Further Studies of the Postnatal Change in Chemical Heterogeneity of Human Fetal Hemoglobin in Several Abnormal Conditions

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Extract

The fetal hemoglobin of 10 infants has been examined serially from birth to several hundred days of age. In the normal child, the ratio of $^G\gamma$ to $^A\gamma$ chain alters from about 7:3 at birth to about 2:3 at 150–200 days. In children with abnormalities of hemoglobin synthesis, the ratio changes as expected from data from adults with the particular abnormality. Thus, little change in ratio was shown by two β-thalassemia homozygotes, whereas two β-thalassemia heterozygotes showed the same behavior as a normal child; the latter behavior is expected from one of the two classes of β-thalassemia heterozygotes. A heterozygote from the $^G\gamma^A\gamma$ class of hereditary persistence of fetal hemoglobin behaved much like a normal child, but two heterozygotes from the $^G\gamma$ class showed the expected change from approximately 9:1 at birth to 10:0 postnatally. Two children with sickle cell anemia resembled a normal child in change of ratio whereas the third, not unexpectedly, did not show a change in ratio.

Speculation

The mechanisms involved in the gradual change from γ chain synthesis to β and δ chain synthesis are complex and ill defined. The results of analyses of the ratio between the products of the $^G\gamma$ and $^A\gamma$ structural genes in infants with distinct genetic disorders at birth and during the postnatal period underscore the complexity of the changeover. However, differences in the postnatal change in the $^G\gamma$ to $^A\gamma$ ratio in infants with an apparent identical condition may help to further define these mechanisms, and to delineate the conditions involved.

The postnatal changeover from the production of hemoglobin F (Hb-F) to hemoglobin A (Hb-A) takes a far more complex path than is superficially apparent. The nonallelic structural genes for the γ chain of Hb-F (14), of which there may be four (5), appear to be nonsynchronously suppressed (11), with the result that the trace of Hb-F in the adult has a different heterogeneity than the Hb-F of the newborn child. Thus, in the newborn child, the ratio of $^G\gamma$ to $^A\gamma$ chains (19), on the average, is 7:3 (16), whereas it is about 2:3 in the traces of Hb-F in the adult (12). Examination of Hb-F from cord blood and adult blood provides the extremes in these ratios,

but serial postnatal examination in single individuals gives information about the behavior of regulatory mechanisms (11). Not only infants with abnormal Hb-F but also neonates with other abnormal conditions of various types have been studied in this way (11, 12). We have now been able to examine infants with thalassemia major and trait, with the $^G\gamma$ type of the hereditary persistence of fetal hemoglobin (HPFH), with the $^G\gamma^A\gamma$ type of HPFH, and with sickle cell anemia. Patients with thalassemia major or with sickle cell anemia usually are detected only when medical attention is required. Consequently, little is known about the status of such an individual at birth or about the onset of the disease. Present studies provide information about the status of such neonates and their progress, and also test speculations about how the $^G\gamma$ to $^A\gamma$ ratio should change after birth.

MATERIALS AND METHODS

SOURCE OF SAMPLES

EDTA was used as anticoagulant in samples for hematologic examination, and larger samples for isolation of the hemoglobins were placed in EDTA or Alsever's solution.

HEMATOLOGIC DETERMINATIONS

Hemoglobin concentration, packed cell volume, and erythrocyte and reticulocyte counts were determined by standard methods (18).

ANALYTICAL METHODS FOR HEMOGLOBIN COMPOSITION

Hemolysates were examined both by starch gel electrophoresis in Tris-EDTA-boric acid buffer at pH 9.0 (2) and by DEAE-Sephadex chromatography for analytic and isolative purposes (1, 4). Hemoglobin F was determined by a procedure which uses the isoleucine content of chromatographically isolated Hb-F in its relation to leucine and phenylalanine as the basis for calculation (15). These values are presented as percentage of F_{Ile}.

