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Research Article

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ABSTRACT A striking history of familial polycythemia led to a search for an abnormal hemoglobin. None could be demonstrated by routine electrophoretic methods, but the propositus' hemolysate had increased oxygen affinity. Manipulation of the conditions of electrophoresis, and chromatographic methods, permitted identification of hemoglobin Malmö. Studies of hemolysates demonstrated a normal Bohr effect, decreased heme-heme interaction ($n = 1.58$), and a p50 of 1.3 mm Hg at 10°C and pH 7.2. The amino acid substitution occurs in the same position (FG-4) as that of hemoglobin Chesapeake, but in the β-chain rather than the α-chain. The two types of hemolysate have different pathophysiologic properties, and carriers of hemoglobin Malmö exhibit more striking hematologic abnormalities.

INTRODUCTION

Abnormal hemoglobins are now included in most house-officers' lists of causes of secondary polycythemia, but hemoglobin electrophoresis is often the only technique used to detect their presence. Until recently, all abnormal hemoglobins associated with polycythemia have had abnormal electrophoretic mobility (Table I) (1–13), but that finding reflects ease of detection rather than a necessary property of the hemoglobin molecule. Hemoglobins have been suspected to be causes of polycythemia but proof of abnormal molecular structure has been thwarted by inability to identify the aberrant component (12 and personal communication from Dr. Lokich).

We have encountered a kindred with hemoglobin Malmö (Malmö), previously found in four generations of a polycythemic Swedish family by Lorkin, Lehmann, Fairbanks, Berglund, and Leonhardt, but described only in abstract form (11). In our family, abnormal oxygen affinity of the propositus' blood indicated that an abnormal hemoglobin must be present, but none was detected by conventional electrophoretic screening techniques. The anticipated abnormality was found by chromatographic procedures, and subsequently the conditions of electrophoresis were altered to permit its detection by that means as well. Analyses of structure and function of the abnormal hemoglobin are reported here. Descriptions of the clinical status of carriers are published elsewhere.[1]

METHODS

Hematologic examinations were performed in Rochester, and are reported with the clinical data. Blood samples, collected in anticoagulant citric acid dextrose solution and packed in ice, were sent by air to Baltimore for structural and functional analyses. All samples arrived in excellent condition within 24 hr of shipment. Except as indicated, electrophoresis on acrylamide gel, starch gel, cellulose acetate, and agar (citrate buffer, pH 6.0), and denaturation by heat, were performed by standard methods. Details of structural analyses are presented in legends of figures.

Because of delay between sampling and analysis, oxygen affinity of whole blood could not be measured. Accordingly, analysis was limited to toluene extracts which had been dialyzed overnight against 0.1 M phosphate buffers (1). Blood samples from normal controls and from carriers of hemoglobin Chesapeake (Chesapeake) were obtained in Baltimore and studied in the same fashion.

RESULTS

The propositus was a member of a large family in which erythrocytosis occurred in at least 21 individuals. His

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[1] Fairbanks, V. F., J. E. Maldonado, S. Charache, and S. H. Boyer. 1971. Familial erythrocytosis due to a hemoglobin with impaired oxygen dissociation (hemoglobin Malmö, $\alpha_2\beta_2^{97\ gln}$). *Mayo Clin. Proc.* **46**: 723.

TABLE I

Hemoglobins with Increased Oxygen Affinity

Hemoglobin	Substitution	Electrophoretic mobility, pH 8.6	Hemoglobin concentration		Reference
			Men	Women	
			g/100 ml		
Chesapeake	α-92 arg → leu	Fast	15.3–19.9	14.9–18.0	1
J Cape Town	α-92 arg → gln	Fast	13.7	14.6–16.6	2, 3
Yakima	β-99 asp → his	Slow	16.5–22.9	15.9–18.2	4
Kempsey	β-99 asp → asn	Slow	17.2–21.3	17.3–20.2	5
Ypsi	β-99 asp → tyr	Slow*	18–19	12–17	6, 7
Hiroshima	β-143 his → asp	Fast	—	11.2–17.4	8
Rainier	β-145 tyr → cys	Slow‡	21.0	16.2–16.4	9, 10
Bethesda	β-145 tyr → his	Slow‡	20.5	—	7, 10
Malmö	β-97 his → gln	Normal	17.1–21.3	14.3–18.4	11, §
Brigham	Unknown	Normal	16.8–20.0	15.1–18.9	12‖
Little Rock	β-chain	Normal	23	—	13¶

* Multiple bands.

