Globin Chain Synthesis in the Alpha Thalassemia Syndromes

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ABSTRACT Whole blood samples of patients with various forms of alpha thalassemia including hemoglobin H disease, alpha thalassemia trait, and the "silent carrier" state were incubated with leucine-^{14}C for definition of relative rates of production of alpha and beta chains in these disorders. The chains were separated by carboxymethyl cellulose chromatography in the presence of 8 M urea and dithiothreitol. Their absorptions at 280 mμ were determined and their radioactivities measured in a liquid scintillation spectrometer. After correction for differences in extinction coefficients, the specific activities of the widely separated alpha and beta peaks were determined. In 11 nonthalassemic individuals, the alpha/beta specific activity ratios were found to be 1.02 ± 0.07; in nine patients with alpha thalassemia trait, 0.77 ± 0.05; in six patients with hemoglobin H disease, 0.41 ± 0.11; and in four "silent carriers," 0.88 with a range of 0.82–0.95. The results show that in peripheral blood, alpha chain production relative to beta chain production is indeed limited in the alpha thalassemia syndromes. Hemoglobin H disease results from doubly heterozygous inheritance of a gene resulting in moderate depression of alpha chain production (alpha thalassemia trait) and a gene resulting in very mild depression of alpha chain production (the "silent carrier" syndrome."

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This work was presented in part at the Plenary Session of the Annual Meeting of the Society for Pediatric Research, 3 May 1968, Atlantic City, N. J.

Received for publication 20 May 1968.

INTRODUCTION

Alpha thalassemia is a complex disorder with at least four different clinical manifestations (1). The first, alpha thalassemia trait, the heterozygous state, is a mild disease characterized mainly by microcytosis and little if any anemia. The second, hydrops fetalis as a result of homozygous alpha thalassemia, is a lethal disorder in which death occurs in utero or at birth (2). In this disease, there is total absence of hemoglobin containing alpha chains and complete replacement of the newborn hemoglobin mixture by hemoglobin Bart's (γ_4) (3, 4). The third syndrome, hemoglobin H disease, is an hemolytic hypochromic anemia with intraerythrocyte inclusion formation. In such erythrocytes, hemoglobin H (β_4) may comprise up to 40% of the total hemoglobin (5). Such patients are usually the offspring of one parent with alpha thalassemia trait and one with the fourth alpha thalassemia syndrome, the so-called "silent carrier" state. Several different terms have been employed to describe the latter syndrome (6–9). The "silent carrier" is here defined as the hematologically normal parent of an individual with hemoglobin H disease. Theoretically he may also be the offspring of an individual with hemoglobin H disease.

On the basis of gross deficiency of alpha chains, both hydrops fetalis and hemoglobin H disease may be ascribed to reduced alpha chain production. Indeed, evidence for low alpha chain production relative to beta chain production in hemoglobin H disease has been provided by Weatherall, Clegg, and Naughton (10) and, by similar techniques, low beta chain production rela-

tive to alpha chain production has been detected in various beta thalassemia syndromes (10–12). Defective alpha chain production in alpha thalassemia trait is suggested on the basis of family studies and red cell morphology. The "silent carrier" state has been poorly understood and is thought to be a genetic condition which when inherited in association with a gene for alpha thalassemia trait leads to further depression of alpha chain production (7). In this report, we present firm evidence of reduced alpha chain production in three alpha thalassemia syndromes including the "silent carrier" state. This evidence was derived by measurement of the rate of incorporation of leucine-^{14}C into the alpha and beta chains of the peripheral blood hemoglobin of normal individuals, patients with hemoglobin H disease and alpha thalassemia trait, and of individuals considered to be "silent carriers."

METHODS

Subjects

Patients either of Chinese or Italian extraction

Hemoglobin H disease. Six patients with this disorder were studied. The hematologic diagnosis was confirmed by demonstration of hemoglobin H with starch-gel electrophoresis (13).

Alpha thalassemia trait. 9 individuals with microcytosis and (or) mild hypochromia with normal serum iron concentration and normal hemoglobin electrophoresis were included in this group. They were either parents of an hydropic baby with 100% hemoglobin Bart's or parents or offspring of patients with hemoglobin H disease.