CHEMICAL ANALYSIS OF Hb-F

The procedure described previously (14) was used to determine the ratio of the two types of γ chains in Hb-F. The

Table 1. *Hematologic and hemoglobin data*[1]

Subject and condition	Age, days	Hb, g/100 ml	RBC, 10^6/mm^3	PCV, %	Retic, %	Hb-A$_2$, %	Hb-F$_{Ile}$, %	γCB-3 Gly	Ala
MB (hom. β-thal.)	Birth					0.2	99.8[2]	0.73	2.25
	46	9.2	4.69	27.5	4.2	0.0	100.0[2]	0.75	2.32
	77	8.4		25.5	7.4	0.5	99.5[2]	0.68	2.30
	99	7.5		24	22.4	1.1	98.9[2]	0.70	2.28
	112	6.0		20	8.2	1.4	98.6[2]	0.68	2.36
	182[3]	7.6		25		2.6	17	0.67	2.37
	206[3]	9.6		30		3.1	13	0.68	2.33
CK (hom. β-thal.)	109	6.1	2.90	20	4.3	1.8	98.2[2]	0.64	2.37
	128	7.0		23	5.6			0.63	2.45
	191[3]	5.9	2.12	15	2.8		32	0.69	2.34
	228[3]					2.6	8.6	0.66	2.40
TN (A-β thal.)	Birth							0.74	2.29
	50	9.8		27	3.0	0.4	84	0.69	2.30
	135	12.0	5.43	37.5	1.3	3.1	29	0.49	2.56
	230	10.8		35.5	1.4	5.8	10	0.40	2.62
RS (A-β thal.)	56	10.6	4.24	31		2.9	29.9	0.56	2.54
	117	11.4	3.67	37		4.5	4.8	0.45	2.62
	146	11.4	3.98	36		3.9	2.5	0.42	2.70
	439	9.7	5.30	32	1.8	4.4	1.6	0.30	2.72
JTA (A-^{G}γ HPFH)	Birth	16.2	4.63	51	3.2			0.88	2.13
	80	11.3	4.15	32	1.4	1.0	53	0.91	2.10
	147	12.2	4.77	35		1.4	30	0.95	2.09
	164	11.6	4.37	35	2.2	1.4	29		
	302	11.6	4.57	37	0.6	2.2	22	0.98	2.08
NOC (A-^{G}γ HPFH)	Birth	15.0	4.31	45	4.2		76	0.86	2.14
	56	11.3	4.04	34	1.6	1.0	52	0.90	2.13
	112	11.6	4.20	35		2.0	28	0.95	2.08
	133	11.8	4.70	36	0.6	1.5	30	0.97	2.05
	245	12.0	4.75	35	0.4	2.1	19	1.02	2.05
RBe (A-^{G}γ^{A}γ HPFH)	1					0.5	87	0.67	2.29
	63	11.8	4.00	34	1.2	0.5	66	0.66	2.40
	91	13.2	4.57	39	1.2	1.5	44	0.62	2.38
	127	11.2	3.92	36	1.2	1.2	39	0.58	2.38
	157					1.3	35	0.58	2.49
	190	12.8	5.25	38	0.5	1.6	37	0.55	2.45
	203	11.8	4.23	37	0.7	1.3	32	0.58	2.43
	245	10.7	4.81	31	1.2	1.6	33	0.58	2.50
	496					1.6	29	0.53	2.46
LH (hom. SS)	Birth	21.3		65	6.8	0	88	0.74	2.33
	20	15.0		42	0.2	0	81	0.69	2.30
	71	7.6		22	6.5	1.3	56	0.63	2.42
	203	7.6		24	8.7	2.7	7.5	0.47	2.55
	314					2.6	5.6	0.44	2.55
EM (hom. SS)	Birth	22.4		64	4.5	0	82	0.73	2.23
	24	14.6		43.5	0.7	0.3	75	0.76	2.25
	54	8.7	2.46	25	1.5	0.2	71	0.72	2.25
	87	7.6	2.36	22	6.8	1.4	47	0.75	2.28
	159	8.2	3.01	26	20.4	3.0	23		
	330	8.3	3.05	25	4.9	2.7	13	0.75	2.34
WW (hom. SS)	3	17.3		51	7.3	0	91	0.72	2.31
	226	7.3	2.76	23	24.8	1.9	15	0.51	2.54
	268	9.3	3.39	29	17.6				

[1] Hb: hemoglobin; RBC: red blood cell; PCV: packed cell volume; hom.: homozygote; thal.: thalassemia; HPFH: hereditary persistence of fetal hemoglobin.

