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Research Article

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Nature of Fetal Hemoglobin in the Greek Type of Hereditary Persistence of Fetal Hemoglobin with and without Concurrent β-Thalassemia

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Abstract The fetal hemoglobin in the affected members of three Greek families with the hereditary persistence of fetal hemoglobin has only γ-chains of the type with alanine in position 136. Although certain Negro families had been considered to have only this type of γ-chains in their fetal hemoglobin, further studies required that they be reclassified. Consequently, the Greek cases are the sole examples of this class among the heterozygotes for the hereditary persistence of fetal hemoglobin. In Greek double heterozygotes for β-thalassemia and the hereditary persistence of fetal hemoglobin, fetal hemoglobin is increased above the level of hemoglobin F in simple heterozygotes and γ-chains with glycine in position 136 become apparent. In these individuals, γ-chains with alanine in position 136 apparently derive from the chromosome for the hereditary persistence of fetal hemoglobin and are present in the hemoglobin F with γ-chains of both types from the chromosome for β-thalassemia. When these data are correlated with earlier knowledge of the genetic state of the Greek individuals, modifications of our previous ideas about deletions as the genetic basis of the hereditary persistence of fetal hemoglobin must be considered.

INTRODUCTION

As a result of an investigation of human fetal hemoglobin (Hb F) from umbilical cord blood (1), it was concluded that at least two chromatographically and electrophoretically inseparable components were present. These two Hb F's differ at a minimum in the type of amino acid residue at one position of the γ-chain.

As a continuation of these studies, the procedures were applied to Hb F of the condition known as the "hereditary persistence of fetal hemoglobin" (HPFH) which was first observed in the Negro (2) and has since been detected among the Greeks (3) and sporadically in other ethnic groups (4) (Wasi, Pootrakul, and Na-Nakorn [5] give an extensive bibliography). When Hb F of heterozygotes for Negro HPFH was studied (6), this anomaly was shown to be heterogeneous at the molecular level of Hb F. The present paper will describe the results of a study of Greek heterozygotes for HPFH with and without concurrent β-thalassemia.

METHODS

Source of samples. Samples were obtained from two Greek families (families 1 and 2, Fig. 1 and Table I) in which the condition was detected during a survey of approximately 300 families in Kardies and Greece.[1] Other samples derived from family 3 which previously has been termed "family B" (3). The methods of hematologic examination and the criteria for diagnosis of the Greek type of HPFH have been described (3). The hematologic findings for family 3 (Table I) are those already reported (3). Blood was collected in Greece in acid-citrate-dextrose solution and shipped under refrigeration from Greece to Seattle, Wash. and to Augusta, Ga.

Chromatography of hemoglobin components; isolation of Hb F. Chromatography on 0.9 × 45 cm columns of DEAE-Sephadex (A-50, capacity 3.5 ±0.5 mEq/g, particle size 40–120 µ, Pharmacia Fine Chemicals, Inc., Piscataway, N. J.)

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[1] Stamatoyannopoulos, G. Unpublished observations.

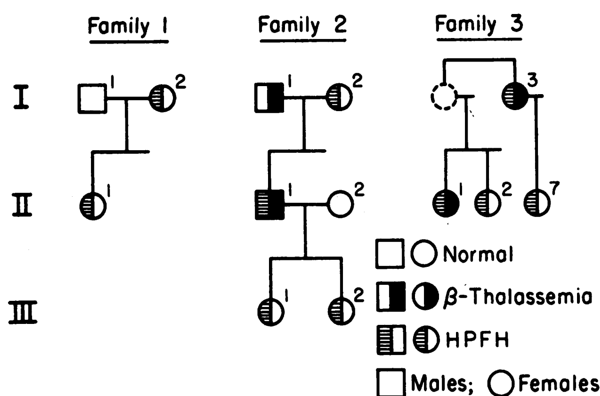

FIGURE 1 Pedigrees of Greek families.

has been used for the analytical determination of the hemoglobin components of hemolysates, whereas the preparative isolation of Hb F for chemical examination was made on larger columns (3.0 × 60 cm) of DEAE-Sephadex (7, 8).