‡ Agar.

§ See footnote 1 in text.

‖ Personal communication from Dr. J. J. Lokich.

¶ Personal communication from Dr. P. A. Bromberg.

hemolysate was normal after agar electrophoresis. Hemoglobin mobility appeared slightly abnormal after electrophoresis on acrylamide gel, cellulose acetate, and starch gel using a standard buffer system, but considerable experience was required for that distinction (Fig. 1, top). Much better discrimination could be achieved after starch gel electrophoresis in a specially modified buffer (Fig. 1, bottom). 19 members of the family were found to have abnormal patterns. In most instances, this abnormality was confirmed by chromatographic analysis as described below. With the exception of one woman who had hypochromic red cells secondary to many phlebotomies, all carriers were moderately polycythemic, and had higher hematocrit values than carriers of Chesapeake (Fig. 2).

Analyses of structure. The abnormal hemoglobin could not be separated by heat precipitation. Its presence could be recognized after chromatography on DEAE-Sephadex (Pharmacia Fine Chemicals, Piscataway, N. J.), but separation from hemoglobin A was not precise enough for quantitative measurement (Fig. 3). The relative amount of the abnormal β-chain could, however, be estimated by chromatography of globin in 8 M urea (16) and subsequent integration (by weighing) of continuous absorbance recordings (Fig. 4, upper right insert). Among 18 individuals, the average proportion of Malmö β-chains was 48.0% (SD 0.43), before correction for "pre-peak" artifacts.[2]

The site of mutation in the abnormal hemoglobin was defined using partially purified samples prepared by DEAE-Sephadex chromatography (Fig. 3, Pool-4). The abnormal β-chain was purified (Fig. 4), aminoethylated, digested with trypsin, and its peptides were separated by fingerprinting. Among all the tryptic peptides, only β-11 was abnormal (Fig. 5): it was shifted to a more anodal position, suggesting loss of about one electrostatic charge measured at pH 4.7. Amino acid analysis indicated that the abnormal β-11 lacked one histidine and had gained either an extra glutamine or glutamic acid residue (Table II). A parallel result was obtained from analysis of amino acids in the whole chain (Table III).

The exact position of mutation in peptide β-11 was established by subtractive Edman degradation. As shown in Fig. 6, histidine was absent from its usual position at β-97 and replaced by glutamic acid or glutamine. Distinction between these two amino acids was made by digestion with leucine aminopeptidase (LAP).[3] This enzyme, unlike the HCl used for hydrolysis in other studies, does not convert amides, e.g. glutamine, to free acids, e.g., glutamic acid. After digestion with LAP for 30 and for 70 min, the three amino acids released in

[2] Although the nature of pre-peaks is unknown, they are found during almost all chain separations. Their position of elution varies with the position of main peaks in a variety of mutant hemoglobins (Boyer, S. H., unpublished observations), and their peptide maps are indistinguishable from that of the immediately following main peak. In the present study, the pre-β^A-peak of A/A homozygotes contained about 18% of the mass in the main peak. Using this factor, the "corrected" proportion of Malmö in heterozygotes averaged 45.5%.

[3] *Abbreviations used in this paper:* LAP, leucine aminopeptidase; p50, oxygen pressure required for 50% saturation.

