BIOSYNTHETIC STUDIES

OF HUMAN FETAL HEMOGLOBIN

Thesis by
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ABSTRACT

Previous investigators have found unbalanced synthesis of Hb F, with γ chain synthesized from 30% to 68% as efficiently as α chain. In this report, biosynthetic studies of 9 umbilical cord bloods and 6 sickle-cell bloods with elevated levels of Hb F gave an efficiency of $47 \pm 3\%$ for γ-chain synthesis relative to α-chain synthesis.

Amino acid analysis of the minor zones in chain separation chromatograms showed that two peaks, one eluting before and one after γ chain, contain products of γ-chain mRNA. These peaks, however, are insufficient to explain the discrepancy in γ-chain synthesis.

A likely explanation for the reduced γ-chain synthesis is that γ-chain mRNA may have a shorter effective lifetime than α- or β-chain mRNA. There would thus be a progressive imbalance in Hb F synthesis after the blood cell leaves the bone marrow and mRNA synthesis stops.

A timed study of a single cord blood, with times ranging from 2.5 minutes to 4 hours, showed that incorporation of radioactivity into acetylated γ chain begins at a high value and decreases with respect to unmodified γ chain. This implies that the γ chain may be modified either on the ribosome or shortly after release, but in any case earlier than 2.5 minutes after the start of incubation.

TABLE OF CONTENTS

INTRODUCTION

Much recent research has dealt with the period of hemoglobin biosynthesis occurring at and immediately after translation. Interest centers on mRNA lifetimes, pools of free hemoglobin chains present in the cytoplasm, assembly of whole hemoglobin, and other post-nuclear processes which may control and balance protein chain synthesis.

Hemoglobin is the hero of a great deal of the original research on protein biosynthesis, since it is readily available and easy to purify. Hb A biosynthesis is fairly well understood, although that of less available hemoglobins such as S or F is not. Several authors have reviewed the biosynthetic process (1-4).

Chain initiation appears to be the rate-limiting step in synthesis. By plotting specific activity of nascent peptides with ^{3}H-lysine incorporated versus the position of the peptide in the chain, Hunt et al. (5) showed that the rate-limiting step does not occur during translation. Subsequent experiments (6-8) confirmed this view, and also showed that chain release is not the rate-limiting step.

Hemoglobin mRNA is synthesized primarily prior to the orthochromic normoblast stage of erythrocyte differentiation (9). Therefore, reticulocytes are dependent on mRNA which is for the most part at least 40 hours old; they do not synthesize their own (10). The decay of mRNA is perhaps critical in translational control, but knowledge of mRNA lifetimes has remained elusive. Indirect evidence indicates that different mRNAs have different lifetimes. In reticulocytes, Hb A_2 synthesis stops sooner than that of Hb A_0 (11-13), indicating that the cells lose their ability to make δ chain sooner than that to make β chain. Similarly, γ-chain synthesis is shorter-lived than β-chain synthesis (14). It is not clear whether the shorter mRNA lifetimes result from mRNA decay, or whether the mRNA becomes unavailable for protein synthesis. Recent evidence (15-17) shows that polyadenine tails on mRNA increase the lifetime. In addition, the mRNA life span depends on how actively the mRNA is synthesizing protein--it is somehow protected from ribonuclease by the attached ribosomes (18).

Besides the possibility of control through mRNA lifetimes, there are two other post-nuclear control mechanisms of interest here. The first is control of the relative amounts of different mRNAs available for protein synthesis, and control of the translation rates on those mRNAs. Rabbits (19) and humans (20, 21) both synthesize β chain on larger polyribosomes than α chain. This is because β-chain mRNA initiates synthesis more efficiently than α-chain mRNA, and so there are more nascent chains attached to β-chain mRNA (22). In order that a balanced synthesis be maintained, there must be more α-chain mRNA (23). Direct assay of α- and β-chain polyribosomes has confirmed this finding (24).

Another possible mechanism for synthesis control uses feedback from a pool of free cytoplasmic α chains which is present in the reticulocyte (25-27). This pool, as well as smaller pools of free β chains (28) and α-β dimers (29, 30), apparently contains the intermediates of hemoglobin assembly (31). Adding α chains to a cell-free translation system decreases production of α chains, but stimulates β chain production (32-35); adding β chain, however, has no effect on α chain production, but does inhibit synthesis of β chains (34, 36). This implies a feedback mechanism in which accumulation of α chains stimulates β-chain synthesis.

Further evidence in this vein comes from experiments with O-methyl-L-threonine (OMT), an isoleucine analog (37). This substitutes for isoleucine if present in the hemoglobin chain and effectively stops synthesis. The α chain of the rabbit, with 141 amino acid residues, has isoleucine at positions 10, 17, and 55 (38), while the β chain, with 146 amino acid residues, has isoleucine at position 112 (39). A mutant rabbit is available with the substitution β^{112} Val; these animals have no isoleucine in the β chain (40). Addition of OMT to reticulocytes of homozygous mutant rabbits retarded α-chain synthesis as expected, but did not, however, retard β-chain synthesis (41). Wolf, Mason, and Honig (42) argued that the α-chain pool might not have been depleted under the experimental conditions employed. They therefore added OMT to cells from the bone marrow of mutant rabbits, which have substantially smaller α-chain pools than reticulocytes (43). The

resulting retardation of both α- and β-chain syntheses implies that the feedback mechanism does indeed exist.

This result is not universally accepted. Garrick, Dembure, and Garrick (44) depleted the α-chain pools in mutant rabbit reticulocytes and performed an OMT experiment, but observed an increase in β-chain synthesis rather than a decrease. They hypothesized that α and β mRNAs might compete for the limiting components of the protein synthetic apparatus, so that when α-chain synthesis stops, more biosynthetic apparatus is available for β-chain synthesis.

Knowledge of the post-transcriptional control of Hb F synthesis is less abundant than that of Hb A. One reason for this is that Hb F usually occurs in combination with large quantities of other hemoglobins, such as A or S. Two distinct control mechanisms may thus operate simultaneously in the cell. Important exceptions to this rule are individuals with homozygous hereditary persistence of fetal hemoglobin (HPFH), who have only Hb F. Several authors have reviewed HPFH (3, 45-47).

There is evidence, however, that the control of Hb F synthesis which occurs in the cytoplasm is substantially different from that of Hb A. Because human γ chains contain isoleucine, OMT inhibits their synthesis. Human α and β chains, however, contain no isoleucine and OMT does not inhibit their synthesis. A cell exposed to OMT produces no γ chain, but accumulates α chain (48, 49). This argues against the α-chain feedback mechanism in cells producing Hb F. In addition, biosynthetic studies of blood from HPFH homozygotes, in which the cell incorporates radioactive leucine into the chains, have shown that the γ chain consistently labels less efficiently than the α chain, so that the ratio of total radioactivity γ/α ranges from 0.30 to 0.68 (50-52). In experiments on HPFH heterozygotes, who usually have 10-40% Hb F, several authors found balanced synthesis (53-55). In another experiment, however, four out of 14 HPFH heterzygotes showed unbalanced synthesis (56). Ringelhann et al. (52) have suggested that HPFH heterozygotes may have unbalanced Hb F synthesis which is masked by balanced synthesis of large amounts of other hemoglobins.

The imbalance of γ-chain and α-chain syntheses is a central problem in the present study. It is possible that individuals who do not have HPFH but still have elevated levels of Hb F, such as newborn children and certain individuals with sickle-cell anemia, may show balanced synthesis only because they produce hemoglobins which mask the Hb F imbalance. Is it possible to dissect out the γ/α total radioactivity ratio when other hemoglobins are present? If so, then it should be possible to tell if the imbalance is a generalized property of Hb F synthesis in reticulocytes.

A second area of investigation in this study was chromatography. Separation of hemoglobin chains on carboxymethyl cellulose produces several minor zones (Figure 1) comprising about 15% of the total globin. Amino acid analysis shows that some of these peaks contain hemoglobin (unpublished results), yet published biosynthetic studies have ignored them. If these zones are hemoglobin chain, then it would be correct to include them in the biosynthetic ratios. This might significantly change the results of the experiments.

Besides the minor zones, there are several other factors which might affect the biosynthetic ratios. DeSimone and Mueller (57), and Lee et al. (58) have suggested that a significant fraction of Hb F is assembled from a pool of α chains labeled to a high specific activity. Non-radioactive α chains from previously existing Hb F might then exchange with radioactive α chains from the pool. A significant fraction of Hb F might thus consist of radioactive α chains and non-radioactive γ chains.

Another possibility is that γ-chain mRNA may have a shorter lifetime than other mRNAs, as Burka and Marks (59) suggested. The reticulocyte, which does not synthesize its own mRNA, may thus contain less γ-chain mRNA than mRNA of other types. Consequently, γ-chain synthesis in the reticulocyte should be retarded.

Yet a third possibility is that the synthetic imbalance is a transient phenomenon. The incubation procedure exposes the reticulocyte to concentrations of amino acids and other substances much in excess of the physiological concentrations. Perhaps this results in stimulation of synthesis, with the requirement that a certain number of α chains

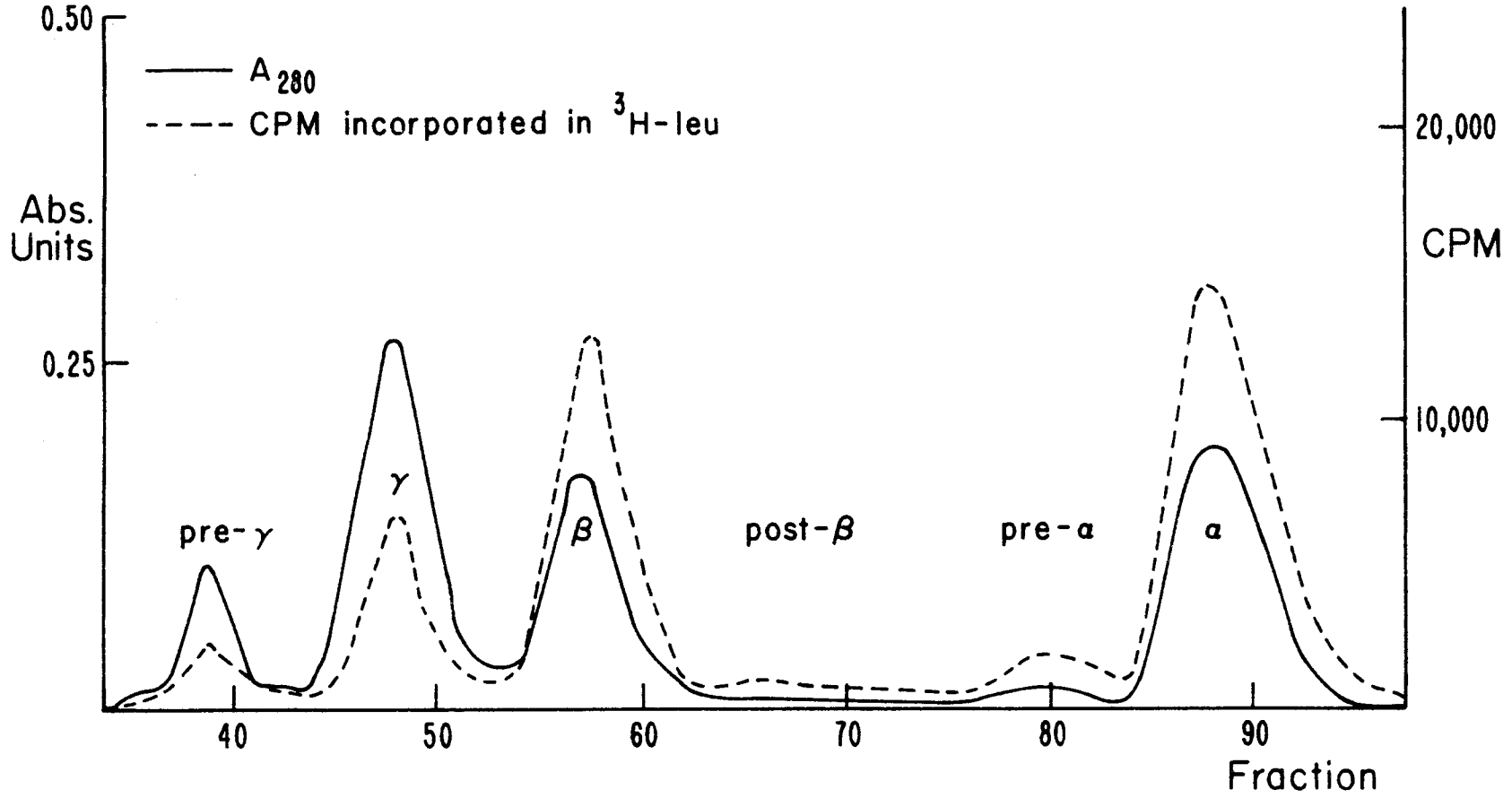

Figure 1. Chain separation on CM-52 cellulose. Zones are pre-γ (frac. 34-44), γ (frac. 45-54), β (frac. 55-64), post-β (frac. 65-75), pre-α (frac. 76-83), and α (frac. 84-96). Fraction size is 3.0 ml.

build up in the cell before increased γ-chain synthesis can begin. In this case, the biosynthetic ratio should become balanced if the incubation continued for a long time.

