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EFFECTS OF A RADIATION-INDUCED α-THALASSEMIA ON THE PRODUCTION OF MULTIPLE FORMS OF HEMOGLOBINS IN FETAL MICE

(thalassemia/embryonic hemoglobins/Mus musculus/yolk sac erythrocytes)

Raymond A. Popp,[1] Blaine S. Bradshaw,[2] and Gerald P. Hirsch[3]

[1] Raymond A. Popp, Ph.D., Senior Staff Scientist, Biology Division, Oak Ridge National Laboratory, P. O. Box Y, Oak Ridge, TN 37830
Phone 615-483-8611, ext. 3-7572 or FTS 850-7572

[2] Blaine S. Bradshaw, Ph.D., Research Associate, University of Tennessee-Oak Ridge Biomedical Graduate School, Oak Ridge National Laboratory, Oak Ridge, TN 37830

[3] Gerald P. Hirsch, Ph.D., Staff Scientist, Biology Division, Oak Ridge National Laboratory, P. O. Box Y, Oak Ridge, TN 37830

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Send proof to: Dr. R. A. Popp

Biology Division

Oak Ridge National Laboratory

Post Office Box Y

Oak Ridge, TN 37830

(615) 483-8611, ext. 3-7572

SUMMARY

Embryonic hemoglobins in α-thalassemic heterozygotes and normal fetuses were compared to study the effects of the deficient α chain on the synthesis of hemoglobins in the nucleated embryonic erythrocytes derived from the fetal yolk sac. Acrylamide gel electrophoresis showed that less hemoglobin EII (α_2y_2) was formed in α-thalassemic heterozygotes between $12\frac{1}{2}$ and $14\frac{1}{2}$ days of gestation. Quantitation of <u>in vitro</u> synthesis between $11\frac{1}{2}$ and $13\frac{1}{2}$ days of gestation also showed that EII was synthesized less rapidly in α-thalassemic fetuses. In contrast, the synthesis of EIII (α_2z_2) was higher in α-thalassemic than in normal fetuses at $12\frac{1}{2}$ and $13\frac{1}{2}$ days of gestation. Measurements of the synthesis of individual chains in EI (x_2y_2) and EII showed that x chain synthesis was normal and that α chain synthesis was deficient in α-thalassemic fetuses at $11\frac{1}{2}$ and $12\frac{1}{2}$ days of gestation. Thus, there is still no proof for close linkage of x- and α-chain genes in chromosome 11. Differences in the electrophoretic patterns of embryonic hemoglobins of α-thalassemic and normal fetuses can be explained by normal synthesis of x chains, deficient synthesis of α chains, and a higher affinity of z than y for the reduced amount of α chain present in the nucleated embryonic erythrocytes of α-thalassemic mice.

The embryonic nucleated erythrocytes, which develop in the blood islands of the yolk sac of the fetal mouse, produce three forms of embryonic hemoglobins (1, 2). The globin chains of these hemoglobins are x_2y_2 for EI, α_2y_2 for EII, and α_2z_2 for EIII (3). All three embryonic hemoglobins are produced in a single erythrocyte and the relative quantitites of these hemoglobins change as the cell differentiates between days 10 and 14 of gestation (2). Of the four kinds of globin chains produced in embryonic erythrocytes, only the α chain is also produced by the anucleated erythrocytes that arise initially within the fetal liver and subsequently from the bone marrow and spleen. The x chain is structurally homologous to the α chain and the y and z chains are homologous to the β chain of the adult (4-6). Gilman and Smithies (7) have shown that the gene controlling the synthesis of the embryonic y chain is tightly linked to the β-chain gene in chromosome 7 of the mouse genome, and Steinheider et al. (8) have shown that the z chain N- and C-terminal regions are homologous with that of the minor β chain and have suggested a close linkage among the β-, y- and z-chain genes in chromosome 7. The α-chain gene is located in chromosome 11 (9) but alleles of the x-chain gene are not available to test by conventional methods whether or not the x-chain gene is closely linked to the α-chain gene (10). Three independent mutations that completely inhibit α chain synthesis (11) have been induced by irradiation (12). Heterozygous animals exhibit a typical α-thalassemia trait (13). DNA hybridization studies are presently being done by Dr. W. French Anderson to determine whether or not the deficient α chain synthesis is due to gene deletion. The deficient α chain synthesis in erythrocytes

of the adult should also be deficient in the embryonic nucleated erythrocytes. If the mutation is a gene deletion and if the x-chain gene is closely linked to the α-chain gene, deficient synthesis of the x chain might also be observed in the nucleated embryonic erythrocytes. This study described the effects of one of the radiation-induced mutations at the α-chain locus on the production of the multiple forms of hemoglobins in fetal mice.