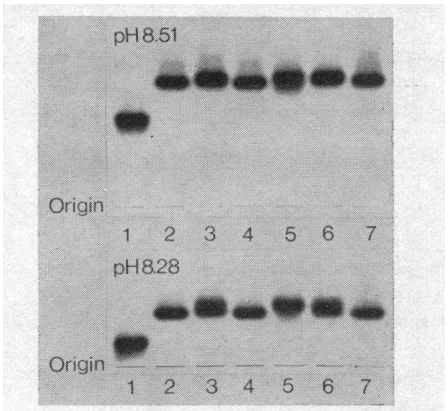

FIGURE 1 Starch gel electrophoresis of hemoglobins stained with amido-black 10B after application of 5 v/cm for 20 hr. In each display, anode was at top of section. Upper gel was performed in a conventional EDTA-boric acid–Tris buffer, i.e. EBT (14), at pH 8.5. In the lower gel, the buffer was changed to 0.001 M EDTA, 0.025 M boric acid, and 0.025 M Tris, giving a final pH of 8.28. Samples (5 mg/ml of cyanmethemoglobin) are: 1, isolated hemoglobin S; 2, hemoglobin A in hemolysate from a normal sibling; 3, hemoglobins A and Malmö in hemolysate from propositus; 4, hemoglobin A in hemolysate from another normal sibling; 5, partially purified hemoglobin Malmö (Fig. 3, Pool-4); 6, hemoglobins A and Malmö in hemolysate from another carrier; and 7, isolated hemoglobin A from a normal subject.

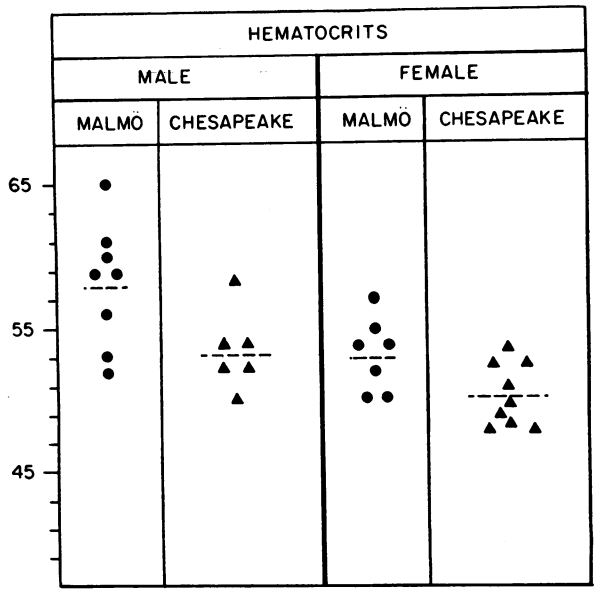

FIGURE 2 Comparison of hematocrit values of adult carriers of hemoglobins Malmö and Chesapeake. One carrier of Malmö, who had hypochromic red cells secondary to many phlebotomies, is omitted.

highest yield were leucine, an amide, and valine. Since digestion with LAP proceeds from the free amino terminus of a peptide, the results of these digestions coupled with the findings of the Edman degradations firmly establish that the substitution penultimate (position β-97) to the amino terminus of variant peptide β-11 is histidine → glutamine. Thus, the abnormal hemoglobin in this family is the same as that reported from Malmö by Lorkin et al. (11).

The substitution of glutamine for histidine in Malmö nicely accounts for its essentially normal electrophoretic mobility at the pH of 8.5–8.6 employed in conventional hemoglobin electrophoresis. In that pH range, the imidazole side chain of histidine is nearly uncharged, and replacement by glutamine effectively produces little change. At slightly lower pH (Fig. 1), the imidazole group becomes more positively charged and Malmö can then be electrophoretically distinguished from hemoglobin A.