[2] Assayed by chromatography; no Hb-A was detectable before transfusion.

[3] Samples were collected after transfusion.

data are presented as the number of glycyl and alanyl residues in the carboxy-terminal γCB-3 peptide and are precise to $\pm$ 0.05 residues (8). The meaning of the data is given in Reference 19.

CASE HISTORIES

Hematologic data are presented in Table 1.

THALASSEMIA MAJOR AND TRAIT

Family B. MB was born August 2, 1971 and weighed 3,120 g. At 42 days of age, she weighed 4,426 g and was 56.5 cm long; liver and spleen were not enlarged. At 77 days of age, the liver and spleen were detectable at the costal margin. Because the hemoglobin level had decreased to 6 g/100 ml, transfusion of *MB* was started on the 112th day. Since transfusion was begun, her hemoglobin has been maintained above 10.5 g/100 ml, and at 2 years of age she receives transfusions every 3 or 4 weeks. The spleen and liver are minimally enlarged. Iron-chelating agents have not been given. Growth and development have been normal.

JB, a 14-year-old sister, also has β-thalassemia major. Her parents and two other siblings have β-thalassemia trait. The family is of Italian descent.

Family K. CK was born October 30, 1971 and was first seen at the age of 109 days. At that time, the boy was well developed and well nourished with a liver and spleen that were both about 2 cm below the costal margin. Transfusions were begun at the age of 132 days. He also has been maintained above 10.5 g/100 ml hemoglobin and at 2 years of age, he requires transfusions every 4–5 weeks. Like *MB*, spleen and liver are minimally enlarged, growth and development are normal, and iron chelating agents have not been given.

His parents are of Greek descent and have the typical stigmata of β-thalassemia trait.

Family N. TN was born December 9, 1970. Her mother was known to have β-thalassemia trait, and the child has also developed these characteristics. She has had no significant illnesses that can be attributed to heterozygosity for β-thalassemia. This child is of Italian descent.

Family S. Data from this Negro girl (*RS*) have been described (12) and are presented again for comparison with those of *TN*.

HEREDITARY PERSISTENCE OF FETAL HEMOGLOBIN

Family A. JTA was born October 28, 1972 in Bombay. Her mother has the same type of HPFH that *JTA* has developed, but both are clinically normal. Although the father has not been examined, he probably has β-thalassemia. This conclusion arises from the fact that *LTA*, a 3-year-old sibling of *JTA*, is anemic, has mild hepatosplenomegaly, and requires occasional transfusions. *LTA* is believed to have both HPFH and β-thalassemia (17).

Family C. NOC was born December 24, 1972 in Bombay. His mother and a 2-year-old sister both have the type of HPFH that he has developed and all of these individuals are clinically normal. His father has β-thalassemia (Hb-A$_2$, 4.8%) and *NOC*'s 3-year-old sibling has HPFH-β-thalassemia with expression like that of *LTA* of Family A.

Family Be. RBe was born January 19, 1970. Her mother, uncle, two aunts, and grandmother have the same kind of $^G\gamma^A\gamma$-type HPFH. This child has been studied repeatedly over the years and some of the data have been presented previously (10), whereas information on her relatives (described as *Family E*) has been given in an earlier publication (8). Her clinical course has been uneventful.

SICKLE CELL ANEMIA (20)

Family H. LH was born November 16, 1971 and weighed 2,850 g. Her condition was diagnosed at birth. Severe anemia

developed at the age of 2 months. She was admitted repeatedly for infection, and died 4 hr after her last admission on June 7, 1973, of pneumococcal meningitis. Both parents were identified as Hb-S heterozygotes.

