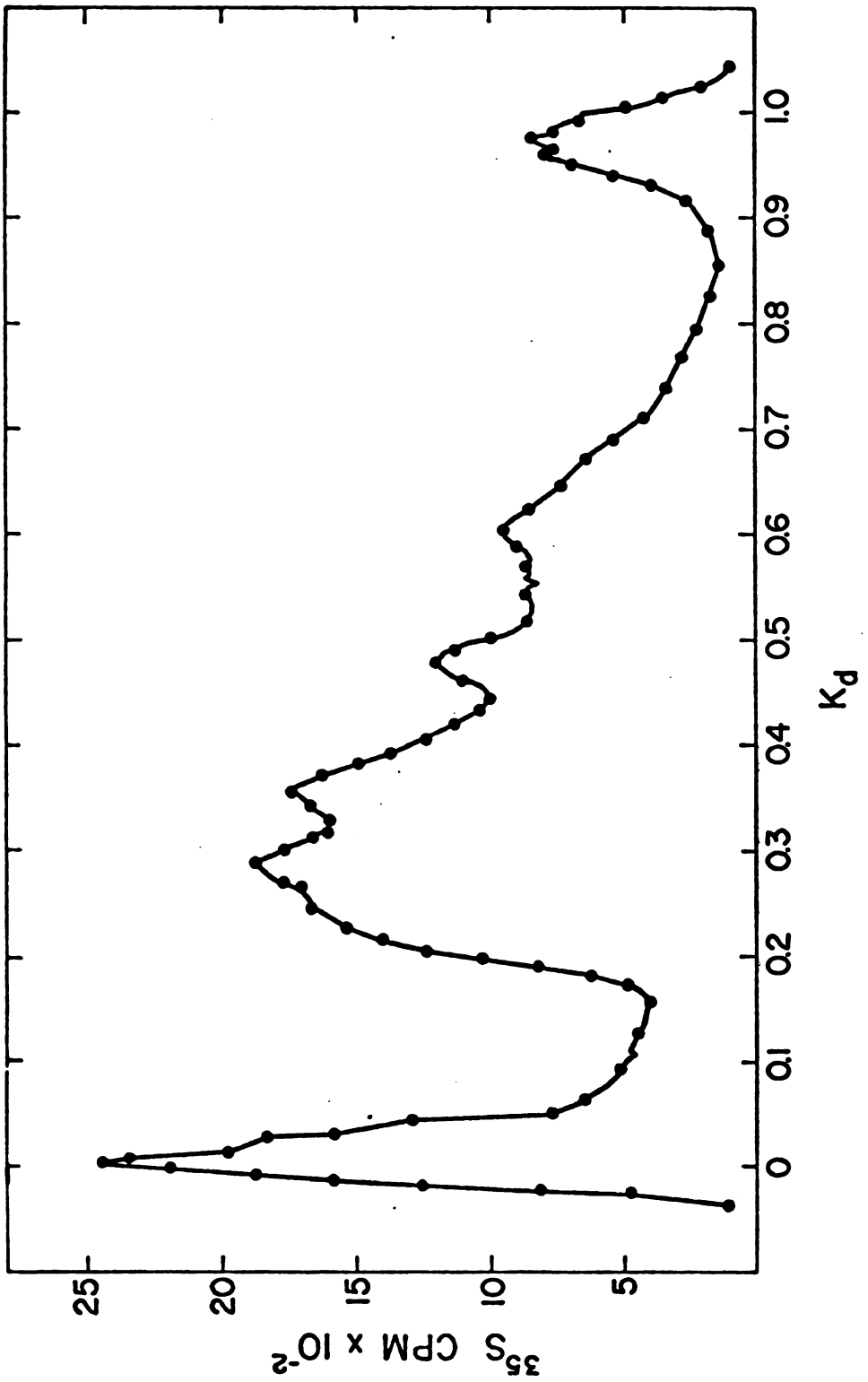

Figure 27

The leading peak of radioactivity, K_d close to 0, has been observed in previous figures.

Comparison of figure 27 with figure 28 in which reticulocytes had been incubated with leucine, equal to 39% of the concentration shown in Table II shows the same peaks. However, the peaks corresponding to the incubation in which a lower leucine concentration was used were very distinctive.