Estimation of Hb F. Three methods were applied to most samples: (*a*) the chromatographic procedure described in the preceding section, (*b*) the alkali denaturation method of Betke, Marti, and Schlicht (9), and (*c*) a newly developed method (10). The latter method bases the quantitation of Hb F upon the ratios of isoleucine to leucine and to phenylalanine in the zone of Hb's A_1 and F_0 which is isolated by DEAE-Sephadex chromatography. From a detailed comparison of analyses by the three methods (10), it was concluded that the results from the new method are the most reliable. Results from the three methods are presented as % F_{Chrom}, % $F_{A.D.}$, and % F_{Ile}, respectively.

Chemical examination of Hb F. After globin had been prepared and reacted with cyanogen bromide, peptide γCB-3 was isolated by Bio-Gel chromatography, purified by Dowex-1 chromatography, and analyzed as previously described (1). The γCB-3 peptide from some samples was degraded by the Edman PTH procedure on paper strips (11).

The manner of evaluating the chemical data is described in Results.

RESULTS

Manner of presentation of the chemical data. The chemical data which take the form previously used (1, 6) record the residues of glycine and alanine from the analysis of the peptide γCB-3 (Table I). This analysis determines whether one or the other type of γ-chains individually or both are being produced. Thus, peptide γCB-3 originates from the C-terminal 13 residues of the γ-chain and has the sequence Val-$\overset{135}{\text{Thr}}$-Gly (or Ala)-Val-Ala-Ser-$\overset{140}{\text{Ala}}$-Leu-Ser-Ser-Arg-$\overset{145}{\text{Tyr}}$-His. The two types of chain differ in the presence of glycine or alanine at position 136. If glycine is present, the chains are designated as $^{G}\gamma$ and if alanine, as $^{A}\gamma$ (see terminology below). If the analysis shows integral numbers of glycyl and alanyl residues such as 1 and 2 or 0 and 3, then $^{G}\gamma$- or $^{A}\gamma$-chains, respectively, are present in the Hb F. Because alanyl residues are always present at positions 138 and 140, each analysis has a minimum of two alanyl residues. If the values for glycine are intermediate between 0 and 1 and those for alanine between 2 and 3, a mixture is indicated and the proportion in the mixture can be calculated. The value for glycine is the simplest indicator of proportion; thus, if glycine is 0.75, 75% of the mixture is $^{G}\gamma$-chains or the ratio of $^{G}\gamma$- to $^{A}\gamma$-chains is 3:1.

Terminology. Because there is more than one type of human γ-chain, a new nomenclature is necessary to describe hemoglobin loci and chains. This nomenclature

TABLE I
Hematological and Chemical Data on Greek HPFH Cases with and without Concurrent β-Thalassemia

Family	Subject	Condition	Hb	RBC	Hb A_2	Hb F*			γCB-3	
						A.D.	Chrom.	Ile	Gly	Ala
			g/100 ml	*$10^6/mm^3$*	%		%			
1	I-1	Normal	13.4	4.24	2.7	—	7.4	—		
	I-2	HPFH	14.8	4.75	2.05	13.6	23.0	17.5	0.13	2.96
	II-1	HPFH	11.6	4.07	1.9	14.4	23.9	17.5	0.11	2.96
2	I-1	β-Thalassemia	12.1	5.27	3.5	0.9	9.2	—	0.74	2.32
	I-2	HPFH	13.0	4.55	1.55	—	18.5	18.5	0.06	2.95
		(Second sample)	—	—	1.55	13.7	22.5	17.1, 14.0	0.06	3.03
	II-1	HPFH and β-thalassemia	12.4	5.47	3.65	—	49.3	43.4	0.33	2.70
	II-2	Normal	14.1	4.90	2.8	—	5.8	—		
	III-1	HPFH	12.6	4.56	1.5	14.4	23.6	18.9	0.07	2.98
	III-2	HPFH	11.8	4.57	2.05	14.4	24.2	—	0.08	2.93
3‡	I-3	HPFH and β-thalassemia	9.3	4.21	4.1	20.9	28.9	26.4	0.24	2.81
	II-1	HPFH and β-thalassemia	9.5	4.66	4.4	22.7	32.1	26.6	0.22	2.86
	II-2	HPFH	—	—	2.1	8.6	24.3		0.05	3.00
	II-7	HPFH	14.6	4.71	1.7	14.0	20.3	16.5	0.11	2.97

* Minor fraction designated as Hb F_1 is also included.
‡ Family 3 corresponds to family B of Fessas and Stamatoyannopoulos (3).