Family M. EM was born August 13, 1973 and weighed 3,110 g. The disease in this patient was diagnosed at birth. Growth and development were relatively normal, but anemia was noted at 3 months of age. At 6 months of age, the spleen was enlarged. The patient is in reasonable health, and attends the out-patient clinic regularly. Both parents are Hb-S heterozygotes.

Family W. WW was born March 4, 1972, weighed 3,140 g, and showed a relatively normal growth and development. However, severe anemia was noted at 6 months of age. The patient had a poor attendance at the clinic and died at home in March 1973 of "infection." The mother was identified as a Hb-S heterozygote.

RESULTS

Although none of the infants who were observed at birth had any physical abnormalities at that time, examination of the hemoglobin from the cord blood of homozygotes for β-thalassemia and sickle cell anemia showed clear abnormality. Thus, MB with a homozygous β-thalassemia, and *LH, EM*, and *WW* with sickle cell anemia had no Hb-A; indeed, *MB* had only Hb-F and a trace of Hb-A$_2$ at birth and produced no other hemoglobins whereas the others all had Hb-S with Hb-A$_2$ and Hb-F. On the other hand, the abnormalities in the other neonates, all of whom are heterozygotes, became apparent only in later samples but were suspected because of the known abnormality in one of the parents.

Figure 1 depicts the chromatographic results of samples from *MB* with homozygous β-thalassemia at birth and at 112 days, before transfusion. CK's hemoglobin composition at 109 days was virtually indistinguishable from that of *MB* at 112 days. Although *MB* showed little change in hemoglobin composition, anemia became progressively worse.

The other children, despite the variety of their conditions, showed the expected decline of Hb-F and increase of an adult
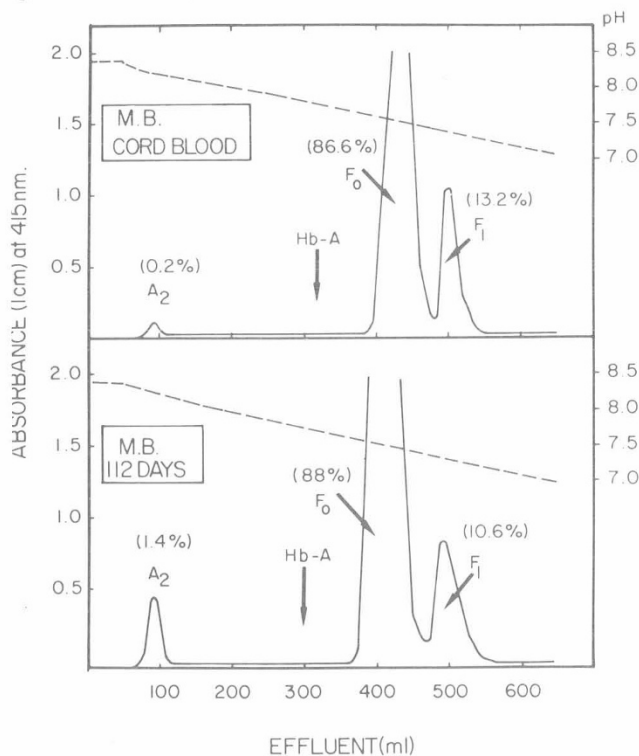

Fig. 1. DEAE-Sephadex chromatograms of the hemoglobins (*Hb*) of *MB* who has thalassemia major.

hemoglobin as well as the onset of anemia if SS disease were present (Table 1). In *JTA* and *NOC,* both with a HPFH heterozygosity, the final level of Hb-F may not yet have been reached; it is significantly lower in *NOC*'s mother and 2-year-old sister, namely 11% and 17%, respectively, but heterozygotes for this type of HPFH show considerable variation in level even within families (17). The Hb-F in *RBe,* the third infant with HPFH trait, at the age of about 500 days has reached the level frequently observed in her mother and other relatives, namely 25–30%. Only in *RS*, heterozygous for β-thalassemia, did the Hb-F decline to almost the adult level within a period of about a year; in the others, Hb-F is in higher percentage than in normal children at a comparable age (12).

