

MASTER

NOTICE

This report was prepared as an account of work sponsored by the United States Government. Neither the United States nor the United States Department of Energy, nor any of their employees, nor any of their contractors, subcontractors, or their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness or usefulness of any information, apparatus, product or process disclosed, or represents that its use would not infringe privately owned rights.

Proceedings of the
Conference on Cellular and
Molecular Regulation of
Hemoglobin Switching in Man
and Animal Models
Seattle, Washington
June 19-21, 1978

EMBRYONIC HEMOGLOBINS IN α -THALASSEMIA MICE

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

Blaine S. Bradshaw, Ph.D., Research Associate, University of Tennessee - Oak
Ridge Graduate School of Biomedical Sciences, P. O. Box Y, Oak Ridge,
Tennessee 37830

Gerald P. Hirsch, Ph.D., Staff Scientist, Biology Division, Oak Ridge National
Laboratory, P. O. Box Y, Oak Ridge, Tennessee 37830

Key words: thalassemia/embryonic hemoglobins/Mus musculus/yolk sac erythrocytes

By acceptance of this article, the publisher or recipient acknowledges the U. S.
Government's right to retain a nonexclusive, royalty-free license in and to any
copyright covering the article.

This work was supported jointly by the National Institutes of Health Grant
HL 20912-02 and the Division of Biomedical and Environmental Research, U. S.
Department of Energy, under contract W-7405-eng-26 with the Union Carbide
Corporation.

DISTRIBUTION OF THIS DOCUMENT IS UNLIMITED

82

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency Thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

DISCLAIMER

Portions of this document may be illegible in electronic image products. Images are produced from the best available original document.

Send proof to: Dr. R. A. Popp
Biology Division
Oak Ridge National Laboratory
Post Office Box Y
Oak Ridge, Tennessee 37830
(615) 483-8611, ext. 3-7572

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 $\alpha_2\gamma_2$ for EI, $\alpha_2\gamma_2$ for EII, and α_2z_2 for EIII (3). All three embryonic hemoglobins are produced in a single erythrocyte, and the relative quantities 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. 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 if 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 W. French Anderson to determine if 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 describes 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.

General Methods

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 (5 1/2 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 Fantoni *et al.* (2). Blood films for hematologic observations were stained with Wright's stain. Separation of globin chains in 8 M urea was done on Whatman CM 23 cellulose (14).

Electrophoretic Observations

The electrophoretic patterns of hemoglobins from normal and heterozygous α -thalassemic mice of the same litter could not be distinguished until 12 1/2 or 13 1/2 days of gestation. At that time the quantity of hemoglobin EII ($\alpha_2\gamma_2$) increased perceptibly in normal embryos but did not increase as much in

(F-1)

α -thalassemic littermates (Fig. 1). Differences in the electrophoretic patterns appear to be due to the reduced production of hemoglobin EII. No γ_4 hemoglobin was found in α -thalassemic embryos; such a hemoglobin would be homologous to the γ_4 , Bart's hemoglobin, of humans (15). Hemoglobin EI, $\alpha_2\gamma_2$, in mice is comparable to hemoglobin Portland I, $\alpha_2\gamma_2$, which is more pronounced in homozygous α -thalassemic humans (16).

Blood Films

We attempted to identify the normal fetuses versus the α -thalassemic heterozygotes by microscopic observations of embryonic blood; their blood films were indistinguishable prior to 14 1/2 days of gestation. At 14 1/2 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.

In Vitro Synthesis of Embryonic Hemoglobins

Owing to our inability to identify the normal fetuses among α -thalassemic heterozygotes prior to 14 1/2 days, studies on the rates of synthesis of hemoglobins in α -thalassemic mice were done using litters containing one-third normal embryos. Nevertheless, the reduced 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 undoubtedly results

(T-1)

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 11 1/2 and 13 1/2 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 chain 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 [^3H]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 [^3H]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 11 1/2 and 12 1/2 days of gestation

(Table 2) suggest that α -chain synthesis is already deficient. The data also indicate that γ -chain synthesis is not deficient in stock 352 HB α -thalassemic mice.

In Vitro Synthesis of Adult Hemoglobin

The above conclusion is based on measurements of the quantities of α and γ 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, reticulocytes were incubated in medium containing [^3H]leucine and 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. Similar measurements of [^3H]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 [^3H]leucine activity in the α chain was now equal to that in the β chain—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 [^3H]leucine activity in the α and β chains, which was 1.10 in hemoglobin obtained directly from reticulocytes or following acrylamide gel electrophoresis (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,

which chromatograph with the β chains of hemoglobin. Although these contribute to the total [^3H]leucine activity in the β chain when globin from reticulocytes is chromatographed on CM cellulose, the soluble pool of excess β chains are apparently removed during the electrophoretic separation of hemoglobin in acrylamide gels.