The K_d values obtained for the tritium markers, 0.486 for β CB-1 and 0.623 for α CB-1 correspond to molecular weights of about 6333 and 3756. These recovered values correspond to chains of 58 and 34 amino acids long compared to the actual values 55 and 32 amino acids long.

Table VII summarizes the results of Bio-Gel A-0.5M filtration chromatography.

Figure 28. Bio-Gel A-0.5M gel filtration of [^{35}S] labeled nascent peptides of rabbit globin, 1 mM leucine. Rabbit reticulocytes (10 ml packed cell volume) were incubated as described in Methods, except that leucine was present at a final concentration of 1 mM in the incubation mixture. At zero time 1 mC of [^{35}S] methionine (4.0×10^{4} µC per µmole) was added resulting in a final exogenous methionine concentration of 0.615 µM. After 11 minutes of incubation the reaction was stopped as described in Methods to prepare peptidyl-tRNA. Bio-Gel A-0.5M gel filtration chromatography was done as described in Methods. Fractions (21 drops) were collected into test tubes. Radioactivity was assayed in the odd numbered fractions by drawing out aliquots (150 µl) and counting in 5 ml Aquasol. Counting for double label was done in a Beckman LS-150 liquid scintillation spectrometer. The [^{35}S] monitor channel was set to detect only 0.072% of the counts appearing in the tritium monitoring channel, as determined by using [^{3}H] toulene standards. The levels of tritium used in this experiment would thus introduce a maximum of 1 cpm in the [^{35}S] channel. Treatment of the data is identical to that of figure 20. The broken lines represent marker peptides β CB-1 at K_d of 0.62 and α CB-1 at K_d o

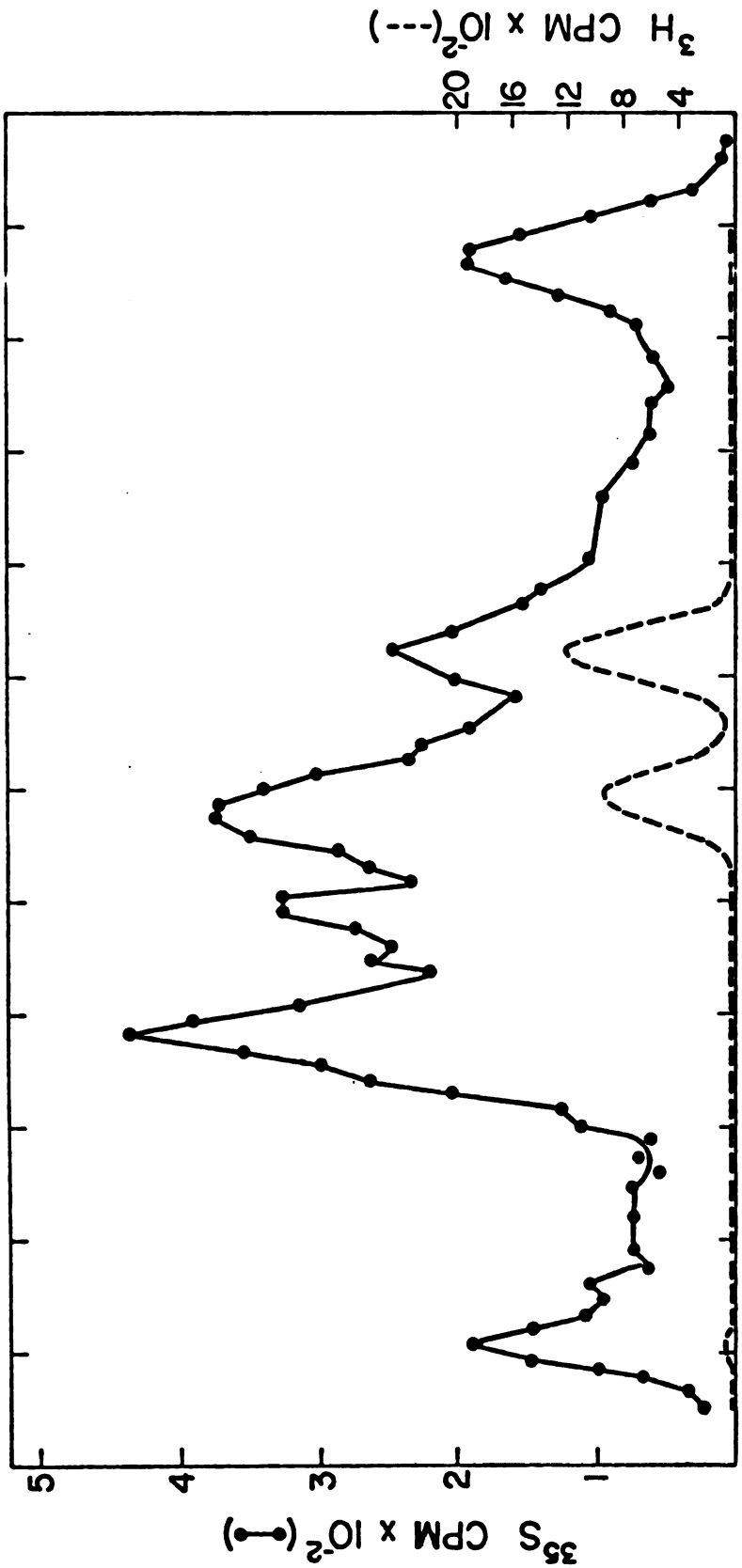
^{35}S CPM x 10^{-2} (—●—)
^{3}H CPM x 10^{-2} (---)
K_d
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1
2
3
4
5
4
8
12
16
20