The chemical examination of Hb-F through the determination of the value for glycine in γCB-3 gave data concerning the ratio of the two types of γ chain in these patients; the interpretation of these data is given in Reference 19. An average glycine value of 0.7 was observed in 108 normal newborn infants (16). A comparable value was seen at birth in the newborn infants of this study with the exception of *JTA* and NOC, both with a HPFH heterozygosity, whose values of 0.88 and 0.86 were at the upper limit of the normal range and of *RBe* whose value of 0.67 was at the lower limit of the normal range. The data for glycine in γCB-3 of various postnatal samples ire recorded in Table 1 and are plotted in Figures 2, 3, and 4 as a function of postnatal age together with the levels of Hb-F observed during the same periods. The initially high value in the cases with $^G\gamma$ type of HPFH (*JTA* and *NOC*) rises to 1.0 postnatally and that in the neonate with $^G\gamma^A\gamma$ type of HPFH decreases only slightly. The value in the two β-thalassemia heterozygotes, *TN* and *RS,* as well as the two SS patients, *LH* and *WW*, declines to that in the traces of Hb-F in the adult. On the other hand, *EM,* another SS patient, has a constant value for glycine in γCB-3 and the two individuals with thalassemia major show a slight decrease.

DISCUSSION

The results of this investigation are in basic accord with those that were anticipated from previous information about Hb-F in these conditions. This discussion will consider each category of disease separately.

β-THALASSEMIA (FIG. 2)

The effect of a β-thalassemia mutation is a reduction in the production of β chains and consequently of hemoglobin A. The consequences in the heterozygote are minor but, in the homozygote, a severe anemia usually develops with attendant bone changes, organomegaly, failure to thrive, and often death in early childhood. Different variants of β-thalassemia exhibit a varying imbalance in chain synthesis (see Reference 3 for references). There is no doubt that *MB* and *CK* suffer from the most severe type, the so-called β°-thalassemia, without detectable production of β chains and inadequate compensatory production of γ chains. Within 3–4 months after birth, they developed a severe anemia which required transfusion for survival. The chromatographic analyses for hemoglobin composition were important for evaluating the genetic condition in these infants who were potential homozygotes because their parents were heterozygotes. These analyses, although decisive in these neonates, cannot be used universally for the diagnosis of β-thalassemia major at birth because homozygous neonates with only a partial suppression of β chain production will have Hb-A and may not, therefore, be strikingly abnormal.

There is a slight but hardly significant decrease in glycine of γCB-3 in postnatal samples from *MB*. Although the γCB-3 of *CK* was first examined at the age of 109 days, the data at that age and later are almost identical with those of *MB*. The values are not influenced by transfusions. Their glycine values of approximately 0.65 as well as that of 0.60 for *MB*'s sibling
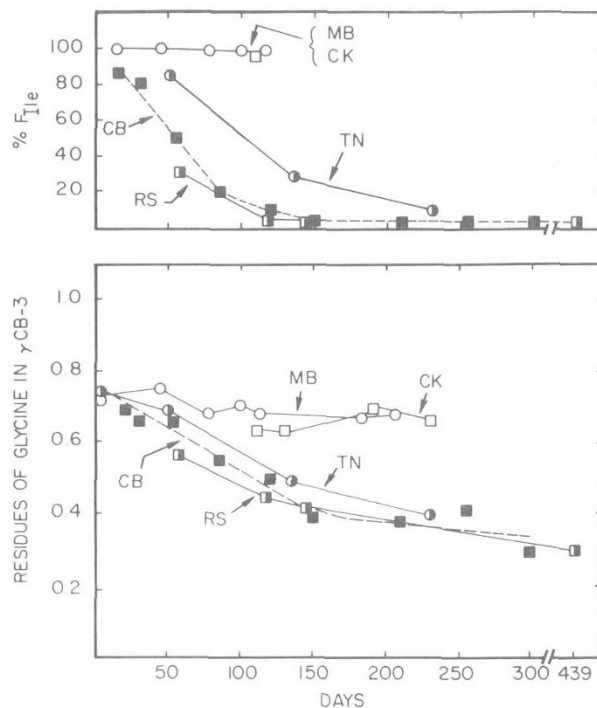

