

Inhibition of Synthesis of Fetal Hemoglobin by an Isoleucine Analogue *

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Abstract. Reticulocytes from newborn infants with Rh isoimmune hemolytic disease actively incorporated radioactive amino acids in vitro into hemoglobins F and A. Approximately 50% of the reticulocytes appeared capable of synthesis of both of these hemoglobins within the same cell, as demonstrated by the selective elution technique of Betke and Kleihauer. An isoleucine analogue, L-O-methylthreonine, inhibited the incorporation of a variety of amino acids into hemoglobin F, without significantly affecting the synthesis of hemoglobin A. The inhibition was prevented upon concomitant addition of L-isoleucine to the medium. These observations suggest that an independent biosynthetic apparatus is present in the cell for the synthesis of each of these two hemoglobins. Because isoleucine is present only in the gamma chains of hemoglobin F, the inhibitory effect of the analogue on the synthesis of this hemoglobin must represent a selective effect on the production of gamma chains.

Introduction

The thalassemias are a diverse group of familial anemias which share as a common feature the defective synthesis of globin chains of hemoglobin (1). The hemoglobin produced in these disorders has no apparent structural abnormality (2) and the defect appears to manifest itself as a quantitative diminution in globin-chain production. Impairment of alpha-chain synthesis, which occurs in alpha thalassemia (3), is associated with abnormal production of Bart's hemoglobin (γ_4), hemoglobin

H (β_4), and hemoglobin δ_4 , which do not contain alpha-globin chains (1). In the beta thalassemias, the effect of the biosynthetic defect is a suppression of production of the beta-globin chains of adult hemoglobin (3), and a characteristic, often extreme increase in the nonbeta-chain hemoglobins F and A₂ occurs. In the beta thalassemias, however, appreciable amounts of soluble alpha chains do not accumulate, despite a severe defect in beta-chain production (4). The significance of these associated effects is not understood, although it has been suggested that they may reflect control mechanisms which operate at the level of regulation of gene action (5) or a coordinated mechanism which regulates globin-chain synthesis on the ribosomes (6).

In order to examine the operation of some of the regulatory mechanisms, we performed the present studies to develop an experimental model in which a controlled thalassemia-like lesion could be produced. For this purpose, we took advantage of the unique presence of isoleucine in the gamma chains of hemoglobin F. Experiments in vitro using erythroid cells from human cord blood (7) or rabbit reticulocytes (8) with isoleucine-deficient

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media have not demonstrated significant inhibition of hemoglobin synthesis, despite the presence of isoleucine in their respective hemoglobins. In our present study the effect of isoleucine deprivation was augmented by the addition of L-O-methylthreonine, an inhibitor of protein synthesis which is an analogue of isoleucine (9).

Methods

Incubation procedure. Blood was obtained from newborn infants with isoimmune hemolytic disease due to Rh incompatibility. The initial 30–50 ml of blood removed at exchange transfusion, which contained small amounts of transfused donor blood, was anticoagulated with heparin and chilled; experiments were begun within 2 hr after collection. We centrifuged the blood at 5000 *g* for 15 min at 4°C and removed the plasma without disturbing the buffy coat. Then we washed the cells twice at 4°C with an isotonic saline solution (sodium chloride 0.140 mole/liter, potassium chloride 0.005 mole/liter, magnesium chloride 0.0015 mole/liter). The washed cells were suspended in an equal volume of the saline solution and filtered through several layers of gauze.