The experimental plan to explore these questions included three stages. First, amino acid analysis and biosynthetic studies helped to clarify the identity of the minor zones, although their exact chemical composition remains unknown. Next, a series of incubations of normal umbilical cord bloods, as well as blood from individuals having sickle-cell anemia with elevated Hb F levels*, gave data on the synthesis of Hb F in these conditions. Finally, an experiment in which aliquots of a cord blood sample were taken at incubation times ranging from 2.5 minutes to 4 hours provided an indication of how the biosynthetic ratios change with time.

*Sickle-cell blood studies were done by Lois Kay, Coleen Warford, and Steve Baker.

EXPERIMENTAL PROCEDURES

PREPARATION OF RADIOACTIVE SAMPLES--. Samples of normal umbilical cord blood drawn at delivery, or blood of individuals with sickle-cell anemia, were drawn by venipuncture into vacutainer tubes containing EDTA. These samples were kept at 4° C during transportation and storage, and were used for incubation not more than 5 hours after they were drawn. In a 5-hour period after drawing, the number of counts incorporated into the hemoglobin decreases by about half.

Incubation with ^{3}H-leucine or ^{3}H-isoleucine followed a modified procedure of Huisman and Jonxis (60). A 5-ml sample was washed three times with reticulocyte saline at 4° C (composition of reagents is given in the published procedure) and 5.0 ml of incubation mixture was added to the packed cells. For isoleucine incubations, isoleucine was omitted from the incubation mixture and 165.0 mg/l L-leucine was added instead. The suspended cells were added to 25 ml Erlenmeyer flasks containing 5.0 mg glucose and 100 μl of 0.5 mg/5 ml solution of human transferrin (Sigma).

The mixtures were preincubated at 37° C for 10 minutes in a metabolic shaker at 50 cycles/minute. A 0.4-ml sample of ^{3}H-leucine (0.5 mCi/ml, Schwartz-Mann) or ^{3}H-isoleucine (2.0 mCi/ml, Schwartz-Mann) was added, and the mixture was incubated for 2 hours. At the end of incubation, samples were washed three times with cold 0.9% NaCl.

Cells used for globin preparation were frozen in dry ice-ethanol and stored at -20° C. Samples destined for separation of whole hemoglobin were hemolyzed as previously described (61) and in some cases were converted to carbonmonoxyhemoglobin by passing a stream of carbon monoxide gas over the hemolysate before storage at 4° C.

In the timed experiment, a single 25-ml sample of cord blood was incubated in one vessel, and quantities of reagents were scaled up five times. Seven 5-ml aliquots were taken, at 2.5 minutes, 5 minutes, 10 minutes, 30 minutes, 1 hour, 2 hours, and 4 hours. These were immediately added to 30-ml portions of 0.9% NaCl at 4° C, which were washed, carbon monoxylated, and stored as above.

DEHEMING--. Preparation of globin followed a modified procedure of Alter et al. (62). The thawed cells were lysed with 4 times the packed

cell volume of 5 mM $MgCl_2$ at room temperature for 5 minutes. Two ml of the lysate was dripped into 5 ml of acid-acetone (100 ml acetone, 2 ml concentrated HCl, and 7 drops of 2-mercaptoethanol) at -20° C with stirring. Thirty-five ml of acid-acetone was added and the mixture was centrifuged at 2,000 rpm (225 x g) for 5 minutes. The globin was washed once with acid-acetone, three times with acetone, and once with ether. It was then dried under nitrogen and stored at -20° C.

CHROMATOGRAPHY--. Most chromatography was done on CM-52 cellulose; some whole hemoglobin separations were done on DEAE-cellulose.

Chain Separation --. Separation of globin chains was carried out on CM-52 cellulose (Whatman) in a procedure resembling that of Clegg, Naughton, and Weatherall (63). A 5-gm portion of CM-52 cellulose was suspended in 100 ml of a starting buffer composed of 8 M urea (deionized by rolling with mixed-bed resin (Dowex AG 501-X8, Bio-Rad Co) overnight), 0.05 M 2-mercaptoethanol, and 0.005 M $Na_2HPO_4 \cdot 7H_2O$, pH 6.8. This was settled for 15 minutes and fines removed. After repetition of the settling operation, a 0.9 x 10 cm column was poured and equilibrated with 45 ml of starting buffer at 30 ml/hr. A 25-mg sample of globin was dissolved in 3 ml of starting buffer and dialyzed versus 3 x 150 ml of the same fluid at room temperature for 3 hours. This sample was applied to the column and allowed to flow in by gravity. Globin chains were eluted at a flow rate of 30 ml/hr and the absorbance was continuously monitored at 280 nm with an ISCO UA-5 absorbance monitor. Twenty 3-ml fractions were eluted with starting buffer. Subsequent fractions were eluted with a linear gradient between 150 ml of starting buffer and 150 ml of another buffer of the same composition but 0.042 M in $Na_2HPO_4 \cdot 7H_2O$. The resulting separation is shown in Figure 1.

Whole Hemoglobin Separation --. The primary method for separation of whole hemoglobin was chromatography on CM-52 cellulose as described previously (61). Most samples were done using a linear gradient between 500 ml of 0.01 M NaCl, 0.03 M Bis-tris [N, N-bis-(2-hydroxymethyl)-iminotris-(hydroxymethyl)-methane], 0.01% KCN, pH 6.1, and 500 ml of the same but 0.07 M in NaCl (Figure 2). Another method used a

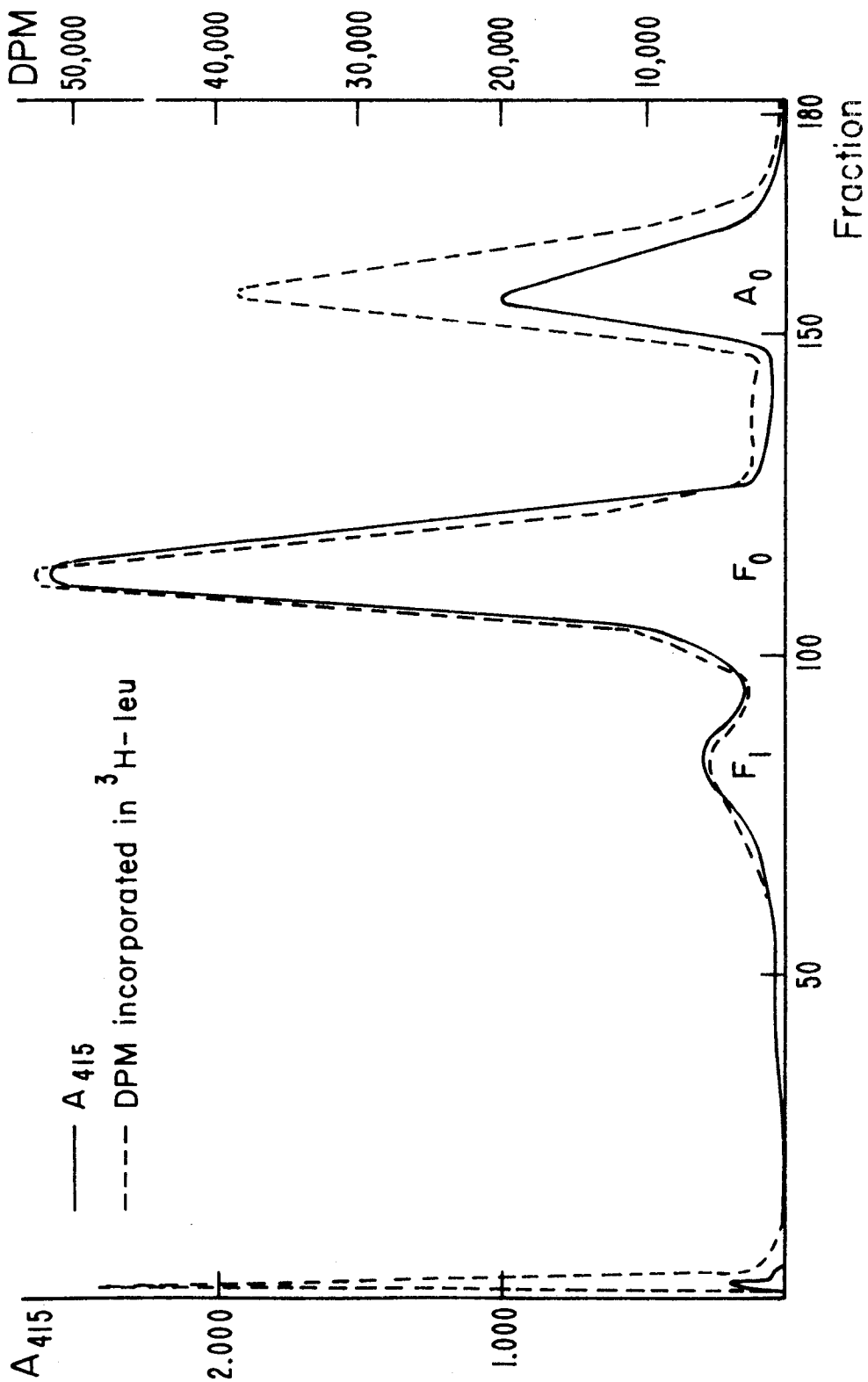

Figure 2. Whole hemoglobin separation on CM-52 cellulose. Zones are Hb Bart's + unincorporated leucine (frac. 2-6), Hb F_1 (frac 65-95), Hb F_0 (frac. 96-135), and Hb A_0 (frac. 147-175). Fraction size is 5.0 ml.

25-ml pregradient of 0.01 M Bis-tris and 0.01% KCN, pH 6.1. This was followed by a linear gradient between 500 ml of 0.03 M Bis-tris and 0.01% KCN, pH 6.1, and 500 ml of the same fluid with 0.08 M NaCl added.

Chromatography of whole hemoglobin on DEAE-cellulose followed published procedures (64).

