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Haemoglobin Inkster (α_2^{85}aspartic acid→valineβ_2) Coexisting with β-Thalassaemia in a Caucasian Family

ROBERT E. REED, WILLIAM P. WINTER* AND DONALD L. RUCKNAGEL*

Departments of Internal Medicine, Veterans Administration Hospital and University of Michigan Medical School (Simpson Memorial Institute), Ann Arbor, and
** Department of Human Genetics, University of Michigan Medical School, Ann Arbor*

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SUMMARY. Haemoglobin Inkster, a new α-chain variant, was discovered in a family which also had the gene for β-thalassaemia. The amino acid abnormality was in αTp-9 which contains 29 amino-acid residues. Structural studies were facilitated by cleavage of the abnormal α-chains with cyanogen bromide followed by tryptic digestion. The substitution was shown to be valine for aspartic acid at position 85 in the α-chain. Affected individuals had no haematological abnormalities. Individuals with both β-thalassaemia and Hb Inkster had slightly lower percentages of Hb Inkster than those found in persons heterozygous for the Hb Inkster gene alone. 'Interaction' between thalassaemia and variant haemoglobin genes involving different haemoglobin loci has been reported in another family with β-thalassaemia and an α-chain haemoglobin mutant, as well as in the converse situation of coexisting α-thalassemia and a β-chain haemoglobin mutant. This decrease in the mutant haemoglobin percentage differs from the more common 'interaction' of thalassaemia and mutant haemoglobin genes involving the same haemoglobin locus, in which the mutant haemoglobin percentage is increased. The mechanism for the 'interaction' is unknown, but the presence of an unusually low percentage of a haemoglobin variant should warrant investigation for coexisting thalassaemia involving a different haemoglobin locus.

Considerable insight into the biochemical nature of thalassaemia has come from study of the amount of different haemoglobins present in individuals carrying genes for both thalassaemia and a haemoglobin variant. When β-thalassaemia coexists with a β-chain haemoglobin variant, the proportion of Hb A is decreased to 0–30% and the proportion of the haemoglobin variant is correspondingly increased in contrast to simple heterozygotes for a β-chain structural gene in which Hb A is usually greater than 50%. This decrease in the proportion of the Hb A in individuals with coexisting genes for thalassaemia and a variant haemoglobin affecting the same globin chain, called 'interaction' (Cohen *et al*, 1959), was one of the first indications that β-chain synthesis is diminished in β-thalassaemia. Similar interaction occurs in heterozygotes for both α-thalassaemia and Hb_α-structural mutants (Atwater *et al*, 1960).

Conversely, when coexisting thalassaemia and structural mutant genes involve different

Correspondence: Dr Robert E. Reed, 2215 Fuller Road, Ann Arbor, Michigan 48105, U.S.A.

haemoglobin loci, the effect is different. For instance, the bloods of heterozygotes for the haemoglobins S, C, D or E and α-thalassaemia contain only 25% of the abnormal haemoglobins in contrast to the 30–40% in simple heterozygotes (Cohen *et al*, 1959; Tuchinda *et al*, 1964; Wasi *et al*, 1967; Weatherall, 1967). This phenotype has been designated as 'non-interacting' (Cohen *et al*, 1959) although this is obviously a misnomer.

The combination of genes for a Hb_{α}-structural variant and β-thalassaemia is rare. However, this combination has been described for Hb L Ferrara (Silvestroni *et al*, 1960), a Hb D_{α} (Lee & Huisman, 1965), Hb Nicosia (Fessas *et al*, 1965), a Hb J_{α} (Atwater *et al*, 1970) and Hb-Q India (Sukumaran *et al*, 1972). We here report the combination of β-thalassaemia with a previously undescribed α-chain variant, $\alpha^{85\ \text{valine}}$, which we designate Hb Inkster from the Michigan community in which the propositus resides.