Incubations were carried out at 37°C in 25-ml Erlenmeyer flasks open to room air. Each flask contained 1 ml of cells in a total volume of 10 ml. The incubation medium contained sodium chloride, 0.140 mole/liter; potassium chloride, 0.005 mole/liter; magnesium chloride, 0.0015 mole/liter; Tris-HCl, pH 7.4, 0.005 mole/liter; glucose, 1 mg/ml; ferrous ammonium sulfate, 0.0001 mole/liter; human transferrin,¹ 0.05 mg/ml; and a mixture of amino acids as described by Lingrel and Borsook (10). Isoleucine was omitted except when indicated. Radioactive amino acids equivalent to 5 μ c of the L-form were added to each flask; the corresponding nonradioactive amino acid was omitted. The radioactive amino acids were purchased from New England Nuclear Corporation (Boston, Mass.) and had the following specific activities: L-arginine-U-¹⁴C, (uniformly labeled), 234 mc/mole; DL-histidine-2-¹⁴C, 1.49 mc/mole; L-isoleucine-U-¹⁴C, 275 mc/mole; L-leucine-1-¹⁴C, 34.1 mc/mole; DL-lysine-1-¹⁴C, 3.66 mc/mole; and L-valine-1-¹⁴C, 22 mc/mole. When L-O-methylthreonine was added, it was at a final concentration of 25 mmoles/liter.

Separation of hemoglobins. Incubation periods were terminated by rapid chilling of the flasks in an ice water mixture. The cells were washed twice with the isotonic saline solution at 4°C, and the erythrocytes lysed by the addition of 4 volumes of cold magnesium chloride, 0.0015 mole/liter, which contained Tris-HCl, pH 7.4, 0.001 mole/liter; after 45 sec 1.7 M sodium chloride was added to bring the solution rapidly to isotonicity. The lysates were saturated with carbon monoxide and centrifuged at 7000 *g* for 15 min at 4°C. The supernatants were further centrifuged at 100,000 *g* for 90 min; the

clarified solutions were decanted, saturated with carbon monoxide, and frozen at -20°C until further fractionated.

Hemoglobins F and A were separated by the chromatographic method of Zade-Oppen (11) with minor modification. Columns of Sephadex G-25, 1 × 30 cm, were equilibrated with 0.05 M sodium phosphate, pH 6.0, which was saturated with carbon monoxide. From 1.0 to 2.5 ml of the lysates, containing 45–60 mg of hemoglobin, was applied to the columns and eluted with the same buffer over a 30 min period. The eluates were applied to columns of Sephadex CM-C 50, 2 × 5 cm, which had been equilibrated with 50 mM phosphate buffer, pH 6.0. The columns were first eluted with 100 ml of the starting buffer, during which time the hemoglobin band remained within 3–4 mm of the top of the column. The effluents contained small amounts of protein having variable, sometimes high specific activities (Fig. 1). Further elution was performed with increasing concentrations of sodium chloride. The first hemoglobin peak was eluted with 250–400 ml of buffer containing 0.05 M sodium phosphate, pH 6.0, and 0.050 M sodium chloride. The remainder of the hemoglobin, which was retained within the upper 1–2 cm of the column, was then eluted with buffer containing 0.110 M sodium chloride. In some experiments this peak was eluted with 0.50 M sodium phosphate, pH 7.0. Chromatography was carried out at room temperature with an elution rate of 0.5–0.7 ml/min.

The completeness of the hemoglobin separation obtained by this method was demonstrated with the use of radioactive hemoglobins. Incubation of cells with leucine-¹⁴C resulted in the labeling of both of the hemoglobin peaks (Fig. 1*a*). Radioactivity derived from isoleucine, which is not present in hemoglobin A (12), labeled only the first hemoglobin peak (Fig. 1*b*), demonstrating that the fetal hemoglobin was confined to the first hemoglobin peak. In another experiment nonradioactive lysate from a newborn infant was mixed with ⁵⁹Fe-labeled hemoglobin obtained from an adult who was undergoing ferrokinetic studies. After chromatography, 97.3% of the recovered radioactivity was present in the second hemoglobin peak, which indicated that the fetal hemoglobin fraction did not contain appreciable amounts of hemoglobin A. Yields of hemoglobin of 95–105% were consistently obtained. This method (11) offers significant advantages over other methods for separation of radioactive hemoglobins A and F (13, 14) in that it is performed at room temperature, gives complete separation of the hemoglobins in a single procedure, and allows removal of radioactive nonheme proteins before elution of the hemoglobin fractions.