conforms to that which has recently been used for the goat α-chains by Adams, Wrightstone, Miller, and Huisman (12). Superscripts and subscripts attached to the γ, thus ${}^{x}\gamma_{z}^{y}$, have the following meanings. The preceding superscript at "x" refers to the type of nonallelic locus. The distinguishing feature of the two γ-chains is the presence of glycine or alanine at position 136. Consequently, the superscripts "G" or "A" will be used to designate the locus, and ${}^{G}\gamma$ and ${}^{A}\gamma$ will refer to chains which previously were termed γ^{136Gly} or γ^{G} and γ^{136Ala} or γ^{A}. The following superscript at "y" designates any mutant of the locus in the usual manner, thus ${}^{x}\gamma^{Texas}$. The following subscript at "z" is used for numbers in the usual chemical sense when reference to a complete hemoglobin molecule might be $\alpha_2{}^{x}\gamma_2{}^{Texas}$. Because the analysis of the γCB-3 peptide identifies the types of Hb_{γ} loci that are operative, the HPFH groups will be defined in terms of the active γ-locus (loci). When the heterozygote produces Hb F with ${}^{G}\gamma$ chains only, this will be referred to as the "$Hb_{G\gamma}$ type" of HPFH. In like manner, there would be the "$Hb_{A\gamma}$ type" and the "$Hb_{G\gamma}$ $Hb_{A\gamma}$ type." This classification uses the chemical data from simple heterozygotes and refers only to *types* and not to *numbers* of operative Hb_{γ} loci. If more than one each of the $Hb_{G\gamma}$ and $Hb_{A\gamma}$ loci exist, if it becomes possible to determine the number of operative Hb_{γ} loci of one or the other type, or when speculation about such a possibility is made, extension to designations such as "$2Hb_{G\gamma}Hb_{A\gamma}$" or "$2Hb_{A\gamma}$" can be made and the numeral would refer to the number of operative Hb_{γ} loci.

Greek HPFH heterozygotes. Hematological and chemical data are presented in Table I. The Greek heterozygotes show the normal red cell indices, the low values of Hb A_2, and the essentially uniform cellular distribution of Hb F that are characteristic of the HPFH. Data from the two new families fall within the ranges for the large series of Fessas and Stamatoyannopoulos (3).

The analyses of the γCB-3 peptide from seven heterozygotes in three families give essentially identical glycine values (0.05–0.13). The virtual absence of glycine (about 0.1 residue) is taken as evidence that these persons are synthesizing ${}^{A}\gamma$ chains only. Hence, they are heterozygotes for the $Hb_{A\gamma}$ type of HPFH.

Data from the Edman PTH degradation. By means of this procedure, amino acid residues may be removed sequentially from the N-terminus of a peptide and identified. The exact nature of the third residue of γCB-3 (which is equivalent to residue 136 of the γ-chain) can thus be examined to determine whether or not the small amount of glycine that appears in all analyses of γCB-3 from the Greek cases is extraneous or is indicative of a small percentage of ${}^{G}\gamma$-chains. For example, although the analysis of γCB-3 from an abnormal Hb F had 0.16

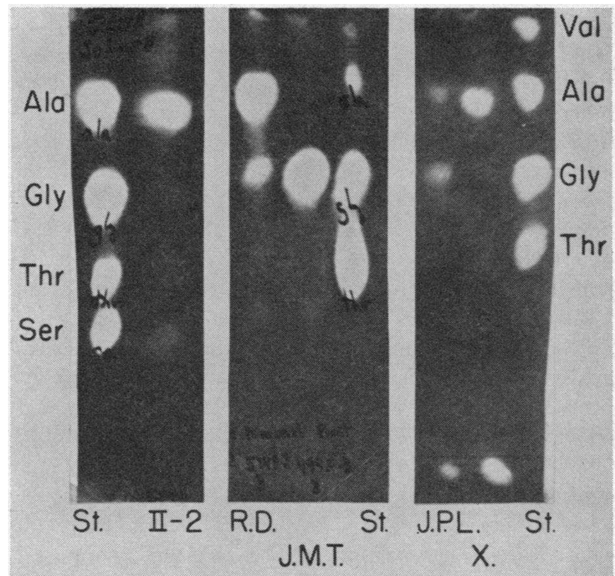

FIGURE 2 Paper chromatographic identification of the PTH amino acid after the third degradation of the γCB-3 peptide from selected cases. St. denotes standard. See text for discussion.

residues of glycine, glycine was absent in the third position by PTH degradation and, therefore, of extraneous origin (1).