MATERIALS AND METHODS

Blood samples anticoagulated with EDTA were examined by standard haematological methods. Toluene-extracted haemolysates were examined by vertical starch gel electrophoresis (Smithies, 1965). The gels were sliced and stained with amido black 10B and orthodianisidine (Owen *et al*, 1958). The proportions of various haemoglobin components were estimated by electrophoresis and elution from starch blocks (Kunkel & Wallenius, 1955) and cellulose acetate membranes (Glynn *et al*, 1968). The fractions were eluted from the unstained strips and the absorbance was measured at 410 nm. The proportion of foetal haemoglobin was determined by alkali denaturation (Singer *et al*, 1951). The abnormal polypeptide chain of Hb Inkster was isolated from the whole haemolysate by carboxymethylcellulose (CMC) column chromatography and a phosphate buffer molarity gradient; the buffer contained 8 M urea and 0.05 M mercaptoethanol (Clegg *et al*, 1968). The isolated chains were aminoethylated (Jones, 1964), the small molecules were removed by gel filtration on Sephadex G-25 with 0.5% v/v formic acid, and the chains were lyophilized.

Cyanogen bromide cleavage (Steers *et al*, 1965) of α-chains was performed by dissolving 16 mg of polypeptide in 2.5 ml H_2O and adding 7.5 ml of 88% formic acid; 5 ml of cyanogen bromide, representing a 90-fold molar excess, was added. The reaction was allowed to proceed for 25 hr at room temperature and the mixture was then diluted with 50 ml of H_2O and lyophilized. The cyanogen bromide peptides or aminoethylated α-chains were then digested with trypsin by dissolving the lyophilized material in 2 ml of 0.2 M $NH_4\ NCO_3$ and adding 100 μg of TPCK-trypsin (Worthington). After reaction for 90 min at 37°C another 50 μg of trypsin was added. The reaction was allowed to proceed for a total of 3 hr and the digest was then taken to dryness in a rotary evaporator. Peptide mapping of the tryptic digest was performed using first descending paper chromatography in the isoamyl alchol–pyridine–acetic acid–water solvent system of Baglioni (1961) and then high voltage paper electrophoresis (Chernoff & Liu, 1961) employing a pH 6.4 buffer system (Ingram, 1958). The chromatograms were stained with either 0.1% ninhydrin or trinitrobenzenesulfonic acid (TNBS) (Kotaki *et al*, 1964). Automatic peptide chromatography was performed (Jones, 1964) on PA-35 (Beckman) ion exchange resin using a 500 ml double linear gradient. The first gradient was from 0.05 M pyridine acetate, pH 2.4, to 0.50 M pyridine acetate, pH 3.75. The total volume was 250 ml. At the end of the first gradient, the system automatically switched to a

TABLE I. Haematological values in family with Haemoglobin Inkster and β-thalassaemia