The α-thalassemic stock of mice used for this study are called 352 HB; ♂ 352 was a progeny of an irradiated SEC male mated to an unirradiated 101 female (12). The stock has been partially inbred by backcrossing to strain SEC mice; the mice are homozygous for the single β-chain allele and the tightly linked y^1 allele (10). Matings between α-thalassemic heterozygotes produce litters composed of one-third normal and two-thirds α-thalassemic mice because homozygous α-thalassemic mice die at the late blastocyst or early egg cylinder stage (5½ days of gestation). Embryo ages were determined by inspection for vaginal plugs. Removal of embryos, collection of embryonic blood, in vitro incorporation of ^{3}H-leucine and ^{14}C-phenylalanine, and electrophoresis of hemoglobin in 7 percent acrylamide gels were basically as described by Fontani et al. (2). Blood films for hematologic observations were stained with Wright's stain. Separation of globin chains in 8M urea was done on Whatmann CM 23 cellulose (14).

The electrophoretic patterns of hemoglobins from normal and heterozygous α-thalassemic mice of the same litter could not be distinguished until 12½ or 13½ days of gestation. At that time the quantity of hemoglobin E11 increased preceptibly in normal embryos but did not increase as much

in α-thalassemic littermates (Fig. 1). Differences in the electrophoretic patterns appear to be due to the reduced production of hemoglobin EII. No y_4 hemoglobin was found in α-thalassemic embryos; such a hemoglobin would be homologous to the γ4, Bart's hemoglobin, of humans (15). Hemoglobin EI, x_2y_2, in mice is comparable to hemoglobin Portland I, x_2y_2, which is more pronounced in homozygous α-thalassemic humans (16).

We attempted to identify the normal fetuses versus the α-thalassemic heterozygotes by microscopic observations of embryonic blood; their blood films were indistinguishible prior to 14½ days of gestation. At 14½ days of gestation the nucleated embryonic cells of normal and α-thalassemic heterozygotes were similar; however, eosinophilic inclusion bodies were observed in the anucleated erythrocytes derived from the fetal liver of α-thalassemic heterozygotes. These eosinophilic inclusions presumably are due to precipitated excess β chains.

Owing to our inability to identify the normal fetuses among α-thalassemic heterozygotes prior to 14½ days, studies on the rates of synthesis of hemoglobins in α-thalassemic mice were done using litters containing one-third normal embryos. Nevertheless, the incorporation of ^{14}C-phenylalanine into the three embryonic hemoglobins of normal versus α-thalassemic litters confirmed our electrophoretic observations that the relative synthesis of EII was depressed in α-thalassemic mice (Table 1). The reduced quantity of EII in α-thalassemic mice most certainly results from the reduced synthesis of α chain, which is approximately 70 percent of normal in erythrocytes of the adult (Fig. 2A). It should be noted that the synthesis of EIII in α-thalassemic embryos does not decline between 11½ and 13½ days

of gestation (Table 1); the reason is not clear because both EII and EIII contain α chains. Perhaps the z chain has a higher affinity than the y chains for the reduced quantity of α chains available in the α-thalassemic embryos. We have shown previously that such competition does occur among the multiple β chains for the reduced quantity of α chain synthesized in erythrocytes of α-thalassemic adult mice (17).

The relative quantities of newly synthesized α, x, y and z chains in the embryonic hemoglobins were determined following the in vitro incorporation of ^{3}H-leucine and ^{14}C-phenylalanine and the separation of the three embryonic hemoglobins by acrylamide gel electrophoresis. The x and α chains have leucine at position 2 and the y and z chains have phenylalanine at position 3. Thus, measurements of ^{3}H-leucine at positions 2 and ^{14}C-phenylalanine at position 3 for each of the three embryonic hemoglobins at successive days of development reveal the relative rates of assembly of these chains. Globins of each of the embryonic hemoglobins were subjected to automated Edman degradation (18) and the phenylthiohydantoin forms of the amino acids at each of the first four positions were recovered for radioactivity measurements. In a few experiments the PTH-amino acids were purified by high pressure liquid chromatography to show that all the radioactivity at steps two and three of the Edman degradation were indeed associated with leucine and phenylalanine, respectively. The ratios of the newly synthesized chains in the three embryonic hemoglobins at 11½ and 12½ days of gestation (Table 2) suggest that α chain synthesis is already deficient. The data also indicate that x chain synthesis is not deficient in stock 352 HB α-thalassemic mice.