Figure 28

Table VII Molecular Weights of the Peaks in Figures 19, 22, 24, 25, 27 and 28

Figure	Incubation	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
28	Methionine Low Leucine	12550	10482	6707	*	4088
27	Methionine High Leucine	12905	8854	6333	*	3702
24	Tryptophane RNAse	12137	9880	5891	4573	3154+
25	Whole Blood	12448	9685	6154	4994	3118+
22	Tryptophane No RNAse	12550	9257	**	**	3068
19	Tyrosine Low Leucine	12934	10190	6200	*	*

*Not observed

+Shoulder

**Not observed due to loss of the sample at this point.

DISCUSSION

Evidence presented in this thesis indicates the existence of a here-to-fore undetected accumulation of α globyl-tRNA on rabbit retioulocyte polysomes. In the case of β globyl-tRNA there is no such accumulation. Evidence is also presented to show that this phenomenon is not limited to the completed α-chain. Different sizes of nascent chains accumulate at specific points during the translation of the mRNA of rabbit globin.

Accumulation of the Completed α Chain

If one were to assume that the size distribution of nascent globin chains is uniform (Hunt et al., 1968a) then the α globyl-tRNA might be expected to be 1/141 th or 0.71% of the total nascent chains. In contrast to this prediction, the percentage of α nascent peptides present as α globyl-tRNA was found to be more than 6 times higher than predicted. Similar calculations for the nascent β chains would predict 1/146 th or 0.69% β globyl-tRNA in the β nascent peptide peptide fraction. In this case the analytical results coincide closely with the predicted value for the β nascent peptides. A 6.4-fold excess of α globyl-tRNA to β globyl-tRNA exists in the peptidyl-tRNA fraction (Table IV). In spite of the high ratio of α globyl-tRNA to β globyl-tRNA the

overall ratio of total nascent β chains to total nascent α chains as determined from the intercepts of Figure 6, was found to be 1.04-in close agreement with the finding reported by others (Lodish, 1971; Hunt et al., 1968a,b). Various authors have, in the past, measured the percentage of nascent globin chains in the rabbit reticulocyte polysome corresponding to completed α globin chains or β globin chains. However, these experiments were carried out using preparations which contained varying levels of contaminating soluble hemoglobin. This has led to a wide range of often conflicting results. Luppis et al. (1970) found no evidence of accumulation of completed α or β chains on the polysome. Hunt et al. (1968a) found equal numbers of completed α and β chains attached to the polysome, with results varying from experiment to experiment. The latter authors reported the presence of from one complete globin chain per 7 nascent globin chains to one completed globin chain per 60 nascent globin chains (Hunt et al., 1968a). Colombo and Baglioni (1966), found an excess of α globin chains in a reticulocyte preparation. On the basis of the time required for the termination and release of globin chains Lodish and Jacobsen (1972), have predicted that 5% of the nascent globin chains on the reticulocyte ribosome should be completed α and β globin chains, both chains being present in equal proportions.