Subject	*PCV* (%)	*RBC osmotic fragility*	*RBC morphology*	*Haemoglobin components* (%)				*Genotype*
				Ink	*F*	A_2	Ink_2	
II-1	37.5	Decreased	Hypochromia	—	1.2	4.5	—	β-thal. trait
-2	44	—	Normal	—	1.0	2.5	—	Normal
-3	—	—	—	21.4*	2.3	1.6	0.6	Hb-Ink trait
-4	45	—	Normal	—	1.0	2.2	—	Normal
-5	51.5	—	Slt. polychromasia	22.1*	0.9	2.0	0.6	Hb-Ink trait
-6	39.5	Decreased	Hypochromia	18.9*	0.8	3.5	1.0	Hb-Ink-β-thal.
-7	40	Decreased	Hypochromia	20.7*	—	4.0	1.3	Hb-Ink-β-thal.
III-1	43	—	Slt. anisocytosis	—	1.0	1.9	—	Normal
-2	41	—	Normal	—	0.9	1.8	—	Normal
-3	41.5	—	Normal	—	1.0	2.2	—	Normal
-5	47	—	Slt. poly.	21.7*	1.3	2.0	0.6	Hb-Ink trait
-6	46	—	Slt. poly.	22.2*	0.7	2.0	0.7	Hb-Ink trait
-7	46	Normal	Normal	22.5	—	2.4	—	Hb-Ink trait
-8	38	Normal	Normal	22.9	—	2.2	—	Hb-Ink trait
-9	40	Decreased	Hypochromia	—	1.1	4.8	—	β-thal. trait
-10	47	—	Normal	—	0.5	2.6	—	Normal
-11	46.5	Normal	Slt. poly.	22.4*	0.7	1.9	0.8	Hb-Ink trait
IV-1	40.5	—	Normal	—	1.0	2.8	—	Normal
-2	37.5	Normal	Normal	—	1.3	2.5	—	Normal
-3	—	Normal		—	1.1	2.5	—	Normal
-4	45	—	Normal	—	1.0	2.2	—	Normal
-5	41	—	Slt. poly.	—	0.9	2.9	—	Normal
-6	43	—	Occ. schistocytes	21.4	0.8	2.2	—	Hb-Ink trait
-7	45	—	Slt. poly.	22.9	1.8	2.4	—	Hb-Ink trait
-8	41.5	—	Normal	—	1.2	3.1	—	Normal
-9	40.5	—	Normal	—	2.5	2.8	—	Normal
-10	41	—	Normal	—	2.4	1.7	—	Normal
-11	41	—	Occ. schist.	22.0	0.5	2.6	—	Hb-Ink trait
-12	—	—	Normal	21.3	0.7	2.7	—	Hb-Ink trait
-13	40	—	Normal	—	0.6	2.8	—	Normal
-14	42.5	—	Normal	22.3	1.0	2.7	—	Hb-Ink trait
-15	37	Normal	Normal	—	—	1.7	—	Normal
-16	38	Normal	Normal	—	—	1.7	—	Normal
-17	38	Normal	Normal	—	—	2.2	—	Normal
-18	37	Normal	Normal	—	—	1.8	—	Normal
-19	—	Normal	Normal	—	—	2.1	—	Normal
-20	37.5	Normal	Normal	—	—	2.5	—	Normal
-21	41	Decreased	Hypochromia	—	1.0	5.2	—	β-thal. trait
-22	42.5	—	Normal	—	1.2	2.7	—	Normal
-23	39.5	—	Normal	—	1.4	2.5	—	Normal
-24	41	—	Normal	—	0.8	2.1	—	Normal
-25	35	Normal	Normal	—	1.8	2.8	—	Normal

* Mean of triplicates analysed by cellulose acetate electrophoresis. The remainder were analysed by starch block electrophoresis.

second linear gradient of identical size which was from 0.50 M pyridine acetate, pH 3.75, to 2.0 M pyridine acetate, pH 5.0. Tryptic peptides were either eluted from the paper peptide maps or recovered from the column fraction collector, hydrolysed *in vacuo* with constant boiling HCl at 110°C, and analysed on the Beckman 120C Automatic Amino Acid Analyser.

RESULTS

Family Data (Fig 1)

The propositus (III-9) attended the University of Michigan Medical Center for evaluation of anaemia that had not responded to iron therapy. This confirmed the presence of a microcytic, hypochromic anaemia with many target cells. The serum iron was normal and the