Question of Linkage of γ - and α -Chain Genes

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 -thalassemic trait also caused deficient γ -chain synthesis in the fetal mouse. Our data indicate that γ -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 11 1/2 days of gestation. The altered electrophoretic pattern of embryonic hemoglobins in α -thalassemic fetal mice can be explained on the basis of normal γ -chain synthesis, of deficient α -chain synthesis, and of a higher affinity of γ than δ chains for the reduced quantity of α chains available for assembly of the tetrameric molecules of embryonic hemoglobin. Higher affinity of γ than of δ chains for α chains seems plausible because the γ -chain gene is likely to be the ancestral embryonic non- α -chain gene since only the γ chain combines with the embryonic α chain (8). The prolonged and elevated expression of EIII 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 γ -chain gene.

Normal expression of the γ -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 γ -chain gene. In such a case, the γ -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 whether the x- and α -chain genes are closely linked.

Summary. Embryonic hemoglobins in heterozygous α -thalassemic 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. Visual inspection of embryonic hemoglobins following acrylamide gel electrophoresis suggested that less hemoglobin EII ($\alpha_2\gamma_2$) was formed in α -thalassemic heterozygotes between 12 1/2 and 14 1/2 days of gestation. Quantitation of in vitro synthesis between 11 1/2 and 13 1/2 days of gestation confirmed 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 1/2 and 13 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 1/2 and 12 1/2 days of gestation. 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.

REFERENCES

1. Craig ML, Russell ES: A developmental change in hemoglobin correlated with an embryonic red cell population in the mouse. *Dev Biol* 10:191-201, 1964
2. Fantoni A, De Le, Chapelle A, Marks PA: Synthesis of embryonic hemoglobins during erythroid cell development in fetal mice. *J Biol Chem* 244:675-681, 1969
3. Fantoni A, Bank A, Marks PA: Globin composition and synthesis of hemoglobins in developing fetal mice erythroid cells. *Science* 157:1327-1329, 1967
4. Melderis H, Steinheider G, Ostertag W: Evidence for a unique kind of α -type globin chain in early mammalian embryos. *Nature* 250:774-776, 1974
5. Steinheider G, Melderis H, Ostertag W: Embryonic ϵ chains of mice and rabbits. *Nature* 257:714-716, 1976
6. Gilman JG: Mouse haemoglobin beta chains. *Biochem J* 155:231-241, 1976
7. Gilman JG, Smithies O: Fetal hemoglobin variants in mice. *Science* 160:885-886, 1978
8. Steinheider G, Melderis H, Ostertag W: Evidence for Hb Lepore-like hybrid globin β genes in mice. *Nature* 257:712-714, 1976
9. Russell ES, McFarland EC: The genetics of mouse hemoglobins. *Ann NY Acad Sci* 241:25-38, 1974
10. Stern RH, Russell ES, Taylor BA: Strain distribution and linkage relationship

- of a mouse embryonic hemoglobin variant. *Biochem Genet* 14:373-381, 1976
11. Popp RA, Stratton LP, Hawley DK, Effron K: Hemoglobins of mice with radiation-induced mutations at the hemoglobin loci. *J Mol Biol*, in press
 12. Russell LB, Russell WL, Popp RA, Vaughan C, Jacobson KB: Radiation-induced mutations at mouse hemoglobin loci. *Proc Natl Acad Sci USA* 73:2843-2846, 1976
 13. Popp RA, Enlow MK: Radiation-induced alpha thalassemias in mice. *Am J Vet Res* 38:569-572, 1977
 14. Clegg JB, Naughton MA, Weatherall DJ: An improved method for the characterization of human haemoglobin mutants: Identification of $\alpha_2\beta_2$ 95GLU, haemoglobin N (Baltimore). *Nature* 207:945-947, 1965
 15. Hunt JA, Lehmann H: Haemoglobin 'Bart's': A foetal haemoglobin without α -chains. *Nature* 184:872-873, 1959
 16. Weatherall DJ, Clegg JB, Boon WH: The haemoglobin constitution of infants with the haemoglobin Bart's hydrops foetalis syndrome. *Brit J Haematol* 18:357-361, 1970
 17. Popp RA, Bradshaw BS: Altered concentrations of β -chain polypeptides in hemoglobins of α -thalassemic mice. *Fed Proc* 37:1909 (abstr), 1978
 18. Edman P, Begg G: A protein sequenator. *Eur J Biochem* 1:80-91, 1967
 19. Weatherall DJ, Clegg JB: In vitro hemoglobin synthesis in the thalassemic syndrome. *Exp Pathol* 13:117-159, 1974

Table 1. Synthesis of embryonic hemoglobins in normal and α -thalassemic fetal mice. 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. 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, and the percentage of total hemoglobin synthesized was calculated.

Age of Fetus (days)	Normal						α -Thalassemic					
	EI		EII		EIII		EI		EII		EIII	
	cpm	% total	cpm	% total	cpm	% total	cpm	% total	cpm	% total	cpm	% total
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

Table 2. Synthesis of individual globin chains in the embryonic hemoglobins of normal and α -thalassemic fetal mice. 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. Embryonic erythrocytes were incubated with [^3H]leucine 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 [^3H]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 as a ratio and the relative synthesis of x versus α among hemoglobins with y chains and y versus z among hemoglobins with α chains was calculated.