"Silent carriers." Three individuals whose spouses had alpha thalassemia trait and who had at least one offspring with hemoglobin H disease were included in this group. They had no anemia, microcytosis, or hypochromia. Rare poikilocytes were observed in their stained blood smears. One child of a patient with hemoglobin H disease was also included in this group.

Nonthalassemic controls

Eight normal individuals, including one of Chinese and two of Italian extraction, and three patients with hypochromic anemia as a result of iron deficiency were selected as controls. Two of the latter patients were studied while they were undergoing mild reticulocytosis during iron therapy.

Methods

Hematologic values were determined by standard methods (14).

Incubation

5 ml of heparinized peripheral blood to which dextrose (2 mg/ml) was added were preincubated in a 25 ml Erlenmeyer flask at 37°C for 10 min in a Dubnoff metabolic shaker. 15 μc of uniformly labeled L-leucine-^{14}C [1] was then added. Incubation was continued for 2 additional hr. The red blood cells were then thrice washed with cold isotonic sodium chloride. The packed red cells were hemolyzed by the method of Lingrel and Borsook (15). The hemolysate was then centrifuged at 20,000 *g* for 20 min to remove stroma.

Preparation of globin [2]

Hemolysate containing 100 mg of hemoglobin was added to 80 ml of 0.05 M barbital buffer, pH 8.6, which also contained 0.015 M 2-mercaptoethanol. The hemoglobin was precipitated by the addition of 4 ml of 0.4 M zinc acetate and was centrifuged at 2000 *g* for 10 min. It was solubilized in approximately 10 ml of cold acid acetone (1 part 12 M HCl to 90 parts acetone) to which was added 2-mercaptoethanol in a final concentration of 0.015 mole/liter. The globin was then precipitated quantitatively when an additional 80 ml of the acid acetone was added. It was centrifuged at 2000 *g* for 10 min and then thrice washed with cold acetone.

Separation of alpha and beta chains and determination of their specific radioactivities

The method was that of Clegg, Naughton, and Weatherall (16) with minor modifications. The starting buffer contained 8 M urea,[3] which had been deionized by passing through a column of mixed bed resins,[4] 2.6×10^{-3} M Na_2HPO_4 and 6×10^{-4} dithiothreitol,[5] the pH of the buffer adjusted to 7.2 with 10% phosphoric acid. Globin was dissolved in 2–3 ml of this buffer and then dialyzed against five changes of the same buffer for 2½ hr. 75 mg was then applied to a 1 × 20 cm carboxymethyl cellulose column [6] previously equilibrated with the same buffer. It was eluted with a linear gradient system delivered from a gradient mixing apparatus.[7] The first chamber contained 150 ml of the starting buffer and the second chamber held 150 ml of a buffer containing urea and

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[1] Supplied by Tracerlab Div., Laboratory For Electronics, Inc., Waltham, Mass., with a specific activity of 200 mc/mmole.

[2] Gerald, P. S. Personal communication.

[3] Supplied by J. T. Baker Chemical Co., Phillipsburg, N. J., and by Mallinckrodt Chemical Works, St. Louis, Mo.

[4] Rexyn I-300, supplied by Fisher Scientific Company, Pittsburgh, Pa.

[5] Supplied by Nutritional Biochemicals Corporation, Cleveland, Ohio.

[6] Whatman CM52, supplied by Reeves Angel & Co., Clifton, N. J.

[7] Contigrad, manufactured by Metaloglass, Inc., Boston, Mass.

dithiothreitol, at the same concentrations but with 2.8×10^{-2} M Na_2HPO_4, the pH adjusted to 7.2 with H_3PO_4. The flow rate was regulated at approximately 0.4 ml/min with a Technicon proportioning pump, and 6 ml fractions were collected. The optical density at 280 mμ of each of the collected fractions was determined in a Hitachi-Perkin-Elmer spectrophotometer. In later experiments, improved resolutions of the protein peaks were obtained by a somewhat different elution system. Four chambers of the gradient mixer were each filled with 100 ml of buffer. Each contained urea and dithiothreitol as above. In the first chamber, the concentration of Na_2HPO_4 was 2.6×10^{-3} mole/liter, in the second and third 1.5×10^{-2} mole/liter and in the fourth 3.2×10^{-2} mole/liter. All of the buffers were adjusted to pH 6.7 with 10% H_3PO_4.