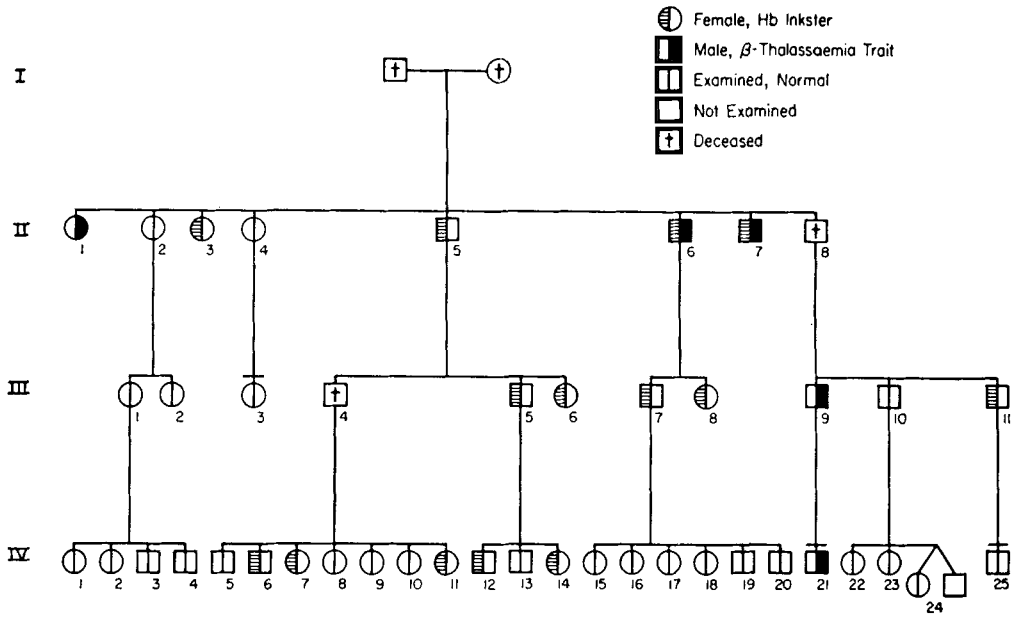

FIG 1. Pedigree of family containing β-thalassaemia and Hb Inkster variants.

bone marrow contained normal iron stores. The clinical impression of thalassaemia was confirmed by demonstrating decreased RBC osmotic fragility (Table I), and an increased proportion of Hb A_2 on electrophoresis; similar findings were present in the blood of his son (IV-21) and his paternal aunt (II-1). His brothers were studied and, while neither had thalassaemia, an abnormal Hb was found in one. The electrophoretic pattern at pH 8.6 was nearly identical to that of sickle cell trait, except that the abnormal component, moving cathodally to Hb A in the position of Hb S, was only 20% of the haemoglobin. This abnormality is henceforth referred to as Haemoglobin Inkster (Hb Ink). Also, a minor component (Hb Ink_2) moved in a comparable relationship to Hb A_2, indicating that the abnormality in Hb Ink was located in the α-chain.

Family studies were performed to assess the haematological consequences of the abnormal haemoglobin and to seek coexistence of the two abnormalities. The pedigree is shown in

Fig 1. The family is of English and German extraction. Fourteen members had Hb Ink. All but two of these had, at most, only minimal RBC morphological abnormalities (Table I). Packed cell volumes varied between 38 and 51.5%; three who were tested had normal RBC osmotic fragilities. The proportion of Hb Ink, estimated initially by starch block electrophoresis (Kunkel & Wallenius, 1955) of haemolysates from 11 subjects, was $21.7 \pm 1.6\%$. The mean proportion of Hb A_2 pooled with Hb Ink_2 (α_2 Ink δ_2) was $2.4 \pm 0.3\%$. The exceptional heterozygote (II-6), a paternal uncle of the propositus, had perceptibly greater red cell morphological abnormalities and decreased osmotic fragility. The proportion of Hb $A_2 + Ink_2$ in II-6 was 4.5%, suggesting that the morphological abnormality was due to inheritance of the β-thalassaemia gene along with Hb_α Ink. The proportion of Hb A_2 in the blood of 28 relatives who did not have either mutant gene was $2.3 \pm 0.4\%$.

After the completion of these studies, blood was obtained from an additional paternal uncle of the propositus (II-7), living outside the state of Michigan, who was also judged to possess