Analyses of function. Oxygen affinity of the propositus' hemolysate was increased (the p50, i.e. oxygen pressure required for 50% saturation, was decreased to 1.3 mm Hg at 10°C and pH 7.2), and varied in

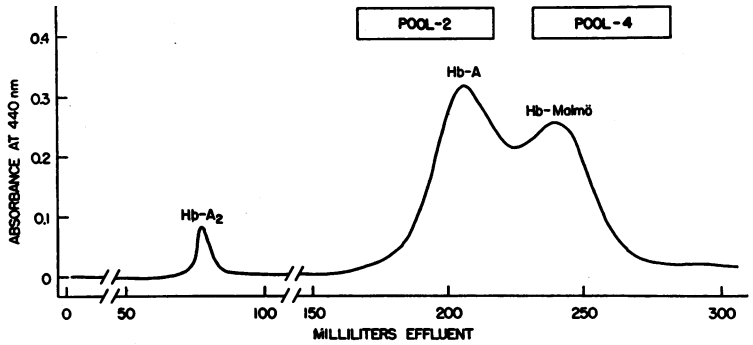

FIGURE 3 Column (1.5 × 20 cm) chromatographic separation on DEAE-Sephadex (A-50, Pharmacia Fine Chemicals, Inc.) of 15 mg whole hemolysate from propositus using gradient elution (pH 8.00–7.60) at 14 ml/hr with 0.05 M Tris-HCl, 0.001 M KCN buffers (15). Sample and column were equilibrated with pH 8.5 buffer before application.

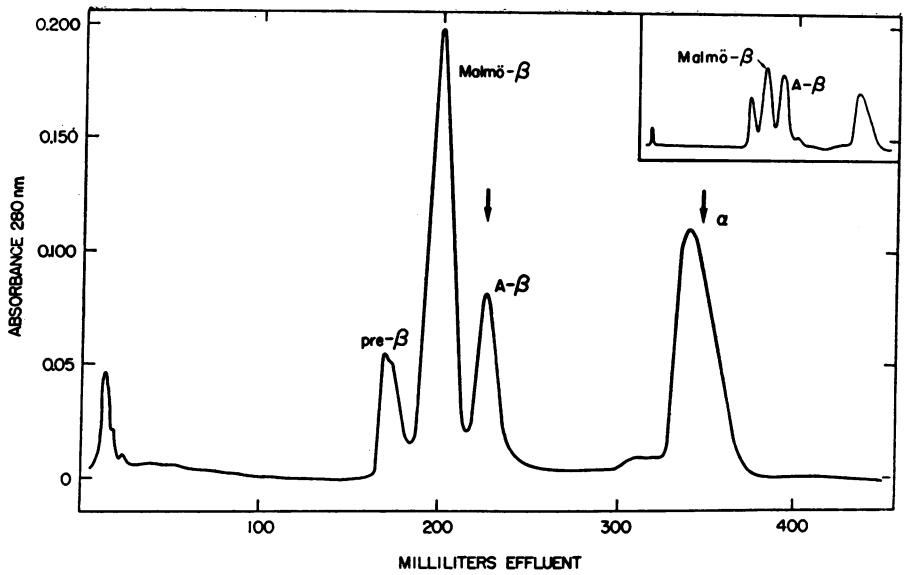

FIGURE 4 Column (0.9 × 10 cm) chromatographic separation of globin from pool 4 (Fig. 3) on carboxymethyl cellulose (Whatman CM 32) as described by Clegg, Naughton, and Weatherall (16). The β-chain of Malmö is eluted before the β-chain of residual hemoglobin A. Arrows above A-β and α denote peak of principal components from hemoglobin A separated simultaneously on a parallel column. Upper right inset represents corresponding analysis of 40 mg of whole hemolysate from propositus.