Results of the application of the PTH procedure to γCB-3 of several HPFH heterozygotes is depicted in Fig. 2 which is a montage of paper chromatograms for the identification of residues as PTH derivatives at the third position. Because individual chromatograms were made at different times, each result must be compared with its own standard. J. M. T. is a Negro heterozygote of $Hb_{G\gamma}$ type (6); only glycine and not a trace of alanine was observed in the third position. J. P. L. is representative of the $Hb_{G\gamma}Hb_{A\gamma}$ type in which both glycine and alanine are observed in approximately equal amounts as anticipated on the basis of the amino acid analysis (6). (X. is unrelated to the present study.) R. D. (another Negro heterozygote for HPFH) also has both glycine and alanine, but alanine is preponderant by far. Approximate quantitation suggests a ratio of 1:10 glycine to alanine in agreement with previous data (6). A similar result (not depicted in Fig. 2) was obtained from siblings V. R. and J. H. of R. D. (6), as well as from C. W. of a previously unreported family. The glycine in the analyses of their γCB-3 is real. However, its source is not the small percentage of Hb F that is normally present in the adult. Calculation shows that Hb F normally present in the adult would contribute only 0.01 residue of glycine to the analysis of γCB-3 if the HPFH determinant were producing only ${}^{A}\gamma$-chains. Consequently, these individuals who had been

considered to be $Hb_{A\gamma}$ type must be placed in the $Hb_{G\gamma}Hb_{A\gamma}$ category. The results of an extensive reinvestigation of this family will be published elsewhere. On the other hand, when γCB-3 of II-2 of Greek family 3 and of I-2 of Greek family 2 was examined, only alanine and *not a trace* of glycine was observed; only the result from II-2, family 3 is presented in Fig. 2. Despite superficial similarity in the amino acid analyses, the Greek heterozygotes truly express the $Hb_{A\gamma}$ type of HPFH and the presumed Negro examples of this type do not.

Greek HPFH heterozygotes with concurrent β-thalassemia. The three individuals with this double heterozygosity synthesize both types of γ-chain (Table I). The proportion of $^G\gamma$- to $^A\gamma$-chains in II-1 of family 2 is about 1:2 and in I-3 and II-1 of family 3 about 1:3. The fraction of $^G\gamma$-chains increases in approximately direct proportion to the increase in Hb F in the double heterozygote as compared to their HPFH heterozygous relatives. The proportion of $^G\gamma$- to $^A\gamma$-chains in I-1, family 2, an individual with β-thalassemia trait, is 3:1 as is observed in Hb F of the newborn infant (1).

DISCUSSION

The Greek and Negro HPFH conditions have been considered to be different on the basis of two features: (*a*) the percentage of Hb F, and (*b*) the production of β-chains in *cis* to the HPFH determinant in the Greek but not in the Negro cases (3). It is necessary to reevaluate these conclusions on the basis of the present as well as recently published data (6).

The homogeneity of expression in Greek HPFH is in contrast to the heterogeneity of expression in the Negro in whom more than one group may be distinguished (6 and unpublished). Negro heterozygotes in 23 of 24 families make both types of chains whereas a single family may be classified in the $Hb_{G\gamma}$ group. The PTH results require that two Negro families that had been classified as $Hb_{A\gamma}$ type must be removed from that category. All Greek samples fall into the $Hb_{A\gamma}$ type.

On the basis of the percentage of Hb F, Negro heterozygotes for HPFH may also be classified into two groups which produce roughly 15–25% or 30–35% of Hb F (6). Greek heterozygotes (all of whom are $Hb_{A\gamma}$ type) fall into the class with the lesser Hb F as do also Negro HPFH heterozygotes of the $Hb_{G\gamma}$ type. However, this class also contains those Negro HPFH heterozygotes whose Hb F contains mainly $^A\gamma$-chains with a small amount of $^G\gamma$-chains. The percentage of Hb F, therefore, is not a distinguishing feature of the Greek and Negro expressions of HPFH.