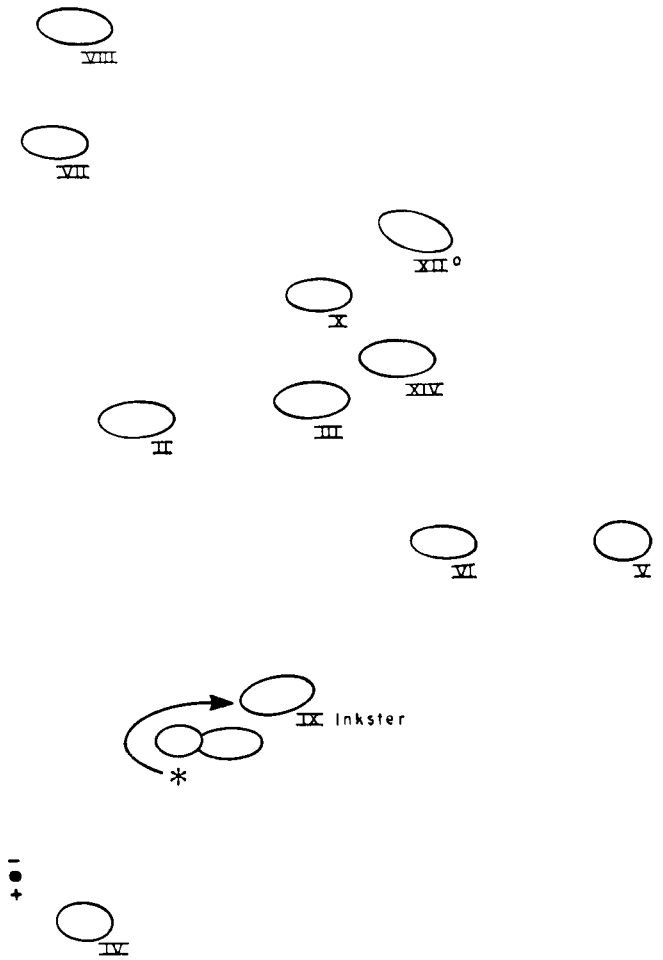

Fig 2. Two-dimensional paper peptide map of α-chains of Hb Inkster: Horizonal dimension: descending chromatography in a buffer (Baglioni, 1961) containing butanol, pyridine, acetic acid and water (15:10:3:12); vertical dimension: electrophoresis at pH 6.4. Stained with 0.1% ninhydrin in ethanol.

both mutant genes. Since we were then utilizing a reproducible cellulose acetate technique (Glynn *et al*, 1968) for estimation of the haemoglobin components a subsample of the family was re-examined to determine whether the proportion of Hb Ink was different in those heterozygotes with and without the β-thalassaemia gene. Accordingly, the haemoglobins of the two persons heterozygous for both genes (II-6 and II-7) were compared with those from five relatives having only Hb_α Ink. Each specimen was examined in triplicate using the cellulose acetate technique. The mean proportion of Hb Ink in the persons having both abnormalities was $19.8 \pm 1.3\%$, in the simple heterozygotes it was $22.0 \pm 0.6\%$. This difference was tested for statistical significance by employing an analysis of variance technique such that the three replicate measurements for each person were taken into account. The difference was significant, $F(1.5) = 15.48$, $0.01 > P > 0.025$.

The proportion of Hb A_2 in the persons having both mutant genes was $3.8 \pm 0.4\%$ and in those having only Hb_α^{Ink} $1.9 \pm 0.2\%$. The proportion of Hb Ink_2 in the two groups was