SCINTILLATION COUNTING--. Into polyethylene scintillation vials (Beckman) were pipetted 2.0 ml of each fraction from the columns, and 10 ml of Aquasol-2 (New England Nuclear). In cases where 8 M urea was used in the developer, 1.5 ml water was added to each vial to prevent the contents from separating into two phases. Samples were counted for either 2 x 5 minutes per vial (chain separations) or 1 x 5 minutes per vial (other experiments) on a Beckman LS-350 scintillation counter. Measurements of samples containing heme groups were corrected for color quenching by the external standard ratio method (65). This method is particularly convenient, as the Beckman scintillation counter automatically prints out the channels ratio (66).

DATA ANALYSIS --. Counts from chain separations were averaged, while those from other separations were corrected for color quenching as explained above. The latter results were corrected to 100% counting efficiency, and are thus expressed in dpm rather than cpm. Counts contained in each chromatographic zone were added to give the total radioactivity for that zone. The graphs of absorbance from each chromatogram were photocopied, and zones cut out and weighed. It was then possible to determine the relative specific activities of the zones by dividing total radioactivity by peak weight. Zones measured at 280 nm were corrected for the different molecular extinction coefficients of the chains, caused by different tryptophan and tyrosine contents, by dividing the weights of γ-chain zones by 2.00 and β-chain zones by 1.52.

Final results were expressed in terms of ratios of zones. This compensates for such things as different absolute amounts of incorporation, radioactive decay of the isotope, and differing amounts of sample applied to columns.

RESULTS

MINOR ZONES--. Amino acid analysis has shown that the zones which elute before fraction 25 of chain separation chromatograms are primarily non-globin protein and unincorporated isotope (unpublished results). The zones of interest here are pre-γ, post-β, and pre-α, as shown in Figure 1. In addition, there appears to be a zone called post-γ, which chromatographs in the same place as the β chain (Figure 3). The primary data referenced in this section are given in the Appendix.

Pre-γ zone--. Amino acid analysis of the pre-γ zone (Table 1) showed γ chain. This is consistent with the assumption that the zone contains acetylated γ chain from Hb F_1. Therefore, the pooled Hb F_1 zones from 8 CM-cellulose columns were dialyzed, concentrated (67), dehemed, and run on a chain separation column. The result is shown in Figure 4. The area under the pre-γ peak is 2.10 times that under the γ-chain peak, representing a great enhancement of the former. In addition, the pre-α zone in Figure 4 is somewhat larger than usual. The α chain of Hb F_1 has not been reported to be modified, and there is no apparent explanation for the unusual pre-α zone.

TABLE 1

AMINO ACID ANALYSIS OF PRE-γ ZONE

	μmoles	Residues	Theoretical γ chain
lys	0.190	11.9	12
his	0.097	6.1	7
arg	0.046	2.9	3
asp	0.213	13.3	13
thr	0.153	9.6	10
ser	0.163	10.2	11
glu	0.206	12.9	12
pro	0.066	4.1	4
gly	0.211	13.2	13
ala	0.181	11.3	11
val	0.203	12.7	13
met	0.031	1.9	2
ile	0.059	3.7	4
leu	0.273	17.1	17
tyr	0.030	1.9	2
phe	0.126	7.9	8

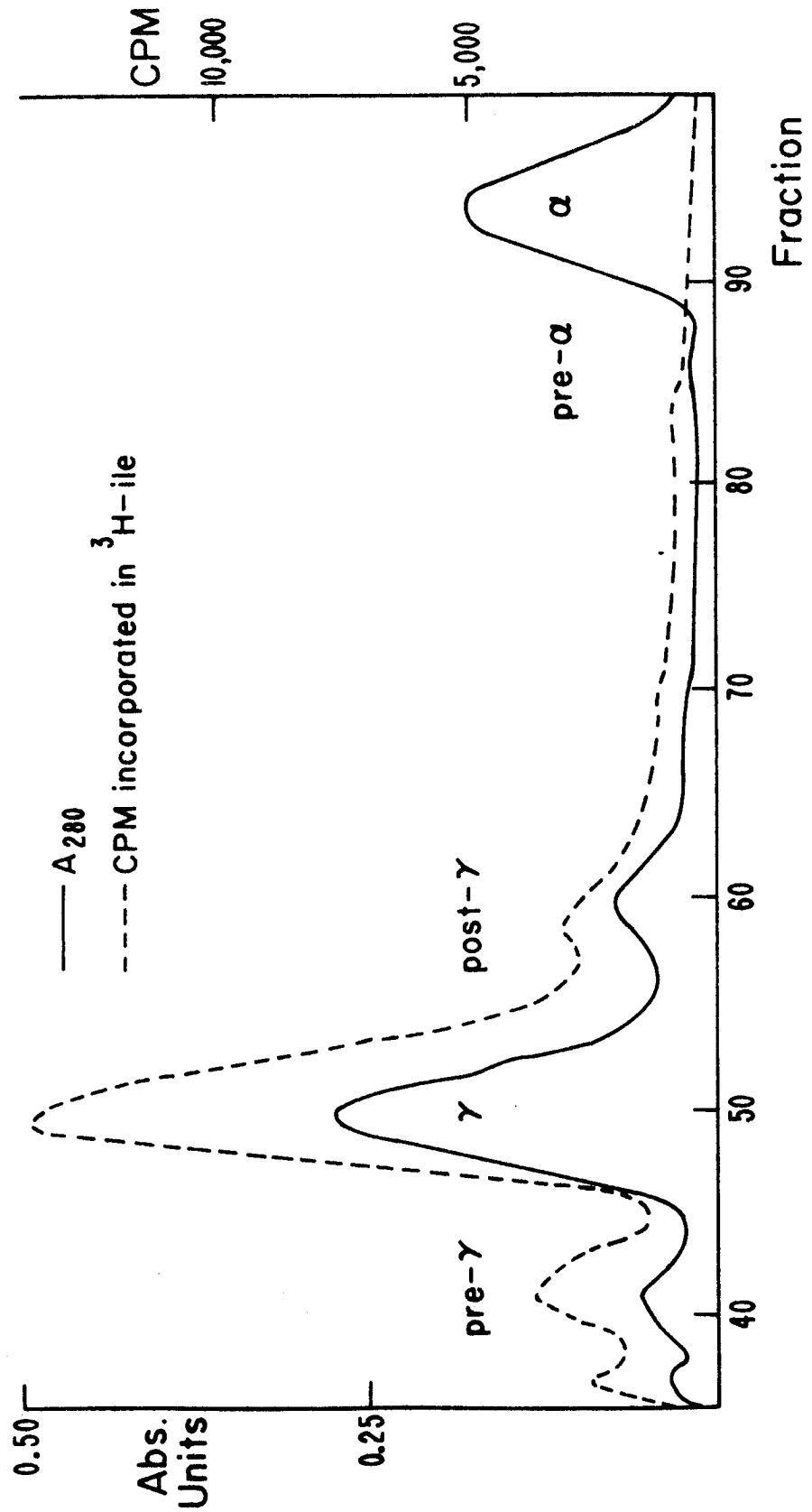

Figure 3. Chain separation of isoleucine incubated whole globin showing post-γ zone (frac. 58-69). Fraction size is 3.0 ml.

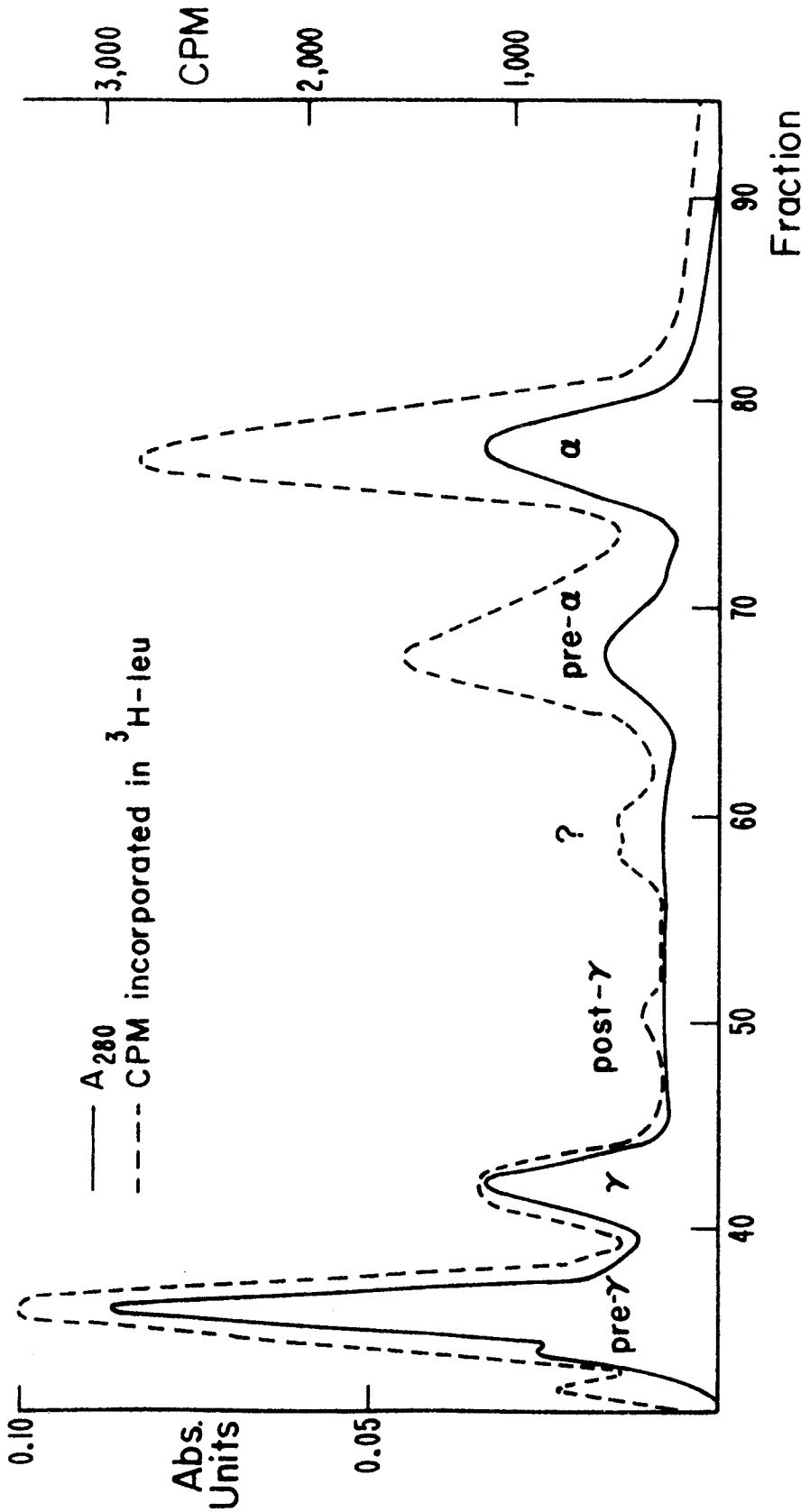

Figure 4. Chain separation of Hb F_1 purified by CM-52 cellulose chromatography. Fraction size is 3.0 ml.

The fact that there is some γ chain present in the chromatogram in Figure 4 is due either to contamination of the sample (since Hb F_0 is not well separated from Hb F_1 on CM-cellulose) or to deblocking of the pre-γ chain. In most chromatograms, the pre-γ zone chromatographs as two incompletely separated zones. Figure 4 shows one of these at fraction 33 and the other at fractions 34-39. The difference between these zones is unknown, although Abraham et al. (68) have suggested that γ chain may be glycosylated as well as acetylated. Thus, there may be more than one modified γ chain present in the pre-γ zone. It is clear, however, that the pre-γ zone is a product of γ-chain mRNA and should be added into biosynthetic ratios as such.