Fig. 2. Values for glycine in γCB-3 and for total hemoglobin F as a function of postnatal age in β-thalassemia. *MB* and *CK* are infants with homozygous β-thalassemia. *RS* and *TN* are infants with heterozygous β-thalassemia. The data on CB with a hemoglobin C trait are from Reference 12.

who also has thalassemia major are comparable with data from many adults with thalassemia major (9). *MB*'s parents have glycine values that approximate that in Hb-F of the normal adult. This is typical of one of the two classes that are observed with β-thalassemia heterozygotes (9, 13); in the other class, the glycine value approximates that in Hb-F of the newborn infant.

On the other hand, the heterozygotes, *TN* and *RS*, have a progressive postnatal decline in the value of glycine in γCB-3. The final value is much like that of the parents of *MB* and places them in the same class. If either of these heterozygotes had been in the other class, their glycine value would have been about 0.70–0.75 at birth and would have remained essentially constant in postnatal life.

HEREDITARY PERSISTENCE OF FETAL HEMOGLOBIN (FIG. 3)

The HPFH condition may be subdivided into three major types: the $^G\gamma$ type, the $^A\gamma$ type, and the $^G\gamma^A\gamma$ type in which, respectively, the Hb-F has only $^G\gamma$ chains, only $^A\gamma$ chains, or both (see Reference 3 for summary). Infants *JTA* and *NOC* belong obviously to the $^G\gamma$ type: their Hb-F, which at birth contained both $^G\gamma$ and $^A\gamma$ chains, has only $^G\gamma$ chains in the Hb-F that persists in adult life. Infant *RBe* belongs to the $^G\gamma^A\gamma$ type.

In the normal child, the activity of both $^G\gamma$ and $^A\gamma$ genes is suppressed postnatally, and their ratio of production is altered. In *JTA* and *NOC*, we observe the suppression of the $^G\gamma$ and $^A\gamma$ chain loci on the normal chromosome, whereas the suppression of γ chain loci on the abnormal chromosome which has the HPFH determinant is less complete and does not involve one of the $^G\gamma$ loci. As a result, the $^A\gamma$ chain disappears from the Hb-F and the persistent Hb-F has only $^G\gamma$ chains. The various classes of HPFH have been considered to result from deletions of δ, β, and one or more γ loci (see Reference 9 for a detailed discussion). In these two children, the deletion would involve the δ, β, and $^A\gamma$ loci. Because of the production
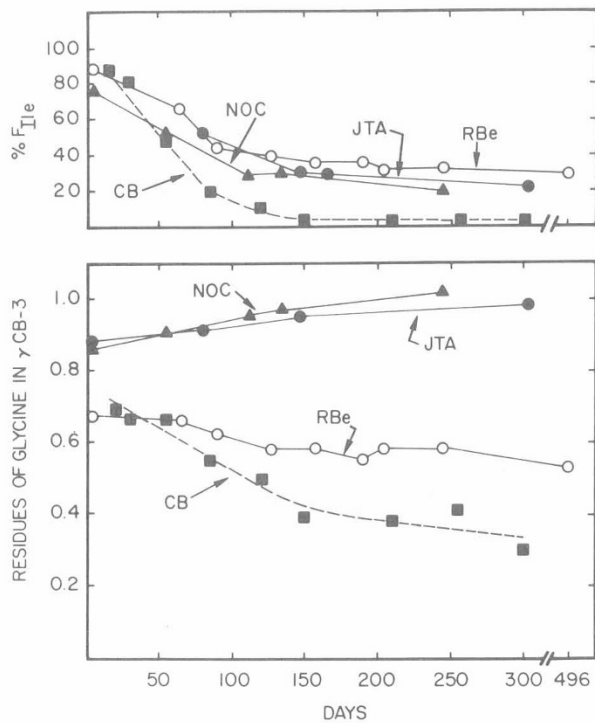
Fig. 3. Values for glycine in γCB-3 and for total Hb-F as a function
f postnatal age in hereditary persistence of fetal hemoglobin (HPFH).
TA and NOC have a $^G\gamma$ type of HPFH trait and RB has a $^G\gamma^A\gamma$ type
f HPFH trait. The data on CB with a hemoglobin C trait are from
eference 12.