We determined hemoglobin concentrations by measuring the absorbancy at 522 or 542 *m* μ of the carboxyhemoglobins (15).

Radioactivity determination. For determination of the specific activities of the isolated hemoglobins, the chromatographic fractions were combined and trichloroacetic acid (TCA) added to a final concentration of 10%. The precipitates were collected and washed two times with 5% TCA, once with 5% TCA at 90°C for 15 min, once with 5% TCA at room temperature, two times with absolute

¹ Pentex, Inc., Kankakee, Ill.

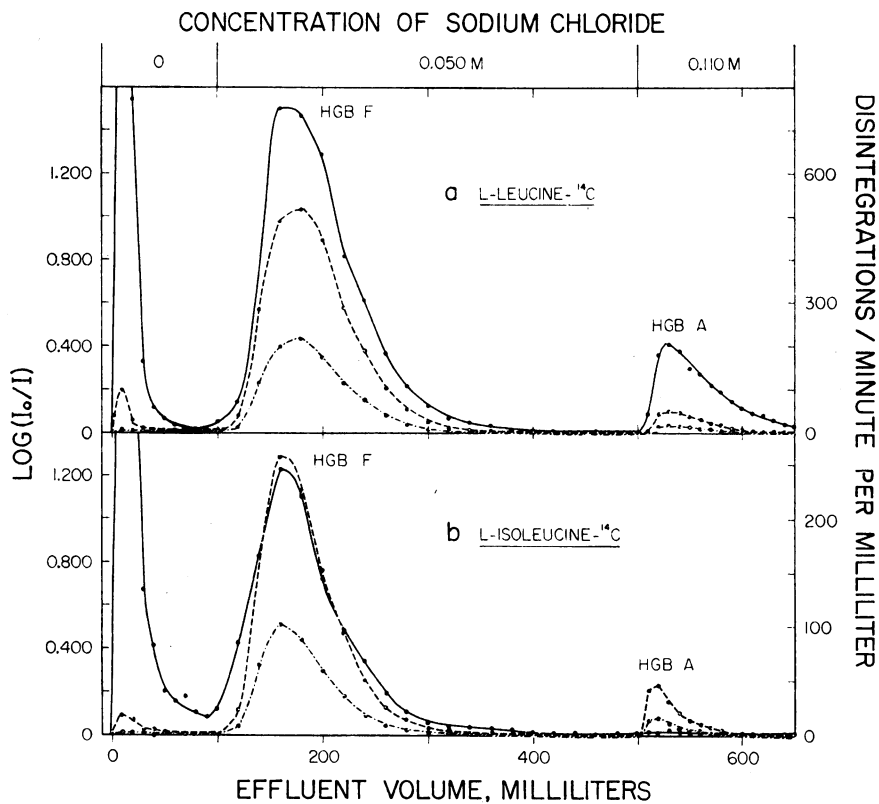


FIG. 1. CHROMATOGRAPHIC SEPARATION OF HEMOGLOBINS (HGB) F AND A. Cells from cord blood of a normal newborn infant were incubated for 2 hr with uniformly labeled radioactive amino acids as indicated. ---- absorbancy at 280 $m\mu$; - · - · - absorbancy at 542 $m\mu$; ——— radioactivity.

ethanol, two times with ethanol-ether 3:1 (v/v) at 62°C, and three times with ether. The dried precipitates were plated on tared aluminum planchets and counted in a Nuclear-Chicago gas flow counter with a Micromil window (16). Corrections were made for self-absorption of the samples. For counting of individual chromatographic

fractions, aliquots containing less than 1 mg of hemoglobin were precipitated with 10% TCA, heated at 90°C for 15 min, then collected on Millipore filter discs (Millipore Filter Corp., Bedford, Mass.), and washed with 5% TCA. The discs were dried, then placed in scintillation counting vials containing 10 ml of a 2,5-diphenyloxazole-