Evidence has been presented to show that the amount of free hemoglobin contaminating the preparations of peptidyl-

tRNA used to obtain the results reported here does not contribute significantly to the observed results. This contamination amounts to not more than 0.30% of the labeled hemoglobin present in the original ribosomal pellet. During the preparation of peptidyl-tRNA from radioactively labeled reticulocytes, such as the preparations used to obtain the data in Table IV, the average radioactivity due to soluble hemoglobin contamination of the unfractionated ribosomal pellet amounted to 6.1×10^6 DPM. This amount of radioactivity in the hemoglobin contaminating the ribosomal pellet will leave not more than 1800 DPM of contaminating hemoglobin in the purified peptidyl-tRNA fraction. Since the supernatant hemoglobin would be expected to be nearly uniformly labeled Dintzis (1961), and since tryptic digestion of globin produces 6 tyrosine-containing peptides, the radioactivity due to each contaminant tryptic peptide would not be expected to exceed 300 DPM. In the analyses reported in Figure 7, tryptic peptide β T16 contained an average of 14,500 DPM. The total radioactivity in all the other tryptic peptides is even greater by at least seven fold or more. Contamination from soluble hemoglobin (300 DPM) would introduce an uncertainty of about 0.014% to the reported value of 0.70% for β globyl-tRNA (Table IV). Similar considerations would introduce an uncertainty due to soluble hemoglobin contamination of approximately 0.015% to the reported value of 4.62% for α globyl-tRNA (Table IV). The

uncertainty due to contamination of the other tryptic peptides is negligible. The results obtained with pactamycin reinforce this conclusion. Figures 9 and 10 show that the preparations of purified peptidyl-tRNA contain true intermediates of globin biosynthesis. In particular, Figure 10 shows quite clearly the effect of inhibiting initiation of new nascent chains. While there is a decrease in the amount of nascent protein attached to the ribosomes the effect is most pronounced among those nascent chains in the early stages of synthesis. Hence, the proportion of globyl-tRNA in the nascent protein fraction is increased markedly even though the absolute quantity of globyl-tRNA is reduced. Contamination of the peptidyl-tRNA fraction by soluble labeled hemoglobin would have obscured these changes.

A consideration of the ratio of α T15 to β T16 found following pactamycin addition to the reaction mixture reveals an average α T15/β T16 ratio of 5.8 during the first 30 seconds after addition of the antibiotic. By 2.2 minutes in the presence of pactamycin that ratio has been reduced to 4.9 and reaches 2.3 after 5 minutes of incubation. These results are incompatible with contamination of the peptidyl-tRNA fraction with free α globin chains which have been reported to be present in the reticulocyte (Baglioni and Campana, 1967).

Accumulation of α globyl-tRNA to the extent of 4.6% of the total number of nascent α peptides present indicates that one ribosome in 23 of those which are actively engaged

in α globin synthesis possess an α globyl-tRNA. From the number of ribosomes per polysome which are synthesizing globin in the rabbit reticulocyte (Hunt et al., 1969). One can estimate that approximately one of these polysomes in 5 contain an α globyl-tRNA. Since α globyl-tRNA would be expected to be the normal substrate for the release steps in α globin biosynthesis the observed accumulation may be a reflection of a limitation of the rate of release of completed α globin chains from the biosynthetic template.

Accumulation of Growing Globin Chains on the Polyribosome

The methodology of agarose gel filtration utilized in this thesis is a very suitable method for the study of populations of nascent peptides ranging from 2 to 200 amino acid residues in length. Swank and Munkres (1971) were able to separate peptide markers in the molecular weight range of 1200 to 10000 by means of electrophoresis in polyacrylamide gel with SDS. These authors used 12% acrylamide to increase sieving properties of the gel. Fish et al. (1969) obtained a useful range of calibration of peptide markers ranging in molecular weight from 1540 to 76600 using agarose gel filtration in 6M guanidine HCL. These authors used Bio-Gel A-5M with a nominal content of 6% agarose as a gel filtration medium. To obtain increased sieving for smaller peptides and thus expand the scale for small peptides, Bio-Gel A-0.5M, 200-400 mesh, with a nominal agarose content of 10% was used in this thesis. The calibration experiments

reported in this thesis indicate that such an expansion of scale was obtained. In the procedure of Fish et al. (1969) peptides having a molecular weight under 18400 appear in the latter 70% of the inclusion volume. In the procedure reported in this thesis, peptides under 18400 appear in the latter 82% of the inclusion volume. There is a further expansion of the scale as the molecular weight decreases. Peptides under 7650 appear in the latter 44% of the inclusion volume as reported by the above authors. Similar peptides appear in the latter 57% of the inclusion volume as reported in this thesis.