The above conclusion is based on measurements of the quantities of α and x chains assembled into hemoglobin rather than of the absolute quantities of chains being synthesized. For the production of normal hemoglobin, synthesis of α and β chains and their assembly into hemoglobin are balanced; however, synthesis of α and β chains are not equal in thalassemia (19). To determine whether this could affect our interpretation, measurements were made of chain synthesis and hemoglobin assembly in reticulocytes from normal versus α-thalassemic adult mice. Figure 2A shows that α-thalassemic reticulocytes synthesized approximately 70 percent as much α as β chains but measurements of ^{3}H-leucine activity in the α and β chains of an aliquot of the same hemoglobin preparation following electrophoresis and elution from acrylamide gels showed that the ^{3}H-leucine activity in the α chain now exceeded that in the β chain, as the ratio of α to β was 1.09 (Fig. 2B). Electrophoresis of labeled hemoglobin from reticulocytes of normal mice did not change the ratio of ^{3}H-leucine activity in the α and β chains, which was 1.10 (Fig. 2C). Apparently all of the newly synthesized α chains are assembled into tetrameric hemoglobin molecules whereas the excess β chains synthesized in α-thalassemic reticulocytes become part of a soluble pool of β chain polypeptides. These contribute to the total ^{3}H-leucine activity in the β chain when globin from reticulocytes is chromatographed on CM cellulose but the soluble pool of excess β chains are removed during the electrophoretic separation of hemoglobin in acrylamide gels.

One reason for doing this series of experiments was to learn whether or not the deficient synthesis of α chain in the adult mouse carrying

α-thalassemia trait also caused deficient x chain synthesis in the fetal mouse. Our data indicate that x chain synthesis in nucleated embryonic erythrocytes of stock 352 HB α-thalassemic mice is normal and that deficient α chain synthesis is already evident by 11½ days of gestation. The altered electrophoretic pattern of embryonic hemoglobins in α-thalassemic fetal mice can be explained on the basis of normal x chain synthesis, of deficient α chain synthesis and of a higher affinity of z than y chains for the reduced quantity of α chains available for assembly of the tetrameric molecules of embryonic hemoglobin. Higher affinity of z than y chains for α chains seems plausible because the y-chain gene is likely to be the ancestral embryonic non α-chain gene in that the y chain does, but the z chain does not, combine with the embryonic x chain (8). Thus the prolonged and elevated expression of Ell1 in nucleated embryonic erythrocytes of α-thalassemic mice appears to be associated with a problem of hemoglobin assembly rather than to a regulatory effect on the z-chain gene.

Normal expression of the x-chain gene suggests that the radiation-induced mutation,which may be a deletion, at the α-chain locus in stock 352 HB did not simultaneously affect the x-chain gene. In such a case, the x-chain gene would be outside the affected segment or it may not be located in chromosome 11. Similar studies on other radiation-induced α-thalassemias will be done to determine further whether the x- and α-chain genes are closely linked.

Table 1. Synthesis of embryonic hemoglobins in normal and α thalassemic fetal mice.

Age of fetus (days)	Normal[a]			α thalassemic[a]		
	EI[b]	EII	EIII	EI	EII	EIII
11 1/2	(990) .40	(892) .36	(594) .24	(3454) .41	(2946) .35	(1935) .23
12 1/2	(16135) .39	(19283) .46	(6242) .15	(20660) .40	(18890) .36	(12670) .24
				(13264) .38	(12221) .35	(9799) .28
13 1/2	(12966) .32	(21923) .53	(6267) .15	(19496) .22	(41468) .46	(29710) .33
				(11910) .32	(14289) .39	(10542) .29

[a]Normal fetuses were obtained from matings between normal mice of stock 352HB and α-thalassemic fetuses were from matings between α thalassemic heterozygotes of stock 352HB. One-third of the fetuses in litters of α thalassemic mice are normal but only large litters or pooled smaller litters were used to minimize the probability of variations that could occur in small litters owing to distorted gene segregation.