In two experiments the absorptions at 280 mμ of isolated alpha and beta chains were compared by dissolving a weighed amount of each chain in buffer derived from that fraction of the ionic strength gradient in which the respective chain emerged. The absorption at 280 mμ/mg of each chain was then determined. It was found that the absorption of beta chains exceeded that of alpha chains by a value of 1.52, both at pH 7.2 and at pH 6.7.

Measurement of radioactivity. 1-ml aliquots of each fraction were added to 15 ml of a counting solution of the following composition: toluene, methanol, ethylene glycol monomethyl ether, and ethanolamine, 3:3:2:1. This contained 2,5-diphenyloxazole (PPO), 0.3 g and *p*-bis [2-(5-phenyloxazolyl)]benzene (POPOP), 0.025 g/100 ml. This constituted a single phase liquid system. There was some precipitation of phosphate in the later fractions, without effect on the counting efficiency. The radioactivity was determined in a Packard model 3375 liquid scintillation counter with 30% gain and window settings of 50–500 divisions. The efficiency for ^{14}C in this system was 50% at 10°C. Automatic external standard counting did not reveal variable quenching in the fractions. The counting error (2 SD of the count rate and background divided by the net count rate) was no greater than 5% and was usually 3%.

Specific activity was computed as counts per minute per absorbance units (cpm/OD). The calculation was performed only on homogeneous fractions which immediately surrounded and included the maximum optical densities of the separated chains. The cpm/OD of each fraction from the same peak agreed with one another within ±5%.

The relative rates of synthesis of the two chains were expressed as the ratio $\frac{\text{specific activity of alpha chain}}{\text{specific activity of beta chain}}$ after correction for the 280 mμ absorption differences of the two chains.

RESULTS

The hematologic values of the patients affected with alpha thalassemia are summarized in Table I. The patients with hemoglobin H disease were moderately anemic; their red cells were hypochromic and microcytic. The predominant alteration in alpha thalassemia trait was microcytosis. None of these patients was anemic, and erythrocytosis was common. The hematologic values for the "silent carriers" were normal. A few irregular elongated erythrocytes were apparent in stained smears of their peripheral blood.

Fig. 1 indicates typical separations into alpha and beta chains on carboxymethyl cellulose columns of the globin derived from a control subject, a patient with alpha thalassemia trait and a patient with hemoglobin H disease. The identities of the alpha and beta chains have been confirmed by Clegg and his coworkers (16) and urea starch-gel electrophoresis [2] performed on our first few columns confirmed their data. Both absorbance and radioactivity are shown in these figures. The lower specific absorption of alpha chains at 280 mμ is revealed by the data derived from the normal subject. This is in agreement with the data of Clegg and coworkers (16) who also found such a difference in absorbance. Fantoni, Bank, and Marks also confirmed the finding in globin obtained from mice (17), but Bank and Marks did not find this difference in human hemoglobin (11). We believe that the difference in absorbance is best explained by the presence of two tryptophans in the beta chain compared to one in the alpha chain in human hemoglobin (18). Tryptophan has the highest molar extinction coefficient in the ultraviolet range (19). At pH 7.2 a small peak which emerged just before the beta chains contained neither alpha nor beta chains at least as measured with urea starch-gel electrophoresis. The radioactivity associated with this peak was close to the beta chain peaks and occasionally "spilt over" to the front portion of the beta chain peak. For uniformity in calculation, only the tubes at the peak and around the descending border of the beta chain peak were used for calculation of beta chain specific activity. The validity of this method of calculation was supported by the similarity of our results in the normal control to those previously reported (10, 11).