This expansion of scale for resolution of oligopeptides makes this method suitable for the study of populations of nascent chains such as those of globin. Furthermore, the method of gel filtration outlined in this thesis complements the method of Dintzis (1961), for the analysis of populations of nascent chains. The method of Dintzis looks simultaneously at the whole population of nascent chains, so that contributions from individual members are obscured. This is illustrated by the following example. Figure 29 illustrates a hypothetical population of 10 nascent peptides uniformly distributed in length. If this population of 10 nascent chains were analyzed for the number of moles of amino acid present at each position along the chain, this analysis would indicate that there are 10 molar multiples or copies of the N-terminal amino acid, since it is carried by all 10 chains. A similar analysis for the amino acid

Figure 29. Hypothetical population of 10 nascent peptides uniformly distributed in size. An amino acid is being polimerized. Direction of chain growth is from the N-terminal end towards the C-terminal end. N-terminal end is indicated by NH_2. The C-terminal end is indicated by COOH.

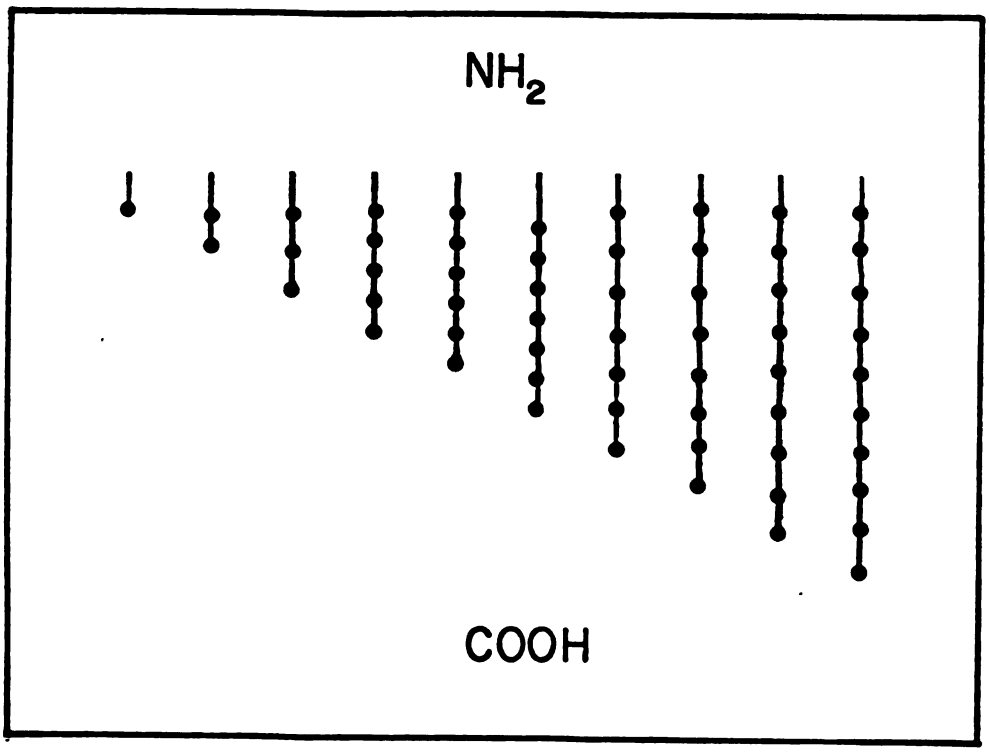

Figure 29

at position 5 would indicate the presence of 5 copies, since it is carried by all 5 chains. If similar analyses were performed for other amino acids along the chain a Naughton-Dintzis plot of this data would give a straight line Englander and Page (1964). The population of nascent chains of β globin comprises peptides of 146 different chain lengths. If the representation of nascent chains belonging to a narrow range of lengths is increased or decreased these variations would go their relative contribution to the cumulative count of residues at each position would be relatively small. Any such variation would go undetected considering that the sampling is usually done at a dozen or less positions. Drastic interference with protein biosynthesis such as incubation with sodium fluoride or tryptophan starvation is necessary to show clear cut alterations in the Naughton-Dintzis pattern (Hunt et al., 1968a).