TABLE I
Incorporation of radioactive amino acids into fetal hemoglobin by erythroid cells in vitro

	% Reticu- locytes	Precursor amino acid	Incorpo- ration	% Radio- activity in hemo- globin F*
				<i>μmoles/g protein per hr</i>
Expt. 1, Rh hemolytic disease	13.1	L-Arginine-U- ¹⁴ C	2.52	81
		L-Leucine-1- ¹⁴ C	46.3	83
Expt. 2, Rh hemolytic disease	11.1	DL-Histidine-2- ¹⁴ C	31.0	42
		DL-Lysine-1- ¹⁴ C	41.0	46
Expt. 3, Rh hemolytic disease	14.2	L-Valine-1- ¹⁴ C	29.0	73
		L-Leucine-1- ¹⁴ C	48.5	77
Expt. 4, normal newborn infant	6.8	L-Leucine-U- ¹⁴ C	1.29	84

* Expressed as percentage of total radioactivity recovered in hemoglobin.

toluene mixture, 4 g/liter, and counted in a Packard scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.).

Results

Incorporation of amino acids into hemoglobin. The incorporation of several radioactive amino acids into hemoglobin F is presented in Table I. Results of a single experiment with cells from cord blood of a normal newborn infant are also included. The radioactivity incorporated into hemoglobin F by cells from erythroblastic infants comprised 42–83% of the total radioactivity recovered in hemoglobin, with a mean value of 67%. These values are in the same range as those from other studies with cells from normal newborns (7, 17, 18). The similarity in the pattern of hemoglobin synthesis by cells from infants with erythroblastosis and those from normal infants supports the suggestion (19) that the increased percentage of hemoglobin A measured in blood of erythroblastic infants (19–21) reflects a selective destruction of cells containing fetal hemoglobin, rather than a relative increase in the synthesis of hemoglobin A in this disorder.

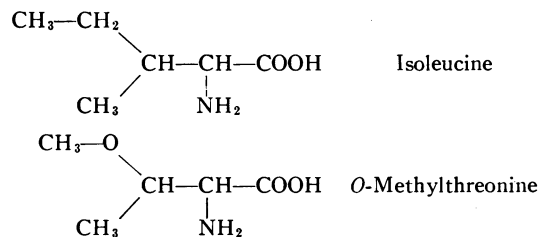


FIG. 2. CHEMICAL STRUCTURES OF ISOLEUCINE AND O-METHYLTHREONINE.

Inhibition of fetal hemoglobin synthesis by L-O-methylthreonine. The isoleucine analogue L-O-methylthreonine differs from isoleucine by an ether linkage which replaces a methylene group of the natural amino acid (Fig. 2). This analogue is a competitive inhibitor of isoleucyl-RNA synthetase, and is not incorporated into protein by mammalian cells (22).

The time course of incorporation of L-leucine-1-¹⁴C into hemoglobins A and F and the effect of L-O-methylthreonine are presented in Fig. 3. The rate of synthesis of hemoglobin F was effectively inhibited by the analogue, whereas the concomitant synthesis of hemoglobin A was not signifi-