f γ chains from the normal chromosome is suppressed
almost) completely and that from the abnormal is at most
artly suppressed, the deletion must also involve one or more
egulatory factors. It appears that this factor which is still
ocated on the normal chromosome can suppress γ chain
roduction in *cis* but cannot control the $^G\gamma$ chain production
n *trans* on the abnormal chromosome. Regulatory factors in
he complex of δ, β, and γ loci are considered in detail in
eference 9.

Similar considerations are valid for the infant RBe with the
$\gamma^A\gamma$ type of HPFH. The production of $^A\gamma$ and $^G\gamma$ chains
om the normal chromosome is suppressed (almost) com-
letely but $^A\gamma$ and $^G\gamma$ chain production from the abnormal
hromosome continues because the deletion involves δ and β
hain structural loci and presumably no γ chain structural loci.
gain it can be assumed that the deletion includes one or more
egulatory factors. Unexplained is the relatively high glycyl
vel (0.53−0.60) which is observed in this infant and also in
er relatives (8). This level is significantly higher than the most
ommon value of about 0.3−0.45 in the $^G\gamma^A\gamma$ type of HPFH.

Further consideration of regulatory factors may be made on
he basis of data from JTA's sibling LTA. LTA presumably has
he $^G\gamma$ type of HPFH and β-thalassemia and produces Hb-F
ith glycine of 0.81 and alanine of 2.18 in γCB-3 (17). When a
ombination of β-thalassemia with the $^A\gamma$ type of HPFH (10)
r the $^G\gamma^A\gamma$ type of HPFH (7) is present, the quantity of
lb-F is much increased over what would be expected if the
ffects of the two genes were simply additive. It has been
oncluded (7, 10) that the increased Hb-F is produced in *cis* to
he β-thalassemia determinant. The same conclusion may be
eached from the data on LTA. The glycine value of 0.81 in
is γCB-3 means that both $^G\gamma$ and $^A\gamma$ chains are present.
ecause the HPFH determinant produces only $^G\gamma$ chains, the
γ chains must derive from the presumed β-thalassemia
eterminant. The sample of hemoglobin from LTA that was
xamined contained only a few percent of Hb-A which may
ave been the residue of a prior transfusion. This suggests that

β^o-thalassemia is present. It may also be calculated in the
manner described previously (7, 10) that the Hb-F from this
thalassemia is the kind that would have a glycine value of
about 0.75 in γCB-3 (9, 13). Apparently, then, the HPFH
determinant (presumably because of deletion of some regula-
tory factor) is able to stimulate production of Hb-F when
β-thalassemia is in *trans* but not when a normal chromosome is
in *trans*.

SICKLE CELL ANEMIA (FIG. 4)

The percentage of Hb-F in individuals with sickle cell
anemia may vary considerably and it is generally considered
that higher amounts ameliorate the effects of the disease. In
the study of Hb-F in sickle cell anemia (6), there is a tendency
for the glycine in γCB-3 to approximate the value of 0.4 for
normal adults. However, a significant number of cases have
values of about 0.7 as does the newborn child. Nevertheless,
these groupings are not as distinct as in thalassemia (see
above). These results are puzzling and would not be the
anticipated result of the mutation of a structural gene as the
cause of sickle cell anemia is considered to be. However, this
study of the three children with sickle cell anemia shows this
very effect. The glycine value in γCB-3 of EM changes not at
all from the newborn value, whereas that of LH and WW has
the expected decline. Although the percentage of Hb-F has
remained higher in EM than in the others, there is no
correlation between quantity of Hb-F and glycine value (6).