The methodology outlined in this thesis displays the spectrum of sizes of the nascent chains. The contribution of each component is weighed only against its nearest neighbors. Only overlapping peaks can interfere.

Significance of Nonuniformity in the Size Distribution of Nascent Peptides

The studies of Luppis et al. (1970), Hunt et al. (1968) using Naughton Dintzis plots indicate that the overall population of nascent rabbit globin chains is uniformly distributed as to chain size. The results presented in this thesis suggest nonuniformities in the size distribution of

an otherwise uniform population. A major nonuniformity represented by the accumulation of the completed α chain on the polysome has already been discussed. Studies with different radioactive amino acids reveal a consistent and very reproducible pattern of peaks and troughs whenever the nascent chains of globin are separated by agarose gel filtration, Table VII. These features of the graphs indicate that sectors of the population of nascent chains are present more frequently relative to others. Thus indicating that ribosomes move at different rates along the mRNA of globin.

Itano (1966) has discussed various models of nonuniform rate of polypeptide chain assembly. Pertinent to the results of this thesis is the model that considers initiation or a step close to initiation as a rate limiting step in the assembly of globin. This model makes plausible the existence of a region or regions where the rate of ribosome movement is slower than average but faster than the rate limiting step. Queues of ribosomes would form at the slow point their length dependent on the rate of ribosome passage past the rate limiting point. These slow points would give rise to local queues that would appear as peaks during agarose gel filtration of the nascent chain of globin.

Winslow and Ingraham (1966) studied the relative specific activity along the α and β chains of human hemoglobin A. Following short pulses of labeling with radioactive lysine, the supernatant hemoglobin was assayed for specific activity at various points along the chain.

Naughton-Dintzis plots of this data revealed the apparent existence of a slow point around residue 90 for both chains. The existence of this slow point was attributed to conformational change of the growing chain after heme insertion. However, no evidence has been for the attachment of heme to the nascent chains of globin (Felicetti et al., 1966; Morris and Liang, 1968). Luppis et al. (1970) and Hunt et al. (1968), however, fail to find any evidence for slow points in their studies of rabbit hemoglobin biosynthesis by means of Naughton Dintzis plots.

The idea of a limiting species of tRNA controlling the rate of globin biosynthesis has been explored by various authors. Gilbert and Anderson (1970) found that when hemoglobin is synthesized in a cell free system in the presence of a limiting amount of tRNA there is a 50% decrease in α chain production relative to β chain production. Several authors have found that for a given specialized cell type there is a correlation between the level of tRNA specific for a given amino acid and the amino acid composition of the specialized protein being synthesized. Thus, Smith and McNamara (1970) compared the ratio of amino acid acceptance activity of reticulocytes to liver in the rabbit. They found this ratio to be high for histidine and very low for isoleucine. These ratios parallel the amino acid composition of hemoglobin in which histidine is unusually common and isoleucine unusually rare. Litt and Kabat (1972) studied the isoleucine acceptance capacity of sheep reticulocytes

during the transition between hemoglobin A and hemoglobin C in the early stages of anemia of A/A homozygotes. They found that isoleucine acceptor capacity is 2 to 3 times higher in tRNA from reticulocytes synthesizing hemoglobin C as compared to reticulocytes synthesizing hemoglobin A. Sheep hemoglobin A contains no isoleucine and hemoglobin C contains two isoleucines.

Different species of tRNA might be rate limiting for the alpha and beta chains of hemoglobin in view of the codon degeneracy that has been shown to be present in hemiglobin. Soll et al. (1966) purified two tRNA Arg species, I and II. Weisblum et al. (1967) found that tRNA I Arg and tRNA II Arg preferentially transfer arginine to positions 141 and 31 respectively of the α chain of rabbit globin.