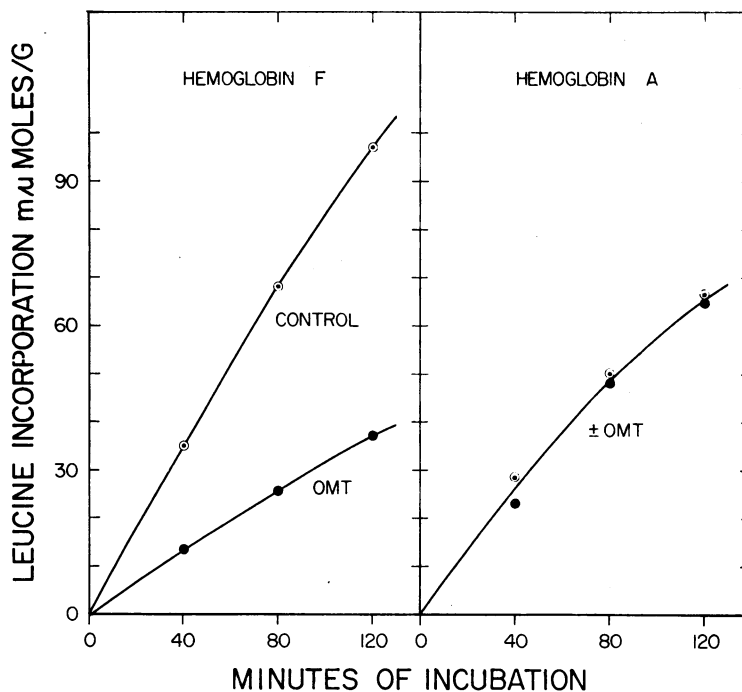


FIG. 3. TIME COURSE OF INCORPORATION OF L-LEUCINE-1-¹⁴C INTO HEMOGLOBINS F AND A AND EFFECT OF L-O-METHYLTHREONINE (OMT). Open circles, controls; closed circles, cells incubated with O-methylthreonine.

TABLE II
Inhibition of synthesis of fetal hemoglobin by L-O-methylthreonine

Precursor amino acid	Addition	Incubation time	Incorporation	
			Hgb F	Hgb A
		<i>min</i>	<i>mμmoles/g Hgb</i>	
L-Arginine-U- ¹⁴ C	None (control)		8.85	4.65
	OMT	210	3.86	4.38
	OMT + L-isoleucine		8.45	4.61
L-Leucine-1- ¹⁴ C	None (control)		161	81.0
	OMT	210	59	82.3
	OMT + L-isoleucine		161*	60.0
DL-Histidine-2- ¹⁴ C	None (control)		31.0	35.9
	OMT		13.5	26.0
	OMT + L-isoleucine	60	25.0	29.8
	L-Isoleucine		30.5	36.8
L-Valine-1- ¹⁴ C	None (control)		57.9	43.4
	OMT		25.8	39.1
	OMT + L-isoleucine	120	55.2†	29.3
	L-Isoleucine		51.5	38.4

OMT, L-O-methylthreonine; Hgb, hemoglobin.

* Because of the inhibition of incorporation into hemoglobin A, the value for hemoglobin F was multiplied by 81/60 for comparison with the other values.

† The experimental value was multiplied by 43.4/29.3.

cantly changed. A similarly selective inhibitory effect on the synthesis of hemoglobin F was observed when other radioactive amino acids were used as precursors, and the addition of L-isoleucine to the incubation media prevented the inhibition (Table II). Whereas synthesis of hemoglobin A was not significantly inhibited by L-O-methylthreonine, in experiments in which radioactive L-leucine or L-valine were used as precursors the addition of O-methylthreonine together with isoleucine inhibited amino acid incorporation into both hemoglobins F and A. For these experiments the values obtained for hemoglobin F were corrected by multiplication by an appropriate factor which corrected the value for hemoglobin A to its control value. This type of inhibition did not occur when isoleucine was added without O-methylthreonine; an explanation for this phenomenon is not apparent, but since these amino acids are closely related chemically to isoleucine, an effect on amino acid transport into the cell may have been involved.

Distribution of hemoglobins A and F in reticulocytes. The reticulocytes comprise the principal cells of peripheral blood which are capable of hemoglobin synthesis (23). The blood samples used for these studies also contained small numbers of normoblasts which synthesize hemoglobin, but mature erythrocytes are inactive in this capacity