Post-β zone--. Amino acid analysis of the post-β zone from a normal cord blood (Table 2) presented a problem of interpretation. The values for ile, val, ser, and his seem to indicate that the zone contains γ chain, but the values for ala, thr, and pro are somewhat different from the expected values for γ chain, and probably indicate that some β chain is present. An analysis of the zone from a chromatogram of normal adult blood (Table 3) indicates β chain. The zone is therefore probably β chain, but it may be contaminated with rather substantial amounts of γ chain in chromatograms of cord blood. Although there is some doubt about the identity of the zone, it is included in biosynthetic ratios as β chain in this study.

Pre-α zone--. The amino acid analysis of a cord blood pre-α zone (Table 4) shows that this zone is mostly α chain. This is apparent in the values for his, glu, and pro. There is, however, some contamination with γ chain, as is apparent from the altered values for gly, ala, and ile. The contamination is not so bad as it is in the post-β zone, and an analysis of the zone from a normal adult blood (Table 5) shows α chain. The pre-α zone is therefore counted as α chain in this study.

Post-γ zone--. Incubation of a sample with ^{3}H-isoleucine, contained in only the γ chain in humans, should have resulted in radioactive incorporation only in the regions of the chromatogram in which γ chain occurs. It was therefore surprising to find isoleucine incorporation in the region of the β chain (Figure 3). In the experiment shown in Figure 3, incorporation in the β chain region was 22% of that in the γ chain

TABLE 2

AMINO ACID ANALYSIS OF POST-β ZONE FROM CORD BLOOD

	μmoles	Residues	Theoretical γ chain	Theoretical β chain
lys	0.069	10.8	12	11
his	0.042	6.6	7	9
arg	0.021	3.2	3	3
asp	0.086	13.4	13	13
thr	0.054	8.4	10	7
ser	0.068	10.6	11	5
glu	0.075	11.7	12	11
pro	0.032	5.0	4	7
gly	0.086	13.4	13	13
ala	0.084	13.1	11	15
val	0.083	13.0	13	18
met	0.008	1.3	2	1
ile	0.015	2.3	4	0
leu	0.108	16.9	17	18
tyr	0.016	2.5	2	3
phe	0.049	7.7	8	8

TABLE 3

AMINO ACID ANALYSIS OF POST-β ZONE FROM NORMAL ADULT BLOOD SAMPLE

	μmoles	Residues	Theoretical β chain
lys	0.125	11.2	11
his	0.091	8.2	9
arg	0.035	3.1	3
asp	0.149	13.4	13
thr	0.077	6.9	7
ser	0.058	5.2	5
glu	0.121	10.9	11
pro	0.079	7.1	7
gly	0.141	12.7	13
ala	0.172	15.4	15
val	0.184	16.5	18
met	0.012	1.1	1
ile	0.004	0.3	0
leu	0.206	18.5	18
tyr	0.032	2.9	3
phe	0.088	7.9	8

TABLE 4

AMINO ACID ANALYSIS OF PRE-α ZONE FROM CORD BLOOD

	μmoles	Residues	Theoretical α chain	Theoretical γ chain
lys	0.121	11.9	11	12
his	0.094	9.2	10	7
arg	0.030	2.9	3	3
asp	0.127	12.4	12	13
thr	0.086	8.4	9	10
ser	0.120	11.8	11	11
glu	0.080	7.8	5	12
pro	0.066	6.5	7	4
gly	0.104	10.1	7	13
ala	0.185	18.1	21	11
val	0.126	12.3	13	13
met	0.016	1.6	2	2
ile	0.010	1.0	0	4
leu	0.179	17.5	18	17
tyr	0.030	2.9	3	2
phe	0.072	7.0	7	8

TABLE 5

AMINO ACID ANALYSIS OF PRE-α ZONE FROM NORMAL ADULT BLOOD SAMPLE

	μmoles	Residues	Theoretical α chain
lys	0.176	10.6	11
his	0.151	9.1	10
arg	0.046	2.8	3
asp	0.211	12.7	12
thr	0.142	8.6	9
ser	0.159	9.6	11
glu	0.106	6.4	5
pro	0.122	7.3	7
gly	0.138	8.3	7
ala	0.338	20.4	21
val	0.215	13.0	13
met	0.029	1.7	2
ile	--	0	0
leu	0.297	17.9	18
tyr	0.048	2.9	3
phe	0.122	7.3	7

plus pre-γ regions, implying that a previously unrecognized zone is hidden under the β chain. Because γ chain and β chain separate incompletely in this type of chromatography, the amount of radioactivity in the γ-chain regions is apparently underestimated by about 20%. Chain separation of Hb F_0 isolated by CM-cellulose chromatography (Figure 5) shows a post-γ zone whose total absorbance at 280 nm is 23% that of the γ-chain plus pre-γ zones. The post-γ zone either chromatographs in the same place as Hb F_0 or Hb F_1 on CM-cellulose, or it is produced by the chain separation procedure. The identity of the post-γ zone was determined by amino acid analysis (Table 6) of the β-chain zone from a nonradioactive cord blood. This analysis is consistent with a sample containing about 33% γ chain and the remainder β chain. The area under the β-chain peak analyzed in Table 6 was 30.0% that of the total area under the pre-γ, γ-chain, and β-chain peaks combined. The post-γ zone thus contains 30.0 x 0.33 x 2.00/1.52 = 13% of the total protein in the three peaks. The γ-chain plus pre-γ protein accounted for 70.0% of the total under the three peaks, so the ratio post-γ/γ + pre-γ is 0.19.

TABLE 6

AMINO ACID ANALYSIS OF β CHAIN ZONE FROM CORD BLOOD

	μmoles	Residues	Theoretical β chain	Theoretical γ chain	Theoretical $1\gamma:2\beta$
lys	0.134	11.2	11	12	11.3
his	0.091	7.6	9	7	8.3
arg	0.035	2.9	3	3	3.0
asp	0.158	13.2	13	13	13.0
thr	0.089	7.4	7	10	8.0
ser	0.079	6.6	5	11	7.0
glu	0.138	11.5	11	12	11.3
pro	0.071	5.9	7	4	6.0
gly	0.158	13.2	13	13	13.0
ala	0.163	13.6	15	11	13.7
val	0.181	15.1	18	13	16.3
met	0.013	1.1	1	2	1.3
ile	0.015	1.3	0	4	1.3
leu	0.216	18.0	18	17	17.6
tyr	0.032	2.7	3	2	2.7
phe	0.096	8.0	8	8	8.0

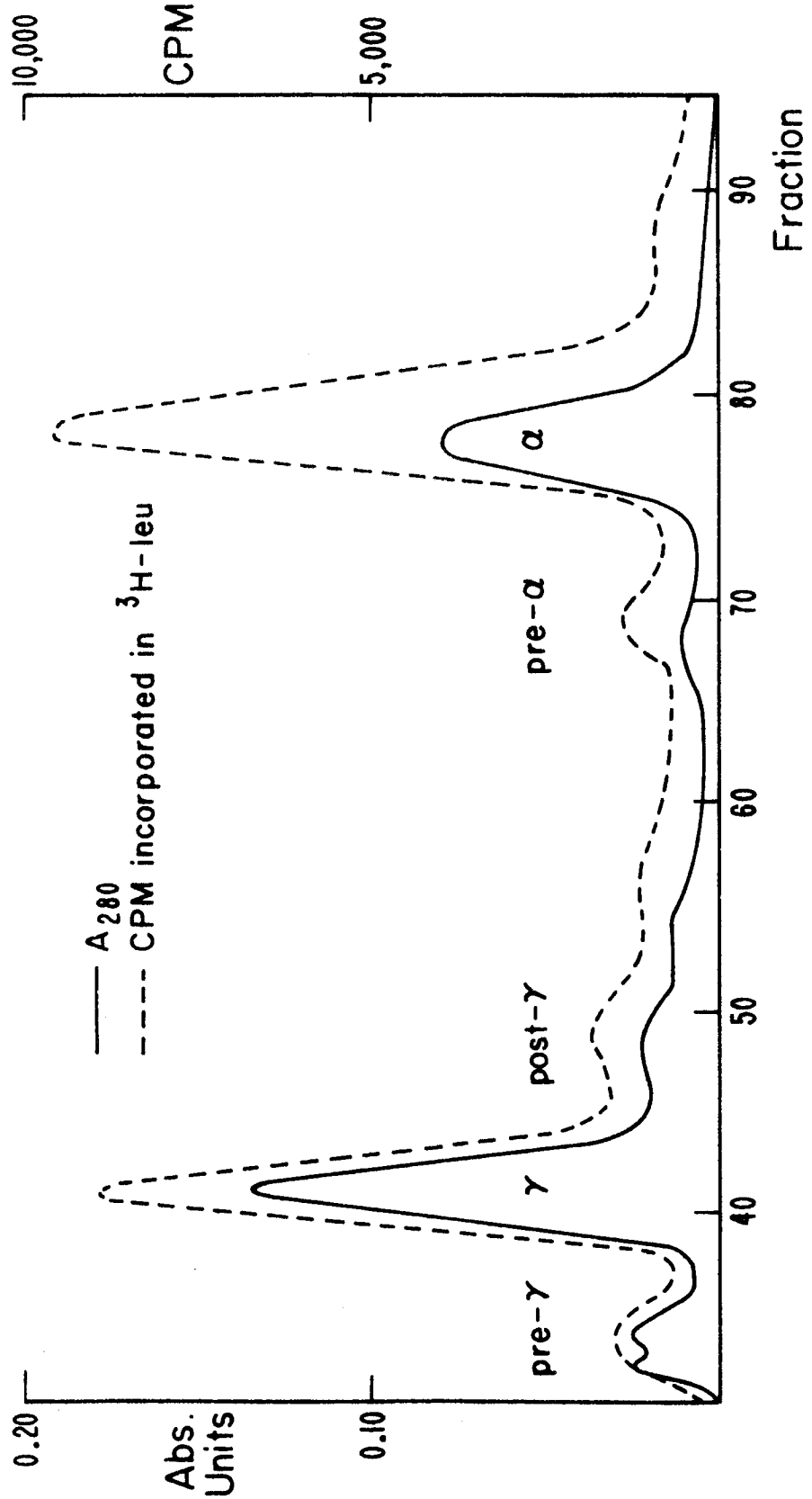

Figure 5. Chain separation of Hb F_o purified by CM-52 cellulose chromatography. Fraction size is 3.0 ml.

It is not clear how to systematically include the post-γ zone in biosynthetic studies. The amount of γ chain which registers in biosynthetic ratios as β chain certainly depends on the goodness of the chromatographic separation. Because of the possible variability of the post-γ zone, further investigation will be necessary before it is possible to include it systematically in biosynthetic ratios.

Because of the indications given in this section that the pre-γ, post-β, and pre-α zones are hemoglobin chains, the biosynthetic ratios presented in the following sections include these minor zones. In addition, it is important to keep in mind that approximately 20% of the γ-chain in cord bloods appears to be hidden under the β-chain peak of the chromatograms, although it is not yet feasible to include this zone in biosynthetic ratios.