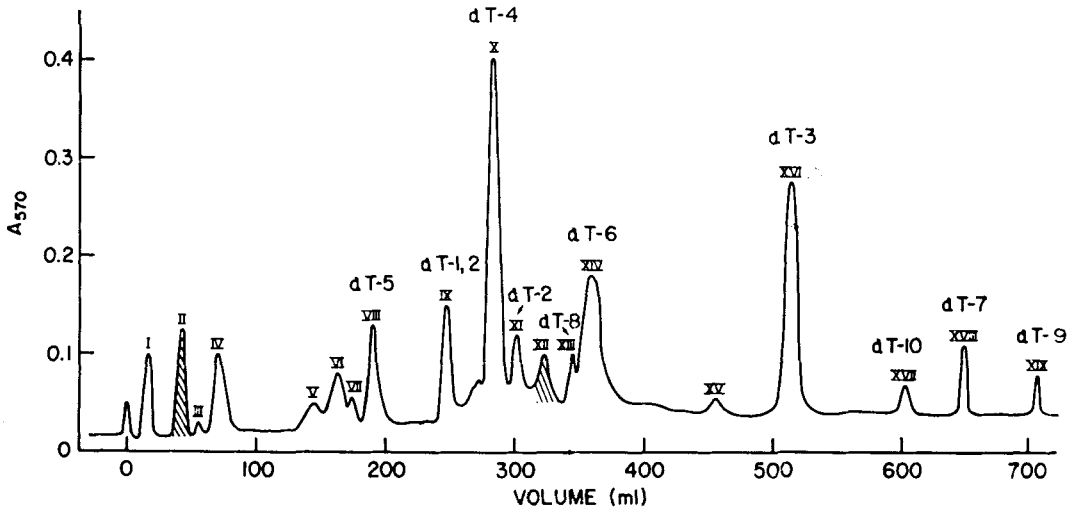

FIG 3. Peptide-column chromatogram of α-chain of Hb Inkster obtained after cleavage with cyanogen bromide and trypsin (see text). The roman numerals refer to pooled fractions. The shaded areas are not present in normal α-chain digests not treated with cyanogen bromide.

1.2 ± 0.2 and $0.7 \pm 0.09\%$, respectively; these results prove that II-6 and II-7 are, indeed, heterozygous for both genes.

Haemoglobin Structural Studies

The variant α-chains were eluted from the CMC column (Clegg *et al*, 1968) following the normal α-chains, as anticipated from the electrophoretic mobility. The peptide map of the tryptic digest of the aminoethylated variant α-chains is compared with that of α^A in Fig 2. αTp-9 is clearly missing and an additional heavy spot (IX-Inkster) is present cathodally. This was eluted from papers stained with TNBS and the amino acid composition determined after acid hydrolysis. The composition of this peptide is shown in Table II. It normally contains four residues of aspartic acid and two asparagine; the latter are, of course, converted

to aspartic acid in the hydrolysis procedure. Taking into account its electrophoretic mobility, one of the aspartic acid residues is substituted by valine in the α^{Ink} chain.

In Hb A, αTP-9 extends from residues 62 through 90. Three of the aspartyl residues are located before the methionine residue at position 76; the remaining aspartyl residue is at position 85. In order to further divide this peptide the α-chains were first treated with cyanogen bromide, cleaving the chain at residue 76, and then digested with trypsin. The results of the peptide column chromatography are shown in Fig 3. Fraction II was found to have a composition corresponding to the amino-terminal cyanogen bromide fraction of normal αTp-9, designated αTp-9a (Table II). Fraction XII corresponded to αTp-9b, the carboxyl-terminal fragment, but was lacking one residue of aspartic acid and now contained a single residue of valine (Table II). At this pH it was so slightly displaced from its normal position that precise comparison with the mobility of the normal equivalent peptide was difficult. These findings made it possible to conclude that Hb Inkster is $\alpha_2{}^{85\ Asp\rightarrow Val}\beta_2$.

TABLE II. Composition of tryptic/cyanogen bromide peptides of Haemoglobin Inkster

	αTp-9 Inkster			*αTp-9$_a$ Inkster*			*αTp-9$_b$ Inkster*		
	Observed residues	*Whole residues*		*Observed residues*	*Whole residues*		*Observed residues*	*Whole residues*	
		Hb Ink	*Hb A*		*Hb Ink*	*Hb A*		*Hb Ink*	*Hb A*
Lysine	0.9	1	1	—	—	—	1.2	1	1
Histidine	2.8	3	3	0.8	1	1	1.7	2	2
Aspartic acid	5.2	5	6	4.0	4	4	1.2	1	2
Threonine	1.1	1	1	1.0	1	1	—	—	—
Serine	1.7	2	2	—	—	—	1.8	2	2
Proline	1.0	1	1	—	—	—	1.0	1	1
Glycine	0.3	—	—	—	—	—	—	—	—
Alanine	6.9	7	7	4.1	4	4	3.0	3	3
Valine	3.9	4	3	3.0	3	3	1.1	1	0
Methionine	0.8	1	1	—	—	—	—	—	—
Leucine	4.1	4	4	1.1	1	1	3.0	3	3
Homoserine	—	—	—	+	1	1	—	—	—
Total		29	29		15	15		14	14

DISCUSSION

Hb Inkster is the first example of this specific amino acid substitution to our knowledge. Normally there are four aspartic acid residues in αTp-9 along with two asparagine residues. The amino acid analysis of the mutant αTp-9 showed that one of the aspartic acid residues was replaced by valine. Cleavage of αTp-9 with cyanogen bromide greatly simplified the structural studies in that there was only a single aspartic acid residue in the 'right hand' cleavage product of that peptide; normally there should be two. This permitted unequivocal assignment of the substitution.