[b]Embryonic erythrocytes were incubated with ^{14}C-phenylalanine and the labeled hemoglobins were separated by electrophoresis in 7 percent acrylamide gels. Each hemoglobin band was eluted separately, an aliquot of each was counted and cpm data are shown in parenthesis, and the percentage of total hemoglobin synthesized was calculated.

Table 2. Synthesis of individual globin chains in the embryonic hemoglobins of normal and α thalassemic fetal mice.

Age of fetus (days)	Normal[a]			α thalassemic[a]		
	EI[b] x/y	EII α/y	EIII α/z	EI x/y	EII α/y	EIII α/z
11 1/2	(12/50)	(14/50)	(7/22)	(29/136)	(23/194)	(20/70)
	x/α=.86	y/z=1.14		x/α=1.80	y/z=2.41	
12 1/2	(58/410)	(83/676)	(59/183)	(70/440)	(70/660)	(120/380)
	x/α=1.15	y/z=2.63		x/α=1.50	y/z=2.98	

[a]See footnote to Table 1.

[b]Embryonic erythrocytes were incubated with ^{3}H-luecine and ^{14}C-phenylalanine and the labeled hemoglobins were separated by electrophoresis in 7 percent acrylamide gels. Each hemoglobin band was eluted, globin was prepared by acid-acetone precipitation and was subjected to automated Edman degradation. The measured radioactivity of ^{3}H-PTH-leucine at position 2 in the x and α chains and of ^{14}C-PTH-phenylalanine at position 3 in the y and z chains are shown in parenthesis and the relative synthesis of x versus α among hemoglobins with y chains and y versus z among hemoglobins with α chains was calculated.

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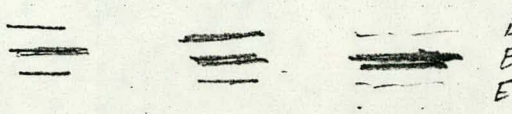
FIGURE LEGENDS

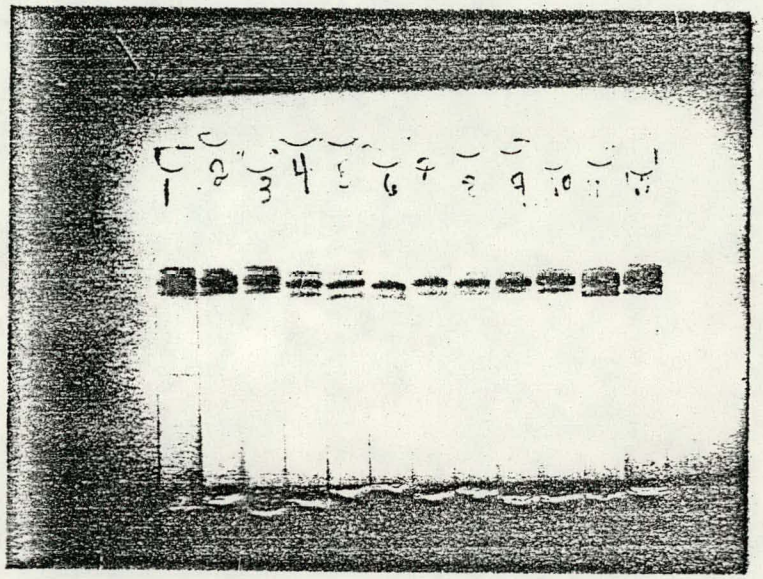
Figure 1. Acrylamide gel electrophoresis of embryonic hemoglobins from fetuses of A, 12½ day α-thalassemic; B, 13½ day α-thalassemic; and C, 13½ day normal mice. Three hemoglobins, EI, EII and EIII, are seen.