CORD AND SICKLE-CELL BLOOD SERIES--. In order to determine the variation in the biosynthetic ratios as the percent Hb F changes, 9 umbilical cord blood samples and 6 sickle-cell bloods were incubated and chromatographed to separate protein chains, and again to separate whole hemoglobin as explained under Experimental Procedures. The cord bloods were randomly sampled, and contained from 89.0% to 73.6% Hb F_0 plus Hb F_1. Sickle-cell bloods were selected so that none of the individuals carried a diagnosis of thalassemia; some samples were also rejected because the ratio of total radioactivity α + pre-α/β + post-β + γ + pre-γ was outside the range 1.00 ± 0.20, even though there was no diagnosis of thalassemia. The sickle-cell bloods contained from 12.8% to 29.1% Hb F_0 plus Hb F_1.

Results are presented as graphs of percent non-γ-chain containing hemoglobin (i.e. percent $A_0 + A_2 + S_0 + S_1$) versus the total radioactivity incorporated into γ + pre-γ/β + post-β (Figure 6), β + post-β/α + pre-α (Figure 7), and γ + pre-γ/α + pre-α (Figure 8).

Figure 6 shows that there is a smooth change from γ-chain synthesis to β-chain synthesis, which is arrested but still in the normal pattern in the sickle-cell bloods shown. Figure 7, too, shows that the sickle-cell bloods are on a smooth curve in relation to the cord bloods. In both curves, the synthesis of β chain reaches half its maximum value before the amount of Hb A in the sample exceeds 20%. This is not

Figure 6. Total radioactivity ratio γ + pre-γ /β + post-β is plotted versus the percent of hemoglobins $A_0 + A_2 + S_0 + S_1$. Cord bloods have 11.0-26.4% non-γ hemoglobin, and sickle-cell bloods have 70.9-87.2%.

Figure 7. Total radioactivity ratio β + post-β /α + pre-α is plotted versus the percent of hemoglobins $A_0 + A_2 + S_0 + S_1$.

Figure 8. Total radioactivity ratio γ + pre-γ /α + pre-α is plotted versus the percent of hemoglobins $A_0 + A_2 + S_0 + S_1$. The data are extrapolated by the method of least squares to 0, giving a theoretical ratio of 0.47 ± 0.03 for individuals with HPFH.

Figure 9. Total radioactivity ratio γ + pre-γ /$(\alpha$ + pre-$\alpha)_\gamma$ is plotted versus percent of hemoglobins $A_0 + A_2 + S_0 + S_1$. Mean ± standard deviation is given for each data set.

Figure 10. Total radioactivity ratio β + post-β/$(\alpha$ + pre-$\alpha)_\beta$ is plotted versus the percent of hemoglobins $A_0 + A_2 + S_0 + S_1$. A straight line is fitted to the data, although they are not necessarily in a linear relationship.

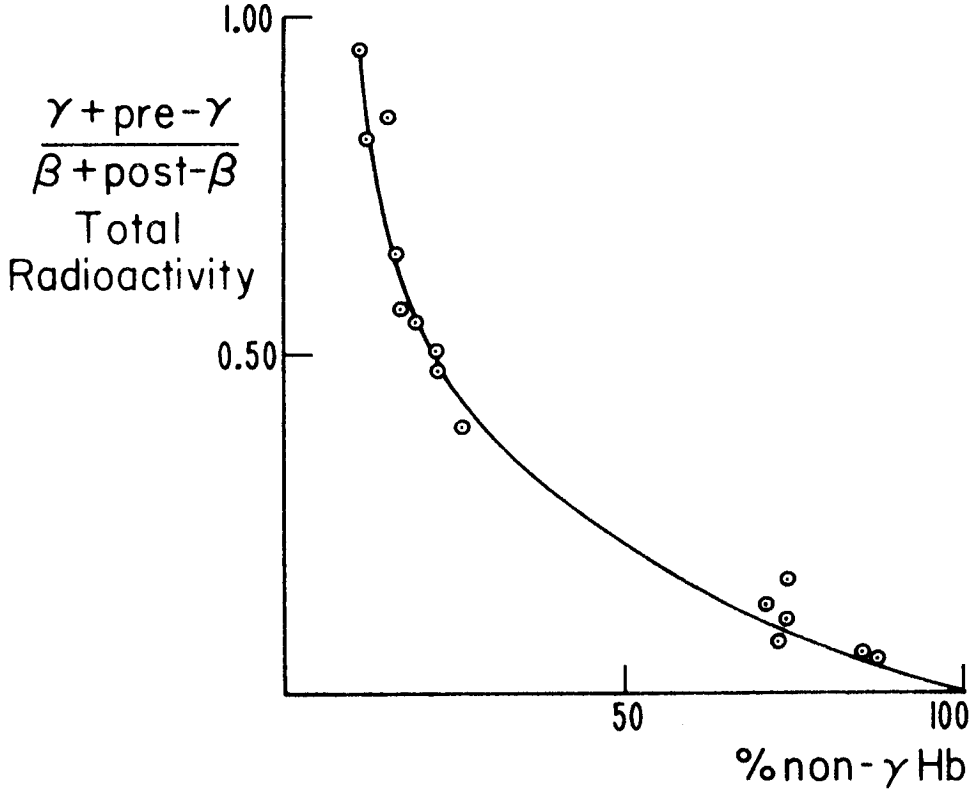

Figure 6

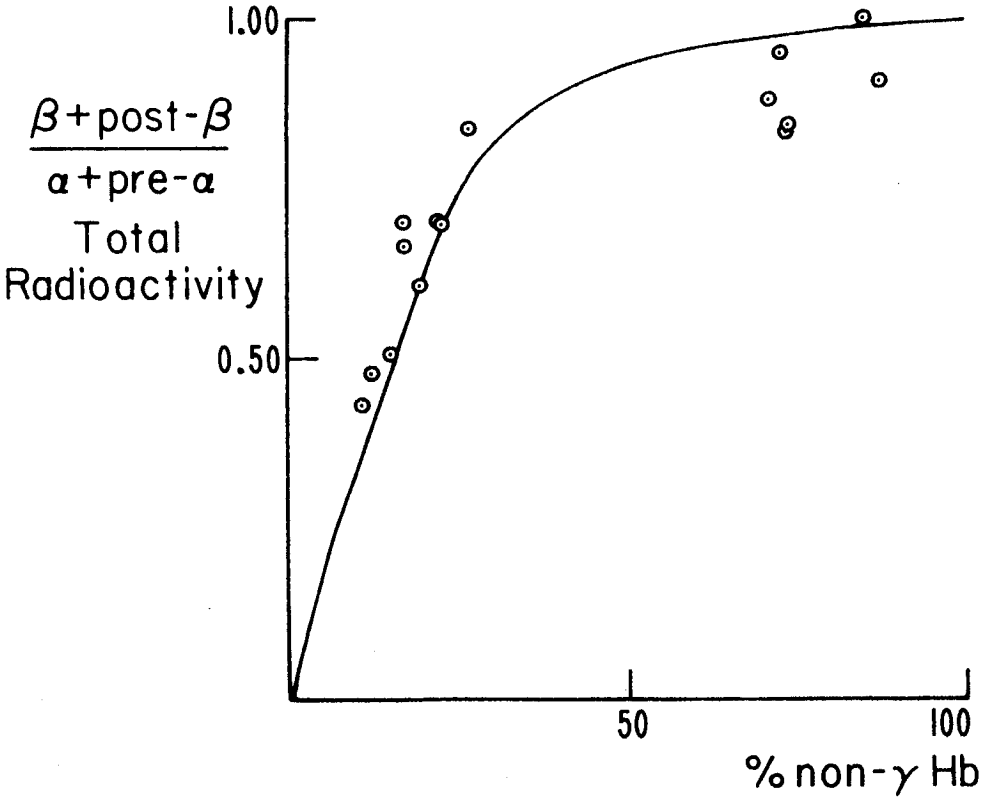

Figure 7

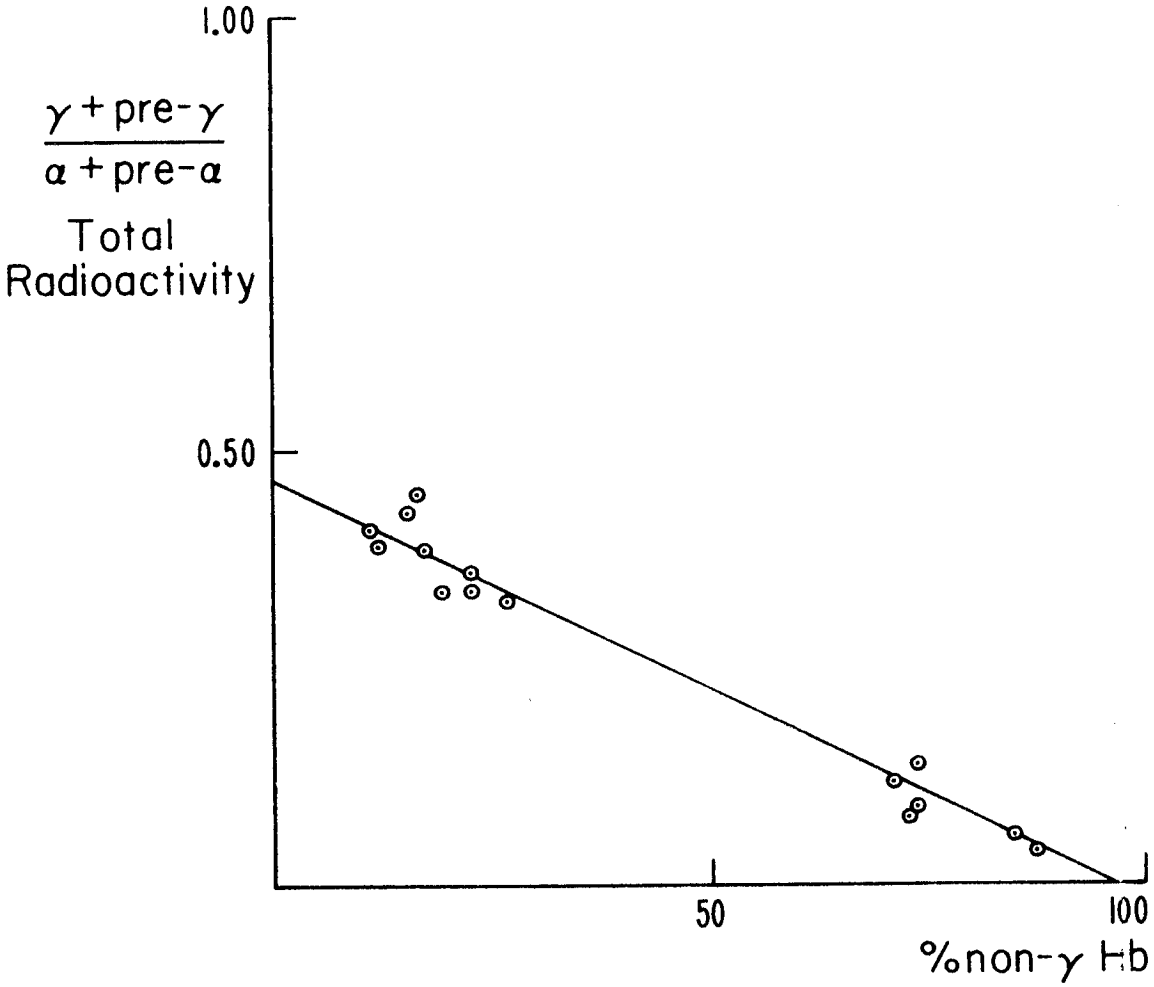
1.00
$\frac{\gamma + \text{pre-}\gamma}{\alpha + \text{pre-}\alpha}$
Total
Radioactivity
0.50
50
100
%non-γ Hb