normal fashion with changes in pH, i.e., the Bohr effect ($\Delta \ln p50/\Delta pH = -1.27$, SE 0.27) did not differ from that of hemoglobin A ($\Delta \ln p50/\Delta pH = -1.35$, SE 0.15) (Fig. 7). The dissociation curve had a more hyperbolic shape than normal, indicating that heme-heme interaction was decreased. Differences in the shape of curves are most easily compared if they are plotted according to the logarithmic form of Hill's equation (20) (Fig. 8). Normal hemoglobin should yield a straight line with a slope (n, or Hill's constant) of about 3; a completely hyperbolic curve, with no heme-heme interaction, has a slope of 1. Straight lines were fitted to experimental data by the method of least squares: the mean value of n for whole hemolysates containing Malmö was 1.58 with a standard deviation of 0.22 (18 samples) (Fig. 8). Hill's constant for normal hemolysates, calculated in the same fashion, was $n = 2.86$ (SD 0.27, 12 samples). Data obtained on hemolysates from carriers of Chesapeake were not randomly clustered about the line obtained in this fashion, but appeared to fall on a biphasic curve (Fig. 8). When straight lines were fit, by eye, to the two portions of these curves, their approximate slopes averaged 2.76 and 1.30 (eight samples) (Fig. 9).

In an attempt to study the properties of pure Malmö, the propositus' hemolysate and that of a normal control were each subjected to starch block electrophoresis at pH 8.6. The front and back running portions of the major hemoglobin band were eluted, and their oxygen affinity was measured after overnight dialysis against 0.1 M phosphate buffer. Front running portions of both hemolysates contained 4% methemoglobin at the end of the experiment, while back running portions (which included the methemoglobin formed during electrophoresis) contained 8–11%. The oxygen pressure required for half-saturation (p50) of the front portion of the

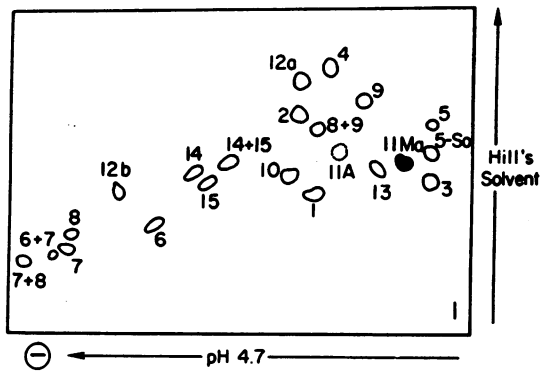

FIGURE 5 Map of ninhydrin developed peptides prepared from tryptic digestion of S-aminoethyl derivative of Malmö-β. Paper electrophoresis at pH 4.7 was followed by descending paper chromatography in Hill's solvent as detailed elsewhere (17). Numbers refer to tryptic peptides designated in Table II. In Malmö-β digests, tryptic peptide β-11 is absent from its usual position at 11A and shifted to a new position at 11 Ma (shown as a solid spot).

TABLE II

Amino Acid Composition of Tryptic Peptides

Tryptic peptide	β-1	β-2	β-3	β-4‡	β-5§	β-5-So	β-6	β-6 + 7	β-7	β-7 + 8
Sequence position¶	1–8	9–17	18–30	31–40	41–59	41–59	60–61	60–65	62–65	62–66
Lysine	0.90	0.83**			0.89	0.64**	0.88	1.36**	0.93	1.27**
AE-cysteine										
Histidine	0.99							1.02	1.10	0.98
Arginine			0.80**	1.08						
Aspartic acid			1.95		3.07	2.96				
Threonine	0.93	0.93		0.89	0.92	0.96				
Serine‡‡		0.80**			1.96	1.91				
Glutamic acid	2.14		2.00	0.86	1.05	1.08				
Proline	0.97			1.00	1.76	1.63				
Glycine		1.01	2.99		2.05	2.08		1.02	1.02	1.02
Alanine		1.92	0.96		1.02	1.02		0.97	0.94	0.66**
Valine	0.92	1.05	2.65**	2.17	1.02	1.10	1.12	0.71**		
Methionine					0.83	0.74				
Leucine	1.07	1.08	0.92	1.76**	0.98	1.06				
Tyrosine				0.74						
Phenylalanine					2.79**	2.42**				
Tryptophan§§		(1)		(1)						
Sum of nearest integers	8	9	13	10	19	"19"	2	"6"	4	"5"

* Peptides were purified on paper (Fig. 5), detected with dilute ninhydrin, eluted with 5.7 N HCl, and, except as noted, hydrolyzed at 120°C for ~20 hr in nonevacuated tubes. All calculations represent the best fit to integrality assuming total residues in each peptide approximate total in homologous A-β peptides. Residues forming <0.10 of an integer are omitted.