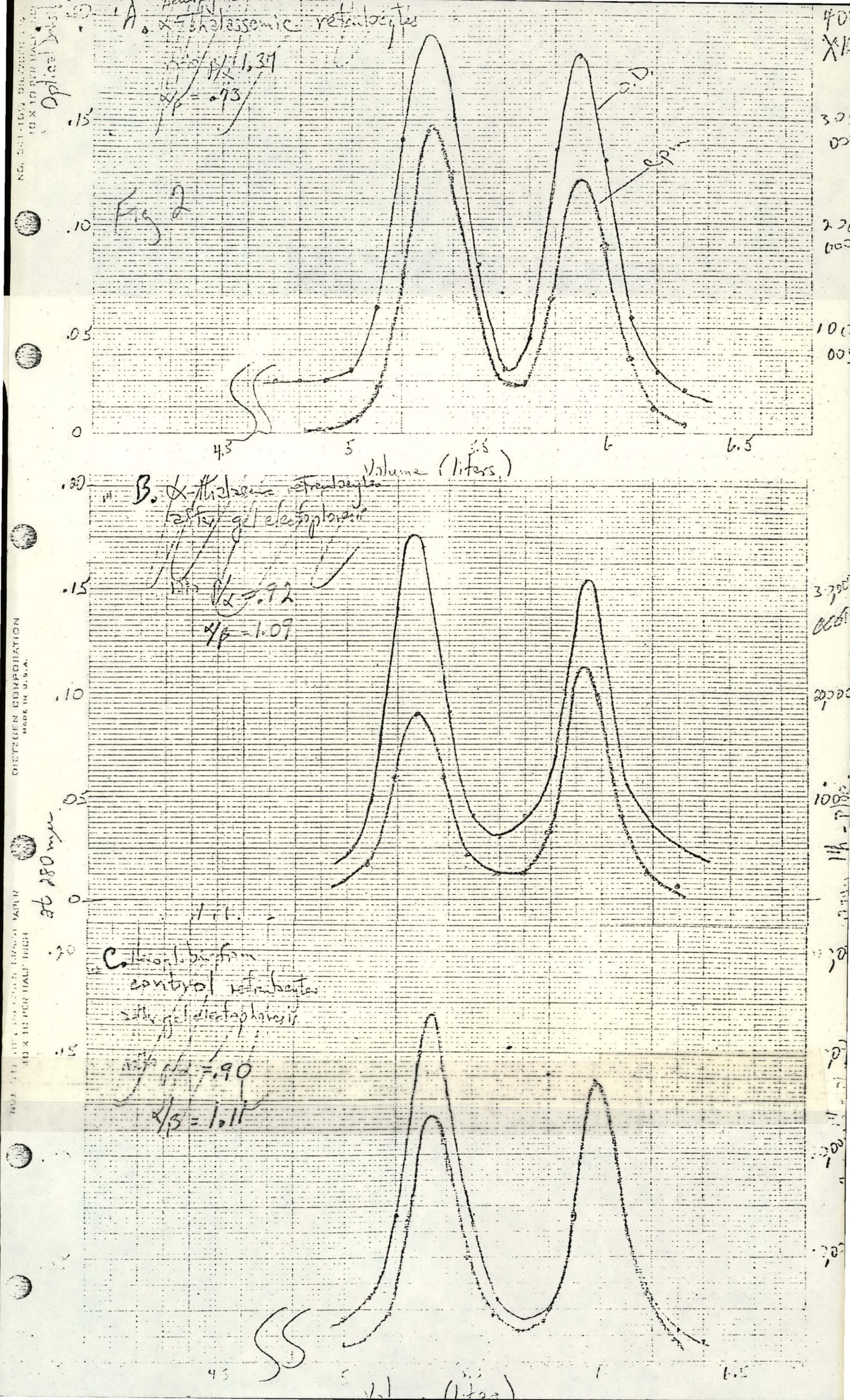
Figure 2. Separation of α and β chains of hemoglobin from reticulocytes of α-thalassemic and normal adults. Reticulocytosis was induced by the subcutaneous injection on days 1, 3 and 5 of 1 mg of recrystallized phenylhydrazine-HCl. Peripheral blood containing 60-70 percent reticulocytes was collected on day 7 and incubated in medium containing ^{3}H-leucine. A. Globin from α-thalassemic reticulocytes. B. Hemoglobin from α-thalassemic reticulocytes was separated by acrylamide gel electrophoresis before preparation of globin. C. Hemoglobin from normal reticulocytes was separated by acrylamide gel electrophoresis before preparation of globin.

Fig 1

A	B	C
4, 5	1, 3	11, 12






A. α-Thalassemic reticulocytes
α/β = 1.39
α/β = .93
Optical Density
O.D.
Fig 2
Volume (liters)
B. α-Thalassemic reticulocytes
Cellulose acetate gel electrophoresis
α/β = .92
α/β = 1.09
at 280 mμ
C. Heterozygous
control reticulocytes
Cellulose acetate gel electrophoresis
α/β = .90
α/β = 1.11