Table I and Fig. 2 summarize the results of all of our studies of the alpha/beta specific activity ratios in the control subjects and in patients with the alpha thalassemia syndromes. In the control group, the ratio was 1.02 ± 0.07 with no differ-

TABLE I
Hematologic Values and Ratios of Specific Activity

Family name	Subject	Race*	Red blood cells	Hemoglobin	Hematocrit	Reticulocyte	Mean corpuscular volume	Mean corpuscular hemoglobin	Mean corpuscular hemoglobin concentration	Ratio of specific activity α/β
Identification			Red blood cell values							Subunit synthesis
			10^{-6}/mm.	g/100 ml	%	%	μ^3	$\mu\mu g$/cell	g/100 ml	
Hemoglobin H disease										
J. E.		C	6.28	10.0	40.5	7.2	64.5	15.9	24.6	0.31
L. U.	II 3	I	4.88	8.8	32.5	6.6	66.6	18.0	27.0	0.62
D. A.	II 4	I	5.02	8.8	38.0	6.0	75.7	17.5	23.1	0.34
D. A.	II 5	I	6.58	11.6	43.0	3.8	65.3	17.6	26.9	0.30
D. A.	II 6	I	6.08	11.2	40.0	4.6	65.8	18.4	28.0	0.49
M. A.	II 1	I	5.26	9.3	36.0	16.4	68.4	17.7	25.8	0.42
										0.41 ($\pm$ 0.11)‡
Alpha thalassemia trait										
L. U.	I 1	I	6.06	13.7	43.0	1.2	70.9	22.6	31.8	0.70
L. U.	III 2	I	4.98	11.2	37.0	1.6	74.3	22.5	30.2	0.81
L. U.	III 3	I	4.87	11.8	38.5	1.6	79.1	24.2	30.6	0.78
D. A.	III 1	I	5.68	11.8	39.0	1.6	68.7	20.5	30.2	0.82
M. A.	I 1	I	6.83	15.5	46.5	0.4	68.1	22.7	33.3	0.69
G. E.	I 1	C	6.35	14.2	44.0	1.1	62.3	22.4	32.2	0.75
G. E.	I 2	C	5.59	12.2	39.5	0.8	70.7	21.8	30.8	0.77
Y. E.	I 1	C	6.85	14.5	43.0	1.0	62.8	21.2	33.7	0.81
Y. E.	I 2	C	5.60	13.3	38.5	1.5	68.7	23.7	34.5	0.80
										0.77 ($\pm$ 0.05)‡
Silent carriers										
L. U.	I 2	I	5.07	14.3	44.0	1.6	86.8	28.2	32.5	0.83
M. A.	I 2	I	4.36	11.3	36.5	0.6	83.0	26.0	31.0	0.82
D. A.	I 1	I	4.45	12.6	42.0	1.2	94.4	28.3	30.0	0.91
L. U.	III 1	I	4.49	15.0	42.5	0.5	94.7	33.4	35.2	0.95
										0.88 ($\pm$ 0.06)‡
Nonthalassemic controls										
	Normal (8) and iron deficiency (3)									1.02 ($\pm$ 0.07)‡

* C, Chinese; I, Italian.
‡ Mean $\pm$ 1 SD.

ences detected between normals and individuals with iron deficiency. In alpha thalassemia trait it was 0.77 ± 0.05, and in hemoglobin H disease it was 0.41 ± 0.11. No attempt was made to correct the value observed in hemoglobin H disease for the large pool of free beta chains which were not present in the other types of cells under study. In the four "silent carriers" the average ratio was 0.88 with a range of 0.82–0.95. These results are highly significant. The nonthalassemic controls and the patients with alpha thalassemia trait and hemoglobin H disease showed no overlap in their ratios and could be separated into three distinct groups ($P < 0.001$). The ratios obtained from the four "silent carriers" lie just below 1 SD of those of the normal group and just above 1 SD of those of the alpha thalassemia trait group. Although individually they cannot be precisely distinguished