The idea of a limiting tRNA species could be used to interpret the nonuniformities in the size distribution of nascent globin chains. For example, Table VII, the weight range between 8854 and 10482 in peak 2 corresponds to chains ranging in length between 81 and 95 amino acids (at 110 per amino acid residue). The amino acid sequence of hemoglobin shows that this is a region rich in leucine (Dayhoff and Eck, 1968). The possibility exists that there is a leucine limitation.

Relevant to this point is the finding of Smith and McNamara (1972), that leucine acceptance of reticulocyte tRNA is particularly low in proportion to its presence in

hemoglobin. The results in Table VII, show that peaks 2 and 5 correspond to regions rich in leucine in both the α and β chains of globin Dayhoff and Eck (1968). No such correlation can be found for peaks 3 and 4, however. Peak 1 is not considered since it appears in the theoretical curves. The possibility of course, exists that more than one amino acyl tRNA is involved in modulation of the rate of movement of the ribosome along the mRNA for globin.

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APPENDIX I

PROGRAM SIZSIM

```
      PROGRAM SIZSIM(INPUT,OUTPUT,TAPE60=INPUT,
     1    TAPE61=OUTPUT)
      REALXM,XKD,XKDI,SUM(1500),BUFF(1500),
     1    YSTAR(200),YGAUSS(300)
      INTEGER N,K,I.J,NCEL,NOCEL,INCK,IOLFAC,
     1    IFAC,IFLAG
C     INITIALIZE SUM AND BUFF
 5    DO 1 I=1,1500
      SUM(I)=0.0
      BUFF(I)=0.0
    1 CONTINUE
C     READ IN J AND IFACT
10    READ (60.2)J,IFACT
    2 FORMAT(215)
C     READ IN GAUSSIAN CURVE
      READ (60.2)(YSTAR(I),I=1,183)
    3.FORMAT(16F5.0)
15    DO 50 I=1,183
      YGAUSS(I) = YSTAR(I)
   50 CONTINUE
      XM=FLOAT(J)*110.0
C     POSITION OF FIRST PEAK
20    XKD=(1.0568147-0.0021011*XM**0.555)**3
      XKDI=XKD*1000.0
      NCEL=1500-IFIX(XKDI)
      K=NCEL-91
      NOCEL=NCEL
25  C   APPROPRIATE FACTOR FOR GAUSSIAN CURVE
      IF(IFACT,EQ,1)GOTO4
    7 DO 10 I=1,183
      YGAUSS(I)=FLOAT(IFACT)*YSTAR(I)
   10 CONTINUE
30    8 GOTO4
C     FILL BUFF ARRAY
    4 DO 5 I=1,183
      BUFF(K-1+I)=YGAUSS(I)
    5 CONTINUE
35  C   FILLS IN SUM ARRAY AND REINITIATES BUFF
      N=K+182
      DO 6 I=K,N
      SUM(I)=SUM(I)+BUFF(I)
      BUFF(I)=0.0
40    6 CONTINUE
C     CALCULATES NEW PEAK POSITION
```

```
PROGRAM      SIZSIM

                 J=J+1
                 IF(J,GT,146)GOT030
                 XM=FLOAT(J)*110.0
45               XKD=(1.0568137-0.0021011*XM**0.555)**3
                 XKDI=XKD*1000.0
                 NCEL=1500-IFIX(XKDI)
                 INCK=NCEL-NOCEL
                 K=K+INCK
50               NOCEL=NCEL
       C         DETERMINES APPROPRIATE FACTOR FOR GAUSSIAN
                 IOLFAC=IFACT
                 IF(J.GE,25,AND,J,LT,35)IFACT=1
                 CONTINUE
55               IF(J,GE,35,AND,J,LT,42)IFACT=2
                 CONTINUE
                 IF(J,GE,42,AND,J,LT,130)IFACT=3
                 CONTINUE
                 IF(J,GE,130,AND,J,LT,140)IFACT=4
60               CONTINUE
                 IF(J,GE,140,AND,J.LE.141)IFACT=5
                 CONTINUE
                 IF(J,GE,142,AND,J,LE,146)IFACT=3
                 IFLAG=IOLFAC-IFACT
65               IF(IFLAG)7,8,7
       C         PRINTS OUT FINAL RESULT
              30 WRITE(61,31)(SUM(1501-I), I=1,901)
              31 FORMAT(1X,10F13,1)
                 STOP
70               END
```