(23). The distribution of hemoglobins A and F in the reticulocytes was studied by a method described by Garby, Sjölin, and Vuille (18). We prepared reticulocyte smears after staining the cells with aqueous new methylene blue (24). The preparations were then subjected to the selective elution procedure of Betke and Kleihauer (25) for demonstration of hemoglobin F. For improved identification of the reticulocytes, the slides were counterstained with Wright's stain. Reticulocytes were classified into three groups: (a) those appearing to contain only hemoglobin F; (b) those appearing to be devoid of hemoglobin F; and (c) those having intermediate amounts, which were presumed to be capable of synthesis of both hemoglobins F and A. Analysis of large numbers of cells indicated that approximately 50% of reticulocytes from infants with isoimmune hemolytic disease contained intermediate amounts of hemoglobin F, with the remainder of the reticulocytes nearly equally divided between the other two groups. It would appear, therefore, that a significant portion of hemoglobin synthesis carried out by these reticulocytes is produced by cells capable of synthesizing both hemoglobins F and A.

Discussion

These studies demonstrate that reticulocytes from newborn infants with isoimmune hemolytic

disease actively incorporate radioactive amino acids into hemoglobins F and A *in vitro*, and that a selective inhibition of synthesis of hemoglobin F can be achieved by the addition of an isoleucine analogue. The isoleucine present in hemoglobin F is confined to the gamma chains, which contain four residues of isoleucine per chain (26). The inhibition of synthesis of hemoglobin F by L-O-methylthreonine would therefore appear to result from a specific inhibitory effect of the analogue on gamma-chain production.

This experimental system may provide a useful model of human thalassemia of a gamma-chain type. The similarity of this chemical lesion to human thalassemia is clearly limited to the decreased rate of synthesis of a single chain of hemoglobin which both of them appear to manifest. The nature of the biosynthetic defect in thalassemia is not known; Itano (27) has suggested the possibility of an anomalous degenerate codon in the RNA template for the synthesis of a single hemoglobin chain. Assuming a relative deficiency of transfer-RNA species to correspond to the altered codon, such a mutation could be expected to inhibit hemoglobin synthesis because of a deficit of suitable activated amino acids at the ribosomal protein-synthesizing sites. A lesion of this type might produce an inhibition of hemoglobin synthesis similar to that seen with the present model.

The observation that the synthesis of hemoglobin A was unchanged by the inhibition of synthesis of hemoglobin F, in a cell population capable of synthesis of both of these species, suggests that these cells do not possess a cytoplasmic compensatory mechanism for increased beta-chain synthesis when gamma-chain production is suppressed. The physiological "switchover" from synthesis of hemoglobin F to that of hemoglobin A in the perinatal period would appear, therefore, not to be a consequence of an inhibition of gamma-chain production, at least at the ribosomal level of protein synthesis. We further conclude that the biosynthetic apparatus for the beta and gamma chains must function in an independent manner.

A common requirement for production of both hemoglobins F and A is the synthesis of alpha chains. Evidence has been presented which suggests that the release of alpha chains from ribosomes, as well as their synthesis, by reticulocytes from rabbits (6) or human adults (3), may re-

quire the prior synthesis of beta chains, as part of a coordinated mechanism for balanced synthesis of the two types of complementary chains. A mechanism of this type might be at least partially responsible for the absence of appreciable accumulation of free alpha chains or subunits in red cell hemolysates in beta thalassemia (4). Fessas, Loukopoulos, and Kaltsoya (28) have recently shown, however, that erythroid cells from patients with beta thalassemia contain inclusion bodies composed largely of precipitated alpha chains, which could account for this finding. Several recent studies (3, 29, 30), moreover, have shown an increase in the rate of alpha-chain synthesis as compared with beta-chain synthesis in reticulocytes from patients with beta thalassemia. It will be of importance to determine whether the unbalanced synthesis of the hemoglobin chains in thalassemia indicates the absence of a control mechanism in erythroid cells for the coordinated synthesis of the globin components of hemoglobin (3, 6) or is a specific manifestation of the biosynthetic defect in thalassemia. The selective inhibition of synthesis of gamma chains which can be achieved by the present system may be applicable for gaining further understanding of this relationship in the synthesis of the component chains of hemoglobin.

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