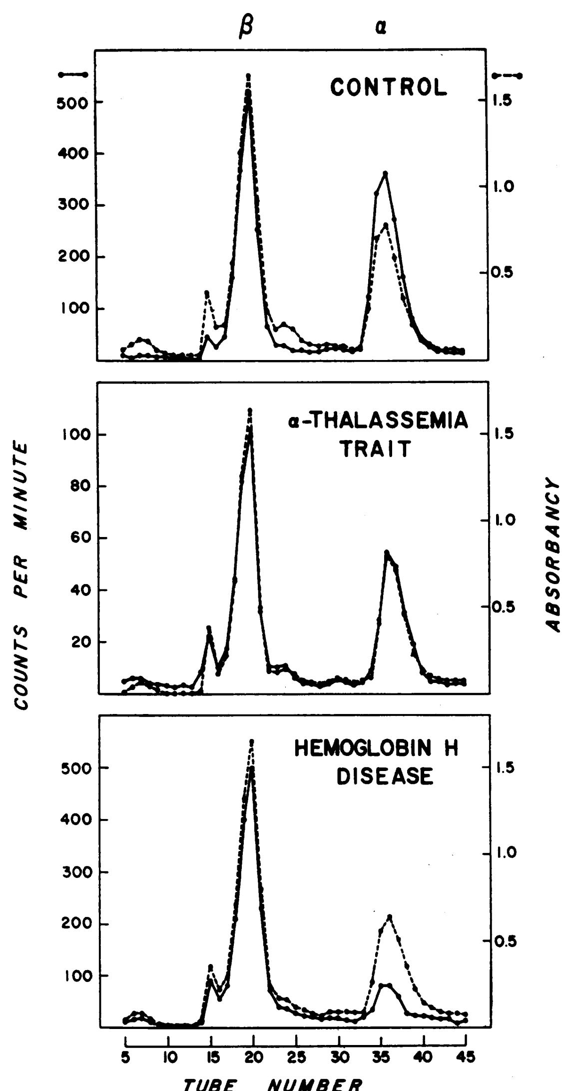

FIGURE 1 Typical separation on a carboxymethyl cellulose column at pH 7.2 of alpha and beta chains from the hemolysate prepared from control, alpha thalassemia trait, and hemoglobin H patients. (Absorbancy is uncorrected.)

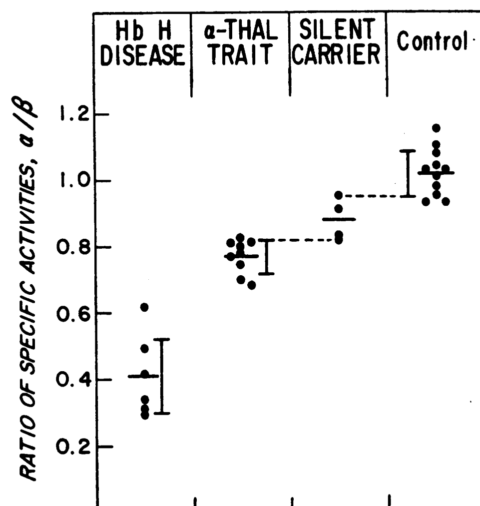

FIGURE 2 Corrected ratios of specific activities alpha/beta in the four groups studied. Mean and 1 SD are shown by the horizontal lines. The ratios for the four silent carriers lie just outside 1 SD of the alpha thalassemia trait and the control groups (dashed line).

from normal individuals or those with alpha thalassemia trait, taken as a whole the ratios obtained in the "silent carriers" are significantly different from normal and from alpha thalassemia trait ($P = 0.01$–0.001).

Family studies of the patients affected with alpha thalassemia are represented in Fig. 3. In this figure, the establishment of the diagnosis of each of the different syndromes was based on clinical and genetic criteria, and the alpha/beta specific activity ratios are noted in those patients who were so studied. These ratios corresponded to the values which were expected from currently held concepts of the mode of inheritance of the alpha thalassemia syndromes. The presumed "silent carrier" in generation III of family L.U. had a synthetic ratio at the low border of normal values. This case is discussed more thoroughly below.

DISCUSSION

The results of this study of globin synthesis in the peripheral blood firmly support the classification of the alpha thalassemia syndromes into distinct clinical and genetic entities: alpha thalassemia trait, hydrops fetalis associated with hemoglobin Bart's, hemoglobin H disease, and the "silent carrier" syndrome. Although it is not known if globin chain synthesis in reticulocytes of peripheral blood is representative of that in the bone marrow precursors, these results clearly distinguish the four groups of subjects studied. The low reticulocyte counts encountered in some of these subjects posed a serious technical obstacle to this study. This was overcome by prolonged radioactive counting of aliquots of the widely separated protein peaks in a highly stable liquid scintillation system.