Figure 8

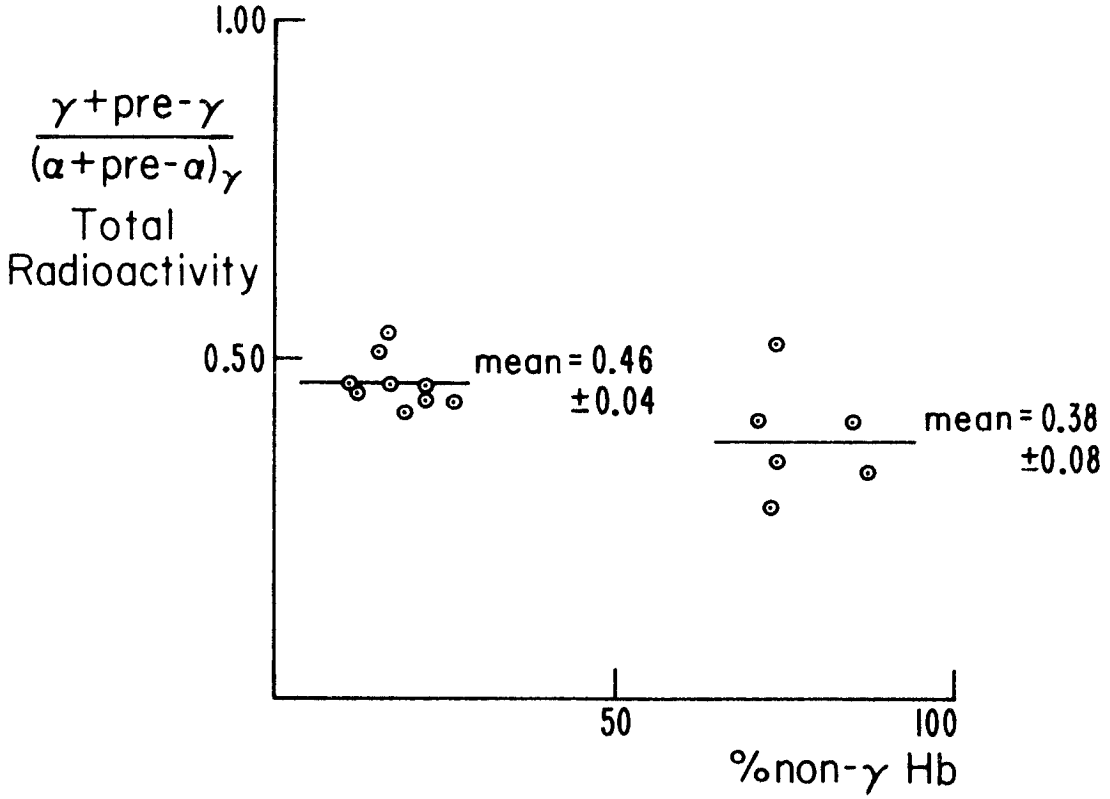

Figure 9

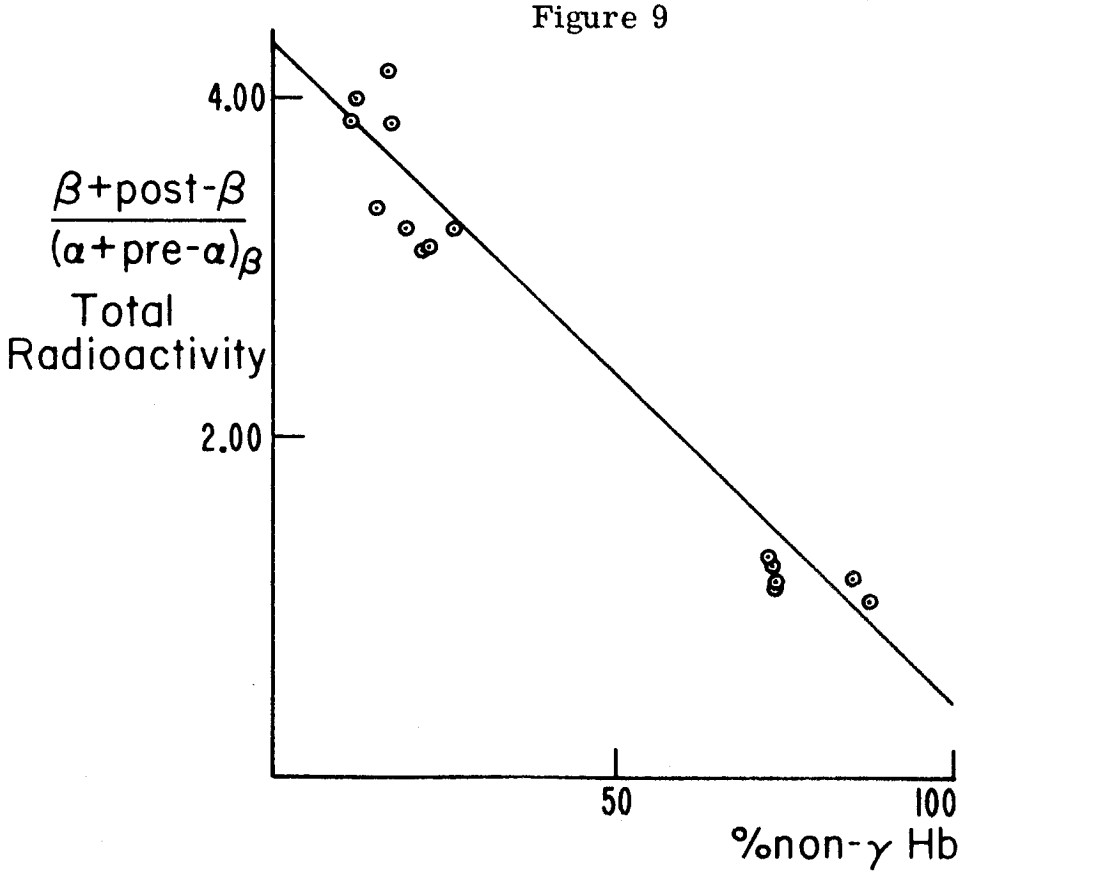

Figure 10

unexpected, as erythrocytes containing γ chain continue to circulate after they have stopped making hemoglobin. Figure 8 again shows the smooth relation of sickle-cell to cord blood synthesis, and is also of interest because the line may be extrapolated by the method of least squares to find the theoretical biosynthetic ratio for 100% γ-chain containing hemoglobin (for example, homozygous HPFH). The graph predicts that an individual with homozygous HPFH should have a total radioactivity ratio γ + pre-γ /α + pre-α equal to 0.47 ± 0.03. Literature values for this ratio range from 0.30 to 0.68, and the value found here represents a median between these figures.

In an attempt to dissociate the biosynthetic ratios from the amounts of Hbs A or S in the sample, the total radioactivity ratios γ + pre-γ / $(\alpha + \text{pre-}\alpha)_\gamma$ and β + post-β /$(\alpha + \text{pre-}\alpha)_\beta$ were calculated. These numbers are found by knowing the number of counts in each chain and the percent of each type of hemoglobin in the sample. It is then possible to calculate the cpm in α chains which are associated with β chains, and the cpm in those associated with γ chains. For example, if chain separation shows 10,000 cpm in the α-chain + pre-α zones, and whole hemoglobin separation shows 80% Hb A, then $(\alpha + \text{pre-}\alpha)_\beta$ = 0.80 x 10,000 = 8,000 cpm, and $(\alpha + \text{pre-}\alpha)_\gamma$ = 0.20 x 10,000 = 2,000 cpm. The total radioactivity ratios γ + pre-γ /$(\alpha + \text{pre-}\alpha)_\gamma$ and β + post-β / $(\alpha + \text{pre-}\alpha)_\beta$ are plotted versus percent non-γ hemoglobin in Figures 9 and 10, respectively. Figure 10 shows a correlation between the amount of β chain produced and the counts incorporated into the α chain. A straight line has been fitted to the points by the method of least squares, although the relation is not necessarily linear. This relationship is not unexpected, as correlation between α-chain and β-chain syntheses has been implicated in the literature (34). Figure 9, however, shows that α-chain synthesis is apparently independent of the amount of γ chain produced. The two mean values shown in Figure 9 are not significantly different.

Ratios of total radioactivity from separations of whole hemolysate on CM-cellulose, such as A_0/F_0 and F_1/F_0, were unrelated to the parameters given in Figures 6 through 10. If there were some simple mechanism for hemoglobin biosynthesis (such as assembly of hemoglobin from chains directly off the ribosomes), then a simple relationship

between ratios found in whole hemoglobin separations and those from chain separations (such as pre-$\gamma/\gamma = F_1/F_0$) should be found. The fact that this does not occur may reflect the presence of pools of free hemoglobin chain in the cytoplasm, through which newly synthesized chain must pass. These pools would be included in chain separations, but not in whole hemoglobin separations.

TIMED EXPERIMENT--. Results of the timed experiment are given for the major hemoglobin chains in Figures 11 and 12, and for minor zones in Figures 13 and 14. Figures 11 and 12 show that the ratios γ/α do not change significantly after 30 minutes of incubation. There is an initial burst of γ-chain synthesis, or perhaps a lack of α-chain synthesis relative to γ-chain synthesis, followed by a smooth change.

The behavior of γ-chain synthesis contrasts to that of β-chain synthesis, shown in the same two figures. In the latter case, there is a sharp dip in β-chain synthesis at 5 minutes, or an excess of α-chain synthesis relative to β-chain synthesis, followed by a rise. The subsequent behavior of the ratios β/α is open to question. The curves would be flat if the 2 hour point were an experimental error. The same results were, however, obtained with two separate sets of columns, although only one sample was studied. There must, therefore, be some doubt as to the validity of the 2 hour point.

The initial changes in the ratios in Figures 11 and 12 could conceivably be interpreted as showing different response times of different chain syntheses to incubation conditions. The γ-chain synthetic apparatus might have an extremely rapid response, followed by that of α chain. This would explain the initially high γ/α ratios, and the sudden dip in the β/α ratios, caused by a sudden increase in α-chain synthesis. β-chain synthesis might then increase in response to increased α-chain synthesis, as suggested in the literature. This would account for the increase in the β/α ratios.

Figures 13 and 14, the results of timed study of the minor zones, show several interesting features. Because the lines for post-β/β and pre-α/α are nearly parallel in both graphs, the dynamics of modification may be similar. The actual chemical changes, however,

Figure 11. Change in the total radioactivity ratios of the major hemoglobin chains of cord blood in a timed incubation.

Figure 12. Change in the specific activity ratios of the cord blood shown in Figure 11.

Figure 13. Total radioactivity ratios of cord blood minor zones compared with the corresponding major zones as a function of time.

Figure 14. Change in the specific activity ratios of the minor zones shown in Figure 13.