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

FIGURE LEGENDS

Fig. 1. Acrylamide gel electrophoresis of embryonic hemoglobins from fetuses of (A) 12 1/2 day α -thalassemic, (B) 13 1/2 day α -thalassemic, and (C) 13 1/2 day normal mice. Three hemoglobins, EI, EII and EIII, are seen.

Fig. 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.

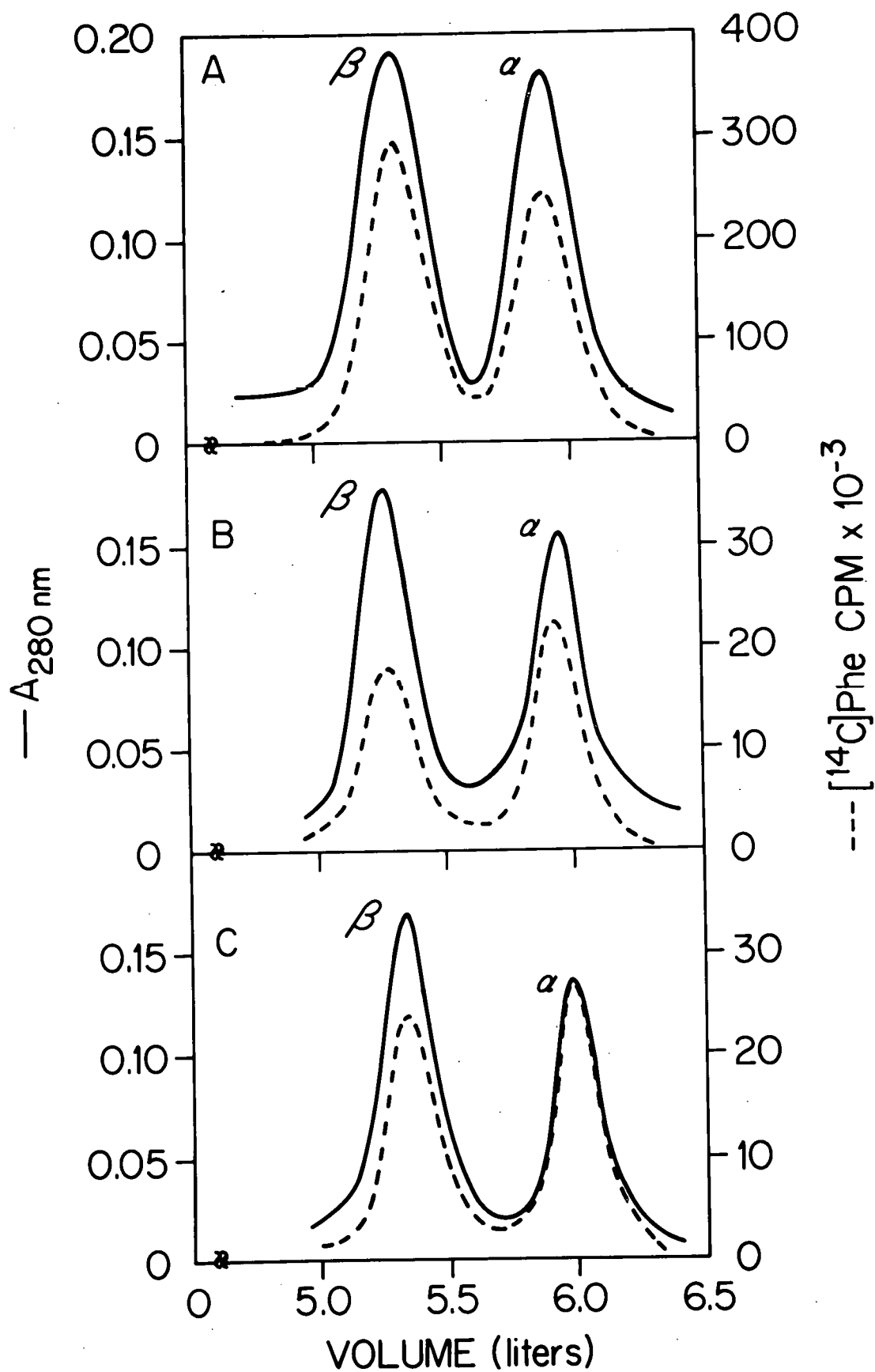


Fig 1

34943

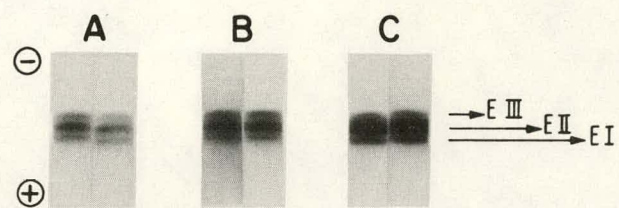


fig. 2