The presence of alpha thalassemia trait is usually recognized on genetic grounds (20). This

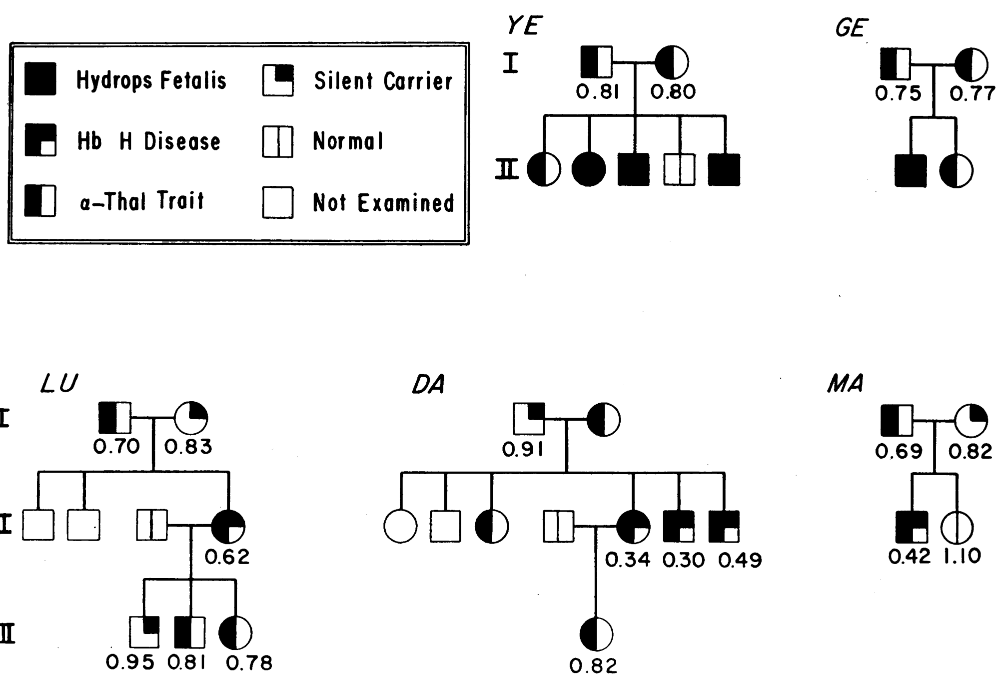

FIGURE 3 Pedigree of the families. The symbols represent diagnoses made on clinical and genetic grounds. The numbers are the alpha/beta specific activity ratios obtained in this study.

diagnosis is ascribed to those patients who exhibit changes in red blood cell morphology similar to mild beta thalassemia trait but who have normal percentages of hemoglobins A and F and are not iron deficient. The patients are found within families in which hemoglobin H disease or hydrops fetalis occurs. They may also be recognized either in family studies in which interaction fails to occur in the presence of a beta chain abnormality such as hemoglobin S (6) or in families in which thalassemia interacts with an alpha chain abnormality (21, 22). Evidence for deficiency of alpha chain synthesis in alpha thalassemia trait has been lacking except in the neonatal period when cord blood concentrations of hemoglobin Bart's may exceed 5% of the total hemoglobin (20). In the adult, precipitable hemoglobin, stainable with brilliant cresyl blue, can be demonstrated in a tiny proportion of the erythrocytes of some individuals (23). This is believed to represent hemoglobin H (1, 23). Despite this minimal evidence of subunit imbalance, our present studies show that a definite reduction of alpha chain synthesis by the reticulocytes to a level of approximately 75% of beta chain synthesis occurs in the heterozygous state. Those individuals in this study who have alpha thalassemia trait on clinical and genetic grounds all have subunit synthetic ratios which confirm this diagnosis (Fig. 3).