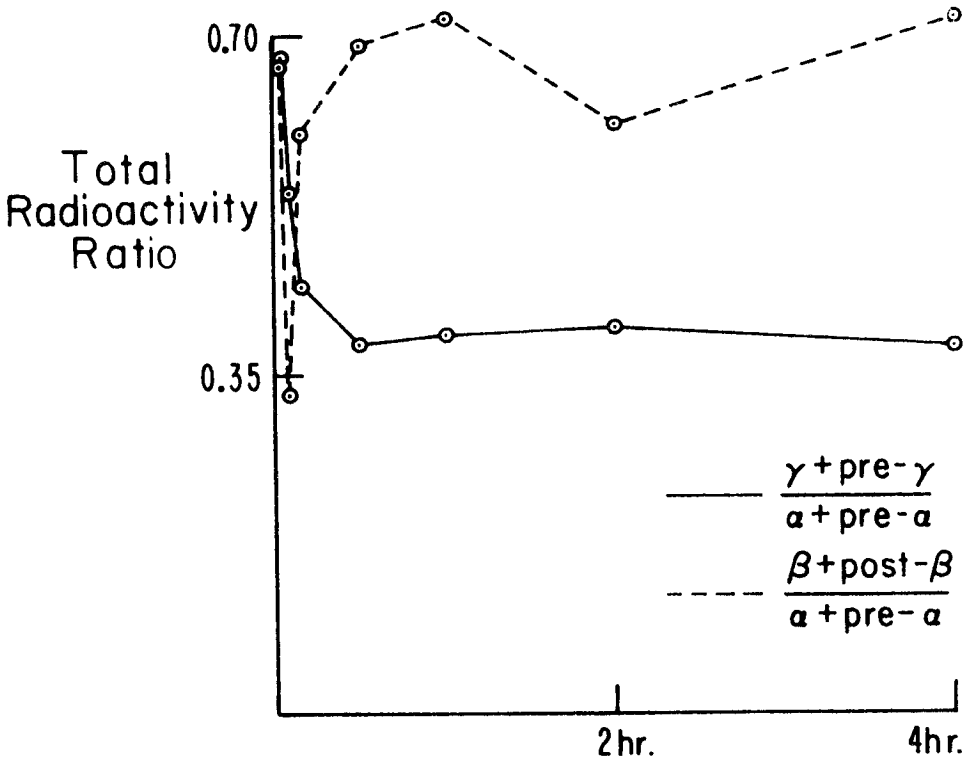

Figure 11

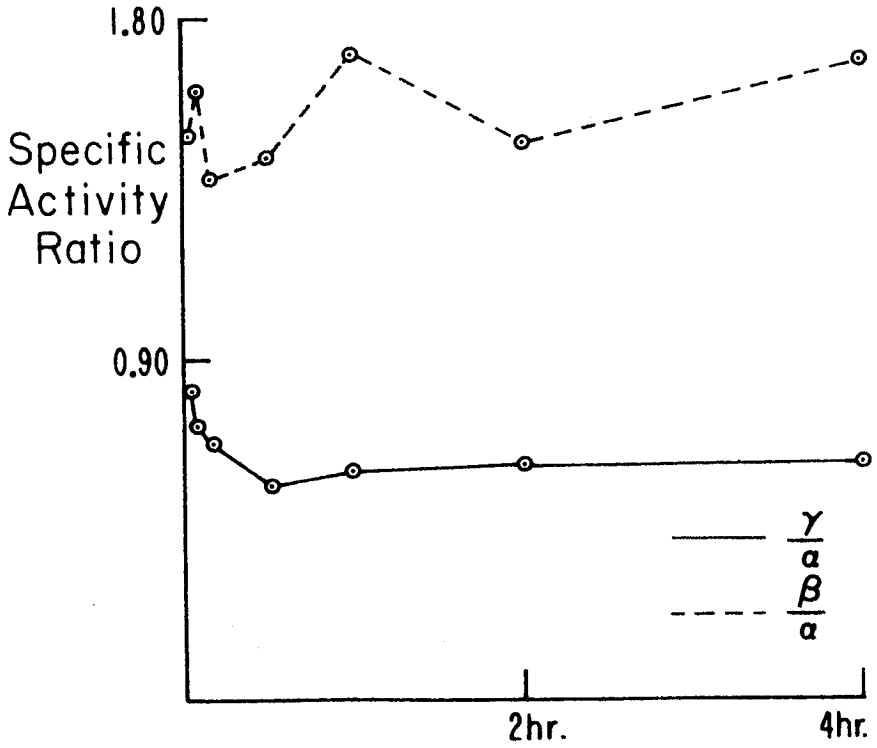

Figure 12

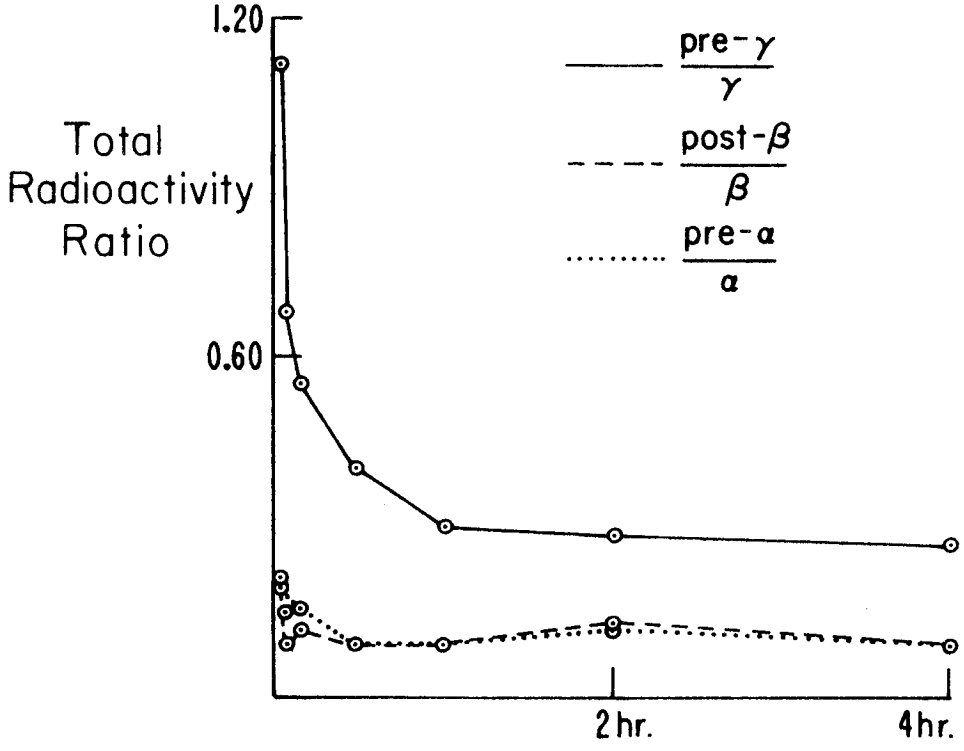

Figure 13

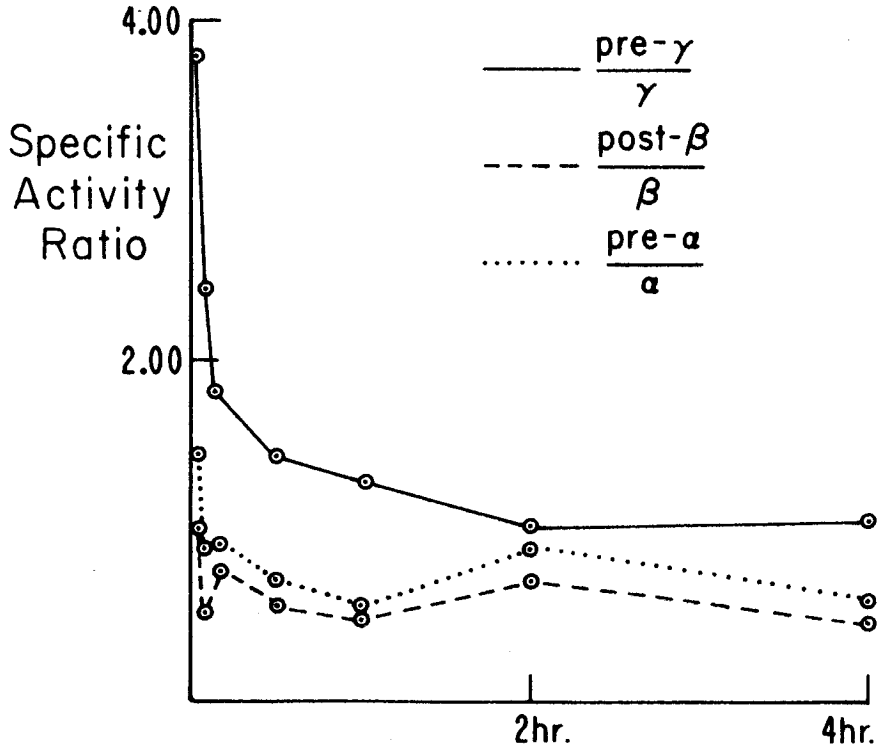

Figure 14

are not necessarily the same, since the post-β zone is chromatographically retarded with respect to its parent peak, while the pre-α zone elutes earlier than its parent peak.

The change in the pre-γ/γ ratios with respect to time presented a puzzle. If the pre-γ zone were a post-translational modification of γ chain, then the ratio pre-γ/γ should start at some low value and increase as γ chain is modified. Figures 13 and 14 show the reverse to be true. This implies that the modification must take place before 2.5 minutes of incubation, perhaps on the ribosome. Abraham et al. (68) came to this same conclusion by means of a pulse-chase experiment and a cycloheximide inhibition experiment. The results of both their studies showed that Hb F_1 synthesis is dependent on Hb F_0 synthesis, and modification occurs very early.

This extremely early modification has rather interesting consequences. The excess modified γ chain found at short incubation times appears to deblock to give γ chain. This would account for the decrease in the ratio pre-γ/γ. The initial high value of 1.12 for the ratio indicates that the translational products of γ-chain mRNA, whether they are modified on the ribosome or shortly after they leave it, are at some point mostly in a blocked form. This blocked γ chain is the material from which Hbs F_0 and F_1 are assembled. However, the initial pre-γ/γ ratio of total radioactivity is 1.12, while the ratio of Hb F_1/Hb F_0 is usually only about 0.25. There must thus be deblocking at some point before the final ratio of whole hemoglobins is reached.

The kinetics of the deblocking process are conceivably rather complicated. It is clearly not necessary for the γ chain to be deblocked before whole hemoglobin is formed, and it may, in fact, deblock after Hb F_1 is formed. One possible model is a steady-state process, in which blocked γ chain is continuously supplied from translation, and depleted by the formation of Hb F_0. The details of the deblocking process must, however, be elucidated through further experiment.

CONCLUSIONS

As shown in Figure 8 and as implicated in the literature on HPFH, there is an imbalance between the synthesis of γ chain and that of α chain in reticulocytes. The total radioactivity ratio γ + pre-γ/α+ pre-α should be 0.47 ± 0.03 in HPFH as predicted by the data in Figure 8; the ratio which previous investigators actually found in this condition ranged from 0.30 to 0.68. It is thus apparent that reduced synthesis of the γ chain in reticulocytes is a property of cord blood and sickle-cell blood, as well as blood from people with HPFH.

There were four ideas advanced in the introduction to explain this imbalance. First, exclusion of the minor zones from the biosynthetic ratios might have altered the ratios by leaving out large amounts of γ-chain mRNA products which should have been included. This hypothesis became especially attractive with the finding that the pre-γ zone and the previously undetected post-γ zone are both apparently products of γ-chain mRNA. The pre-γ zone is included in the γ + pre-γ/α+ pre-α ratio of 0.47 ± 0.03 obtained in Figure 8. While it was not possible to include the post-γ zone in the ratio, the radioactivity incorporated into the post-γ zone is estimated at about 20% of that in the γ and pre-γ zones combined. The ratio with the post-γ zone included should therefore be approximately 0.47 + (0.47 x 0.20) =0.56. While this is an improvement over the previous result of 0.47, it is hardly a balanced synthesis. The minor zones are therefore insufficient to account for the imbalance by themselves.

A second explanation for the imbalance of γ-chain and α-chain syntheses was advanced by DeSimone and Mueller (57). They found a pool of free α chains in the cytoplasm, and hypothesized that this pool labels to very high specific activity during incubation. The radioactive α chain in the pool might then exchange with non-radioactive α chain from previously existing Hb F. There would thus be a substantial quantity of Hb F which had radioactive α chains but non-radioactive γ chains.