Although Negro heterozygotes have previously (6) been placed in the $Hb_{G\gamma}$, the $Hb_{G\gamma}Hb_{A\gamma}$, and the $Hb_{A\gamma}$ categories of HPFH, the results of the PTH degradation make it clear that a Negro example of the $Hb_{A\gamma}$ type has not been observed. When reinvestigation of Negro families with the apparently different $Hb_{G\gamma}Hb_{A\gamma}$ types has been completed, the results will be published elsewhere; at this time, no other reclassification is made than to place the previously supposed $Hb_{A\gamma}$ type into the $Hb_{G\gamma}Hb_{A\gamma}$ category. Consequently, Negro heterozygotes fall into the $Hb_{G\gamma}$ and $Hb_{G\gamma}Hb_{A\gamma}$ categories and Greek heterozygotes with $Hb_{A\gamma}$ type are, therefore, complementary to the former. Although all heterozygotes in the three unrelated Greek families are of the $Hb_{A\gamma}$ type, homogeneity of expression in all Greek HPFH heterozygotes cannot be assumed. In view of the intermingling of nations around the Mediterranean area over the millenia, other types may well be detected among the Greeks and the $Hb_{A\gamma}$ type among the Negroes. The important finding of this study is that the $Hb_{A\gamma}$ type exists.

Although several genetic explanations for the HPFH have been advanced, the heterogeneity of the Negro type on a molecular level has been explained by modifying (6) the ideas of Nance (13) on the deletion of genes. It is generally considered that the synthesis of δ- and β-chains in *cis* to the HPFH determinant is completely absent. This conclusion is reached from two pieces of evidence: (*a*) double heterozygotes for HPFH and an abnormal β-chain hemoglobin (such as S or C) produce no Hb A (that is, no normal β^A-chains), and (*b*) homozygotes for HPFH produce neither Hb A nor Hb A_2 (14, 15). In order to explain the observed types of $Hb_{G\gamma}$, $Hb_{A\gamma}$, and $Hb_{G\gamma}Hb_{A\gamma}$, it was assumed (6) that the arrangement of hemoglobin loci on the normal chromosome is ——$^G\gamma\delta\beta^A\gamma$——. By appropriate deletions of δ-, β-, and γ-hemoglobin loci, chromosomes with remaining functional γ-genes of the types ——$^G\gamma$——, ——$^A\gamma$——, and ——$^G\gamma{}^A\gamma$—— would produce Hb F's of the observed kinds in HPFH (6). It should be pointed out that this explanation requires an arrangement of genes on the chromosome such that the γ-loci are separated by the δ- and β-loci.

Although all Negro and Greek HPFH heterozygotes that have been considered in this and a prior paper (6) have met the hematological criteria for HPFH, direct genetic evidence for absence of normal β^A-chain production in *cis* to the HPFH determinant is not always available. In 11 of 23 Negro families of $Hb_{G\gamma}Hb_{A\gamma}$ type that we have studied, Hb S or Hb C is also present and Hb A is completely absent in one or more double heterozygotes. Unpublished data show that heterozygous relatives of a HPFH homozygote (14) fall in the $Hb_{G\gamma}Hb_{A\gamma}$ class. Consequently, it may be concluded that HPFH heterozygotes of $Hb_{G\gamma}Hb_{A\gamma}$ type that have been studied by us do not produce normal β^A-chains in *cis* to the HPFH determinant. This type of evidence is not yet available for Negro or Greek HPFH heterozygotes who have been classified as $Hb_{G\gamma}$ or $Hb_{A\gamma}$ types: no homo-

zygotes have been observed nor has a double heterozygote for HPFH and a β-chain abnormal hemoglobin been detected. Failure to detect such critical combinations may simply reflect the small number of families that fall into these types. On the other hand, it may be indicative of a fundamental difference between the $Hb_{G\gamma}Hb_{A\gamma}$ and the $Hb_{G\gamma}$ and $Hb_{A\gamma}$ types.