In hemoglobin H disease, the presence of the beta chain tetramers clearly indicates that alpha chains are deficient in the hemoglobin mixture. Our present study confirms the findings of Clegg and Weatherall who showed previously that alpha chain specific activity is diminished in this syndrome (10, 24). Our results, and those of Clegg and Weatherall, show a wide variation in the alpha/beta specific activity ratios among patients with hemoglobin H disease. The alpha/beta ratio in this disorder tends to underestimate the relative rates of synthesis of beta chains, since the large pool of preformed beta chains (hemoglobin H) dilutes the radioactive beta chains that are formed during the relatively short time of incubation with leucine-^{14}C. The observed beta specific activity is affected by the size of this pool which may vary from patient to patient. Little or no influence of unequal pool size is exerted in alpha thalassemia trait and the "silent carrier" state, since excess beta chains are extremely difficult if not impossible to demonstrate in these disorders. Therefore, the alpha/beta specific activity ratios are more

exact functions of relative synthetic rates in the latter conditions.

If hydrops fetalis associated with hemoglobin Bart's represents the homozygous state of alpha thalassemia, the clinically less severe hemoglobin H disease must represent a disorder intermediate in severity between the homozygous and heterozygous state. The concept of the "silent carrier" gene has been proposed to explain this phenomenon (6–9). It is allegedly carried by the hematologically normal parent of a patient with hemoglobin H disease, a disease which represents double heterozygosity for an alpha thalassemia gene and a "silent carrier" gene. Up to now, no evidence for a deficit in alpha chain synthesis has been demonstrated when the latter gene occurs alone in the "silent carrier" state. Our present study of the three "silent carriers," who are the hematologically normal parents of patients affected with hemoglobin H disease, clarifies this issue because all three had very mild reduction of alpha chain synthesis relative to beta chain synthesis in their reticulocytes. Thus, as originally postulated by Sturgeon, Jones, Bergren, and Schroeder (7), the gene for the "silent carrier" state is itself responsible for a degree of alpha chain suppression and contributes to the establishment of hemoglobin H disease in an additive fashion. The reduction of alpha chain specific activity to only 75% of beta chain specific activity in alpha thalassemia trait may result either from incomplete suppression of the one gene affected by alpha thalassemia or from compensatory increase in production by the normal gene. The total suppression of alpha chain synthesis in the homozygous state (hydrops fetalis) (2–4) supports the latter alternative. In hemoglobin H disease, compensation by the gene not affected with alpha thalassemia is limited by the gene responsible for the "silent carrier" state.

One individual (in generation III of family L.U.) who was classified within the "silent carrier" group on genetic grounds requires special attention. He is the hematologically normal child of a patient affected with hemoglobin H disease. His two siblings both have alpha thalassemia trait substantiated by clinical criteria and by their alpha/beta specific activity ratios. His specific activity ratio is at the lower limit of normal. It is not possible in this study to determine absolutely whether he is normal or a "silent carrier," and this differentiation would be necessary for complete understanding of the question of allelism in this disorder. At the present, there is no definite evidence to indicate whether or not the "silent carrier" gene is allelic with the alpha thalassemia gene (8, 25). Indeed in several family studies, hemoglobin H disease has been detected in a parent and a child with the second putative parent hematologically normal (8, 26–28). This would, on the surface, favor nonallelism of the two genes. However, these data do not actually rule out allelism since it has not been possible with heretofore available techniques to detect the "silent carrier" gene in the apparently normal spouse of an individual with hemoglobin H disease. The gene frequency for the silent carrier state is unknown. If a large group of hematologically normal children of hemoglobin H parents are analyzed with the technique reported in this study and the result compared to a large number of known "silent carriers" (viz. the normal parent of a patient with hemoglobin H disease), it will be possible to determine whether the two genes are allelic. If they are allelic, the result of the specific activity ratio determinations in the two groups will be similar. If they are not allelic, many of the hematologically normal offspring of hemoglobin H patients will fall within the normal range of alpha/beta synthetic ratios.

ACKNOWLEDGMENTS

We thank Drs. Thomas Necheles, Shirley Driscoll, and John Craig for referring several of their patients for our studies, and Rebecca Tiedemann, Domenica Paci, and Lynn Esler for technical assistance.

This work was supported by U. S. Public Health Service Grants HD 02777, HE 5255, and K3 AM 35361, and by a grant from the John A. Hartford Foundation.

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