As shown in the present experiments and the previous work on HPFH, however, the imbalance extends to the pool as well as to the whole Hb F. DeSimone and Mueller separated the whole hemoglobin from the pool by

electrophoresis before determining that there was an imbalance. In the other experiments, no separation of the pools by electrophoresis was attempted. Instead, the imbalance was found to be a property of all the hemoglobin in the cell. While it may be true that α chain in completed hemoglobin exchanges with a pool, this appears not to be the explanation for the imbalance.

The third possible explanation, that γ-chain mRNA may have a shorter lifetime than α-chain mRNA, is the most likely one, given the results of the present experiments. Burka and Marks (59) did an experiment similar to the present study and came to the conclusion that mRNA lifetimes probably differ. Pursuant to that study, Prchal and Neuwirt (14) did a 16.5 hour incubation and found that the total radioactivity ratio γ/α decreased as a function of time. In addition, a shorter mRNA lifetime has been implicated in the synthesis of the δ-chain. The hypothesis of different mRNA lifetimes has not been subject to direct experimental test, but is nevertheless very attractive.

It is not clear whether the mRNA actually physically deteriorates, or whether it is simply made unavailable for synthesis by attachment of poly(A) tails (15-17) or some other mechanism. A study of γ-chain synthesis in the bone marrow might be interesting in this context. If the γ-chain mRNA physically decays in the reticulocyte, then it may or may not also do so in the bone marrow. This depends on whether or not the same processes operate in the bone marrow and the peripheral blood to break down mRNA. If so, there would need to be more γ-chain than α-chain synthesis in the marrow to keep a balanced synthesis of Hb F. If, on the other hand, γ-chain mRNA is polyadenylated, then some mechanism in the bone marrow might prevent polyadenylation and insure a balanced synthesis.

The fourth possible explanation for the synthetic imbalance is that a transient phenomenon, caused by the unphysiological conditions of incubation, results in incorrect ratios. This explanation is not susceptible to a test using the present method of incubation. In the timed experiment, however, there was some evidence that all mRNAs do not begin synthesis at the same time, but instead γ-chain mRNA begins before α-chain mRNA, which begins before β-chain mRNA in turn. This phenomenon probably

does not last longer than 30 minutes after the start of incubation, and becomes progressively less pronounced with time (Figures 11 and 12). The question therefore hinges on whether the transient imbalance exists, and whether enough hemoglobin can be synthesized in 30 minutes to throw the ratios off. The latter is probably a correct assumption. However, if there were balanced synthesis after the transient imbalance, then the γ/α total radioactivity ratio should gradually approach 1.0 as more hemoglobin is synthesized. In neither the present experiment nor the 16.5 hour incubation of Prchal and Neuwirt (14) does the ratio show any sign of increase. The existence of a transient imbalance is thus possible, but unlikely.

SUGGESTIONS FOR FURTHER WORK

The present investigation, as well as previous work, has provided indirect evidence of a difference in γ-chain and α-chain mRNA lifetimes. It is desirable, however, to have more direct evidence from investigation of the mRNA itself. Experiments on hemoglobin mRNA might provide quantitative data on its lifetime, and also might clarify what, if any, mechanisms the cell may use to modify the mRNA lifetime.

There are several incubation experiments which might be useful. As mentioned under Conclusions, a study of γ-chain synthesis in the bone marrow might reveal a γ/α total radioactivity ratio greater than 1.0 if it is true that γ-chain mRNA has a shorter lifetime than α-chain mRNA. Such an experiment could be done in the same way as the present study, with samples possibly obtained from stillborn infants.

It might be useful to repeat the timed experiment, especially if extremely short times were included. Figures 11 and 12 show β/α ratios that do not reach a stable state with time. If these data are correct, then there is perhaps some mechanism which takes long periods of time to stabilize β-chain synthesis. On the other hand, this interpretation depends only on the data for 2-hour incubations. The data thus need to be verified.

In addition, data points from incubations shorter than 2.5 minutes might show when the γ chain is modified to pre-γ or vice versa. Figures 13 and 14 show monotonically decreasing pre-γ/γ ratios after 2.5 minutes of incubation. If the γ chain is modified after release from the ribosome, then there may be a break in the curve at some point, as there would at first be more radioactive γ chain than blocked γ chain present, and the ratio would later behave as shown in Figures 13 and 14. On the other hand, if the γ chain is modified on the ribosome, then the curve should remain monotonic. Incubations shorter than one minute would probably be of little value, as before this time the radioactive chains are not complete.

The contents of the cytoplasmic pools of free hemoglobin chain in cord blood may prove to be interesting. DeSimone and Mueller (57), in their work on baboons, found a pool of free α chain in the cytoplasm.

It is not necessarily true, however, that there is a pool of the same composition in the cytoplasm of human cord blood reticulocytes. In fact, the present study shows that after 2.5 minutes of incubation, the pre-γ/γ total radioactivity ratio is 1.12. The normal ratio of Hb F_1 to Hb F_0 is, however, only about 0.25. It is unclear whether the γ chain is deblocked before or after it is joined to α chain. In the former case, deblocking could conceivably occur in a pool of free γ chains. It would thus be of interest to separate the pools in cord blood, if any, and analyze them by chain separation to see if there is any γ chain present and, if so, to what extent it is modified.

APPENDIX

TABLE 7

ABSOLUTE COUNTS (CPM) FROM
TWO-HOUR CORD BLOOD INCUBATIONS

Sample	pre-γ	γ	β	post-β	pre-α	α
24R	5, 169	22, 890	46, 641	11, 236	7, 407	74, 728
26R	9,408	37, 446	38, 407	10, 324	11, 188	102, 072
27R	13, 805	78, 633	163, 290	74, 543	24, 818	257, 601
30R	1, 298	23, 945	8, 952*	--	2, 780	27, 959
33R	6, 486	53, 058	65, 394	26, 314	12, 946	118, 273
34R	12, 855	61, 454	93, 672	35, 642	21, 610	171, 365
37R	13, 832	62, 180	117, 163	21, 437	16, 182	209, 306
44R	10, 344	64, 391	74, 050	13, 606	12, 907	159, 346
46R	12, 572	59, 836	67, 503	21, 286	17, 212	167, 150
47R	9, 175	40, 866	83, 549	14, 422	7, 971	131, 743
50R	6, 028	31, 506	9, 265*	--	6, 644	48, 254
56R	10, 511	4, 534	4, 758*	--	9, 072	15, 933

* indicates post-γ zone only

TABLE 8

ABSOLUTE COUNTS (CPM) FROM
TWO-HOUR SS BLOOD INCUBATIONS

Sample	pre-γ	γ	β^S	pre-α	α
43B	1, 863	4, 262	69, 871	7, 429	71, 444
53B	1, 601	3, 016	29, 550	6, 042	33, 055
57B	1, 364	2, 447	17, 527	5, 189	22, 566
73B	883	1, 125	30, 310	4, 963	26, 975
92B	2, 229	6, 675	183, 301	36, 111	175, 143
137B	2, 147	3, 302	45, 112	5, 717	53, 402

TABLE 9

ABSOLUTE COUNTS (CPM)
FROM TIMED INCUBATION

Time	pre-γ	γ	β	post-β	pre-α	α
2.5 min.	1, 708	1, 523	2, 633	554	741	3, 990
5 min.	1, 247	1, 840	3, 563	338	797	5, 199
10 min.	2, 034	3, 719	7, 969	943	1, 779	11, 439
30 min.	3, 970	9, 595	22, 156	2, 309	3, 027	32, 542
1 hr.	5, 369	17, 918	40, 664	3, 654	5, 449	56, 125
2 hr.	8, 508	29, 650	52, 949	6, 857	10, 337	85, 603
4 hr.	11, 101	41, 310	90, 319	8, 950	11, 745	126, 598

TABLE 10

TOTAL RADIOACTIVITY RATIOS
FROM COUNTS GIVEN IN TABLE 7

Sample	$\frac{\alpha + \text{pre-}\alpha}{\gamma + \text{pre-}\gamma + \beta + \text{post-}\beta}$	$\frac{\beta + \text{post-}\beta}{\alpha + \text{pre-}\alpha}$	$\frac{\gamma + \text{pre-}\gamma}{\alpha + \text{pre-}\alpha}$	$\frac{\gamma + \text{pre-}\gamma}{(\alpha + \text{pre-}\alpha)_\gamma}$	$\frac{\beta + \text{post-}\beta}{(\alpha + \text{pre-}\alpha)_\beta}$	$\%A_o$
24R	0.96	0.70	0.34	0.44	3.13	22.7
26R	1.19	0.43	0.41	0.46	3.85	11.0
27R	0.86	0.84	0.33	0.44	3.23	26.4
30R*	0.88	--	1.14	--	--	--
33R	0.87	0.70	0.45	0.54	4.17	16.5
34R	0.95	0.67	0.39	0.46	3.85	17.1
37R	1.05	0.61	0.34	0.42	3.23	19.2
44R	1.06	0.51	0.43	0.51	3.33	15.2
46R	1.14	0.48	0.39	0.45	4.00	11.8
47R	0.94	0.70	0.36	0.46	3.13	22.3
50R*	1.17	--	0.85	--	--	--
56R**	1.26	--	0.79	--	--	--

* Separation of purified Hb F_o

** Separation of purified Hb F_1

TABLE 11

TOTAL RADIOACTIVITY RATIOS
FROM COUNTS GIVEN IN TABLE 8

Sample	$\frac{\alpha + \text{pre-}\alpha}{\gamma + \text{pre-}\gamma + \beta + \text{post-}\beta}$	$\frac{\beta + \text{post-}\beta}{\alpha + \text{pre-}\alpha}$	$\frac{\gamma + \text{pre-}\gamma}{\alpha + \text{pre-}\alpha}$	$\frac{\gamma + \text{pre-}\gamma}{(\alpha + \text{pre-}\alpha)_\gamma}$	$\frac{\beta + \text{post-}\beta}{(\alpha + \text{pre-}\alpha)_\beta}$	$\%S + A_2$
43B	0.98	0.95	0.08	0.28	1.30	72.7
53B	1.00	0.88	0.12	0.41	1.25	73.0
57B	1.03	0.83	0.14	0.52	1.14	73.4
73B	0.94	1.00	0.06	0.41	1.18	84.8
92B	1.05	0.91	0.04	0.33	1.04	87.2
137B	1.07	0.84	0.09	0.35	1.15	73.4

TABLE 12

TOTAL RADIOACTIVITY RATIOS
FROM COUNTS GIVEN IN TABLE 9

Time	$\frac{\alpha + \text{pre-}\alpha}{\gamma + \text{pre-}\gamma + \beta + \text{post-}\beta}$	$\frac{\gamma + \text{pre-}\gamma}{\alpha + \text{pre-}\alpha}$	$\frac{\beta + \text{post-}\beta}{\alpha + \text{pre-}\alpha}$	$\frac{\text{pre-}\gamma}{\gamma}$	$\frac{\text{pre-}\alpha}{\alpha}$	$\frac{\text{post-}\beta}{\beta}$
2.5 min.	0.74	0.68	0.67	1.12	0.19	0.21
5 min.	1.08	0.54	0.33	0.68	0.15	0.09
10 min.	0.96	0.44	0.60	0.55	0.16	0.12
30 min.	0.94	0.38	0.69	0.41	0.09	0.10
1 hr.	0.91	0.39	0.72	0.30	0.10	0.09
2 hr.	0.98	0.40	0.62	0.29	0.12	0.13
4 hr.	0.92	0.38	0.72	0.27	0.09	0.10

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