The coexistence of Hb Ink and β-thalassaemia provided another opportunity to examine the suppressive effect of one type of thalassaemia on a structural variant in the complementary

chain. Although the degree of suppression is variable, the presence of an α-thalassaemia gene appears regularly to decrease the proportion of a coexistent β-chain structural haemoglobin variant. In the converse situation of an α-chain structural haemoglobin variant and β-thalassaemia this effect is less consistent. Five instances of this type of association have been previously reported and three showed no decrease in the proportion of the α-chain variant (Fessas *et al*, 1965; Lee & Huisman, 1965; Atwater *et al*, 1970) although techniques used at the time of the two earlier studies might have failed to demonstrate small differences in variant-Hb proportions.

In the combination of genes for β-thalassaemia and Hb L Ferrara, however, the proportion of that variant (Silvestroni *et al*, 1960) was clearly lowered. In four families, simple heterozygotes for Hb L Ferrara possessed 22–36% of Hb L Ferrara measured by a less accurate technique than currently available. Individuals heterozygous for both genes had 10–16% of Hb L Ferrara. Two persons having 48% of Hb L Ferrara may have been heterozygous for α-thalassemia as well as Hb L Ferrara. Similarly the proportion of Hb Q India appears to be decreased in heterozygotes for both β-thalassaemia and Hb Q India genes (Sukumaran *et al*, 1972).

In the family we are reporting here, Hb Ink heterozygotes had from 21.5 to 22.4% of the variant haemoglobin while the two individuals doubly heterozygous for the β-thalassaemia and Hb Ink genes had 18.9 and 20.7%. Although the decrease in the proportion of Hb Ink is small, it is beyond the replicability of the method and statistically significant at the 2.5% level.

The mechanism by which a thalassaemia gene decreases the proportion of a mutant structural gene involving a different globin chain is unknown. Tuchinda *et al* (1967) have postulated that this is a consequence of unequal competition between mutant and normal chains for a limited number of complementary chains. A second hypothesis is that thalassaemia genes function analogously to mutant regulator genes in microbial systems, exerting an effect on other loci (Rucknagel, 1964; Zuckerkandl, 1964). Neither hypothesis has been firmly established.

Also unexplained is the apparent inconsistent effect of β-thalassaemia genes when compared with α-thalassaemia. Since, in α-chain structural variants, the proportion of abnormal haemoglobin usually comprises only 15–25% of the total in contrast to 30–40% in Hb_β mutants, a proportionate effect of β-thalassaemia on Hb_α mutants would be expected to be smaller and closer to the resolving power of the electrophoretic methods employed. Hb L Ferrara, having the greatest proportion of abnormal component, demonstrated the greatest decrease, although the electrophoretic method available then was less precise than those now in use. Another variable might be the heterogeneity in β-thalassaemia. In the so-called Mediterranean variant β-chain synthesis is impaired more than in the African variety of β-thalassaemia (Rucknagel & Laros, 1969). Lastly, β-thalassaemia may affect the product of one Hb_α locus more than the other, thus explaining some of the inconsistencies. This explanation is also tenuous, however, because there is evidence that some individuals may have only one Hb_α locus (Abramson *et al*, 1970). Available data does not allow differentiation among these possibilities.

Although we do not understand the pathogenesis of this type of thalassaemia–haemoglobin variant 'interaction', the resulting changes in the proportion of haemoglobin mutants can

alert physicians to the presence of otherwise unsuspected thalassaemia. Thus when individuals with unusually low proportions of abnormal haemoglobins are encountered, or when wide variations in the proportion of abnormal haemoglobins are detected within a family, further studies are indicated. In addition to confirming the diagnosis of coexisting thalassaemia, such investigations may shed further light on the mechanisms responsible for these changes in mutant Hb proportions.

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