Do the data from Greek double heterozygotes for HPFH and β-thalassemia provide any evidence about this possibly fundamental difference? It is well known that some homozygotes for β-thalassemia synthesize Hb A (that is, β^{A}-chains) and others do not; the same effect is apparent in double heterozygotes for Hb S and β-thalassemia (summarized by Weatherall [16]). In all cases the synthesis of β^{A}-chains in *cis* to the β-thalassemia gene is significantly reduced as compared to that directed by a normal β^{A}-gene. All Greek HPFH β-thalassemia double heterozygotes, however, synthesize large amounts of β^{A}-chains (3). This fact presented Fessas and Stamatoyannopoulos (3) with a dilemma if there is no β^{A}-synthesis in *cis* to the HPFH determinant. Because β-thalassemia in the area in which several Greek double heterozygotes live is of the type that does not synthesize β^{A}-chains, Fessas and Stamatoyannopoulos (3) assumed that this type is present in the double heterozygotes. If this conclusion is true, β^{A}-chain synthesis in the HPFH β-thalassemia heterozygote must be in *cis* to the HPFH determinant and an active β-gene must be present on the HPFH chromosome. If the deletion hypothesis for the HPFH condition is correct, the deletion chromosome in the Greek HPFH individuals of the $Hb_{A\gamma}$ type must be ——$\beta\,{}^{A}\gamma$—— or ——$\delta\beta\,{}^{A}\gamma$—— and would be attributed to an unequal crossing over which resulted in the deletion of the ${}^{G}\gamma$- or ${}^{G}\gamma$- and δ-genes. By inference, the $Hb_{G\gamma}$ type may have ——${}^{G}\gamma$—— or ——${}^{G}\gamma\beta$—— or ——${}^{G}\gamma\delta\beta$—— as the remaining chromosome.

There are further implications in the possibilities that δ- or β-genes or both are active in *cis* in the $Hb_{G\gamma}$ and $Hb_{A\gamma}$ types. As was pointed out above, the arrangement ——${}^{G}\gamma\delta\beta\,{}^{A}\gamma$—— is necessary, if simple deletions are to produce ——${}^{G}\gamma$——, ——${}^{A}\gamma$——, and ——${}^{G}\gamma\,{}^{A}\gamma$——. However, if ——$\delta\beta\,{}^{A}\gamma$——, ——${}^{G}\gamma$——, and ——${}^{A}\gamma\,{}^{G}\gamma$——, respectively, describe the chromosome for the $Hb_{A\gamma}$, the $Hb_{G\gamma}$, and the $Hb_{G\gamma}Hb_{A\gamma}$ types, then simple deletions from more customarily arranged loci such as ——$\delta\beta\,{}^{A}\gamma\,{}^{G}\gamma$—— will yield the appropriate remaining genes for the HPFH types.

If these conclusions are valid, they provide an explanation for the difference between the Greek and Negro HPFH β-thalassemia cases (3, 14). The level of Hb F at about 70% in the Negro cases is much higher than in the Greeks. In the Negro individuals of the $Hb_{G\gamma}Hb_{A\gamma}$ type, there can be no question about the total inactivity of the δ- and β-loci on the HPFH chromosome. Consequently, the β-thalassemia must be of the type that produces β-chains, but the production must have lesser efficiency than that of the β-locus *cis* to the HPFH determinant in the Greek cases.

In considering the data from the double heterozygotes, the results from I-1, family 2, a β-thalassemia trait, are of importance. His Hb F has ${}^{G}\gamma$- to ${}^{A}\gamma$-chains in the ratio of 3:1 as in the newborn infant (1). The thalassemia gene must be responsible for this ratio because children over 6 months of age and adults have the chains in a ratio of about 2:3 (17). Hb F with γ-chains in the 3:1 ratio of the newborn is of frequent occurrence in individuals with thalassemia trait.[2] In the double heterozygotes II-1, family 2 as well as I-3 and II-1 of family 3, Hb F is increased over simple HPFH heterozygotes, and glycine is evident in the analysis of γCB-3 (Table I). The Hb F in II-1, family 2 is more than double that in his HPFH heterozygous relatives. Hb F in I-3 and II-1 of family 3 is less than double as compared to II-7, a simple HPFH heterozygote. The greater the increase in Hb F in the double heterozygote, the greater also the increase in glycine in γCB-3. We conclude that the γ-loci on the HPFH and β-thalassemia chromosomes are acting independently in *cis* and that the increase of Hb F in double heterozygotes as compared to HPFH heterozygotes derives from the reactivation of γ-loci on the β-thalassemia determinant. Substantiation of this conclusion comes from the following calculation. In II-1, family 2, we may calculate that 43% of the Hb F is synthesized by the HPFH chromosome and 57% by the thalassemia chromosome. Likewise, the glycine in γCB-3 of Hb F from the HPFH and thalassemia chromosomes should be 0.00 and 0.74, respectively. Hence, glycine in Hb F from II-1, family 2 should be $0.43 \times 0.00 + 0.57 \times 0.74$ or 0.42. This is in reasonable agreement with the observed 0.33. The calculated value for I-3 and II-1 of family 3 is 0.27 as compared to the observed 0.24 and 0.22.

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