CONCLUDING DISCUSSION

These studies bear out the anticipated postnatal behaviors in
the glycine value of γCB-3 in infants with an abnormality in
hemoglobin synthesis. Thus, in β-thalassemia homozygotes,
glycine in γCB-3 has changed only slightly; it would not be
expected to decrease below 0.6 and may approach this as the
children mature. Both β-thalassemia heterozygotes have shown
a decline to the adult level of glycine in γCB-3 and, therefore,
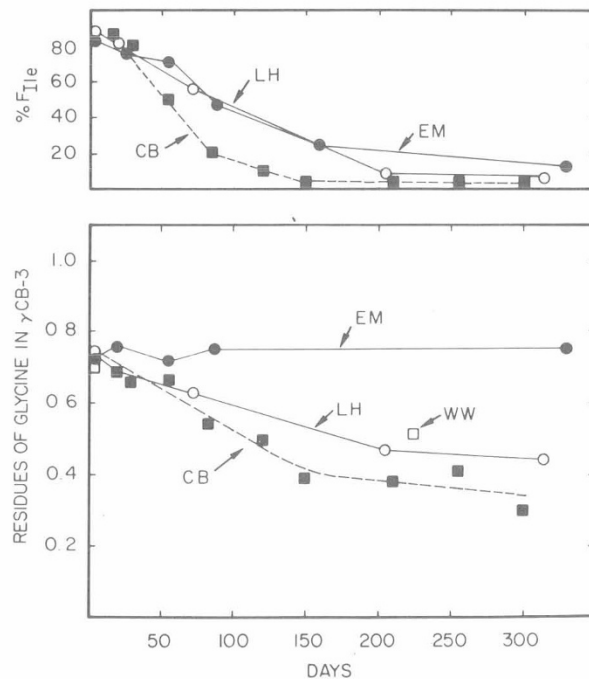are shown to be in that subclass of β-thalassemia heterozygotes
(9, 13).

Fig. 4. Values for glycine in γCB-3 and for total hemoglobin F as a
function of postnatal age in sickle cell anemia. LH, EM, and WW have
sickle cell anemia. The data on CB with a hemoglobin C trait are from
Reference 12.

In HPFH heterozygotes, likewise, the behavior follows the anticipated pattern; glycine in γCB-3, already high at birth in the $^G\gamma$ type of HPFH, increases to 1.0 whereas in the $^G\gamma^A\gamma$ type, it decreases to the adult level.

In sickle cell anemia, most adult cases have the adult level and two children exemplify this by levels which decrease to this value. However, the glycine value in γCB-3 of one child has not decreased postnatally, and some adults have also had values similar to those in the neonate.

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19. $^G\gamma$ refers to the γ chain with glycine in residue 136 and $^A\gamma$ refers to the γ chain with alanine in this position. Chemical examination of Hb-F and the determination of the residues of glycine and alanine in peptide γCB-3 which comprises the carboxy-terminal 13 residues of the γ chain measure the ratio of the $^G\gamma$ and $^A\gamma$ chains. The value for glycine in γCB-3 is the easiest indicator of the ratio of the two types. Thus, if glycine is one, only $^G\gamma$ chains are being produced, and if glycine is 0, only $^A\gamma$ chains are produced. A nonintegral value of, for instance, 0.75 residue shows that both types are present and in a ratio of 0.75:0.25 or of 3:1. The $^G\gamma$ to $^A\gamma$ chain at birth averages 3:1 (6); however, with a further increase in the number of analyses the more accurate average ratio appears to be 7:3.
20. A detailed clinical description of these three patients is in preparation and will be presented elsewhere.
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22. Informed consent was obtained for all subjects in this study.
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