‡ Hydrolyzed in vacuum for ~72 hr.

§ Hydrolyzed in vacuum for ~20 hr.

¶ Sequence positions assigned by homology with peptides from A-β (18).

** Reduced values of expected carboxy- and/or amino-terminal residues stem from excessive reaction with ninhydrin after separation on paper before elution. In a few instances the reduced values are reflected in quotation marks surrounding total of nearest integers.

‡‡ Correction for destruction.

§§ Estimated solely from color reaction on paper.

‖ Underscoring (peptide β-11) emphasizes differences between A-β and Malmö-β.

Malmö hemolysate was 0.75 mm Hg with $n = 1.87$ (cf., 1.58 in whole hemolysates). The corresponding portion of the normal hemolysate had a p50 of 2.3 mm Hg, with $n = 2.87$ (cf., 2.86 in whole hemolysates). Pure Chesapeake, studied under similar conditions, had a p50 of approximately 0.35 mm Hg, with $n = 1.3$ (cf., 1.30 in first section of whole hemolysate). In this single experiment, Chesapeake appeared to be oxygenated more easily than Malmö which in turn was oxygenated more easily than hemoglobin A.

DISCUSSION

Comparison of hemoglobins Malmö and Chesapeake. Oxygen affinity of hemoglobin can be abnormal because of the position of the dissociation curve (oxygen affinity per se), the change in position with pH (the Bohr effect), the shape of the curve, or the degree of heme-heme interaction. Comparisons of these properties between hemoglobins Chesapeake and Malmö are of particular interest: although the two substitutions occur in different chains at apparently different sites (α-92 vs. β-97), they are homologous in three-dimensional space. Both are located at residue FG-4, the fourth position in the connecting region between helices F and G.

Bohr effect. The Bohr effect appears to be normal in Malmö, but our experiments must be interpreted with caution because they were not carried out on the purified abnormal hemoglobin. If subsequent studies confirm this impression, the finding is of some significance: 50% of this change in oxygen affinity with pH can be related to formation of a salt bridge between the C-terminal histidine of the β-chain and residue FG-1-β, only three residues away from FG-4, the site of mutation in Malmö (21). Substitution at FG-4 could affect FG-1 directly, or by distortion of the main chain, which normally

*from the β Chain of Hemoglobin Malmö**

β-8	β-8 + 9	β-9	β-10	β-11‖	β-12a	β-12b	β-13§	β-14‡	β-14 + 15‡	β-15‡
66	66–82	67–82	83–95	96–104	105–112	113–120	121–132	133–144	133–146	145–146
1	1.87	0.60**	0.89			0.91	0.89	0.77**	0.71**	
			0.87		0.55**					
	1.03	0.96	1.05	0		2.00		0.94	1.90	1.00
				0.90						
	2.95	2.98	1.00	2.14	1.02			0.65	1.00	
			1.82				0.98			
	1.04	0.99	0.98							
			1.04	2.09			3.05			
				0.94			1.80			
	2.04	1.97	0.85		1.07	1.03		1.04	1.05	
	2.04	2.06	1.08			1.04	2.06	4.05	4.07	
	0.95	0.81**		0.99	2.00	0.93	1.03	2.88	2.60**	
	3.96	4.02	2.02	0.92	2.90	1.03		1.02	1.04	
							0.92		0.84	0.45**
	0.99	1.06	1.03	1.04		1.06	1.08			
1	17	16	13	9	8	8	12	12	14	"2"

forms a hydrogen bond with the imidazole of FG-4 (*22*). Since the Bohr effect is normal, it is unlikely that the salt bridge is altered, suggesting only a small perturbation of this aspect of structure.

Position and shape of the dissociation curve. The molecular distortion produced by β-97 histidine → glutamine is sufficient to increase oxygen affinity and decrease heme-heme interaction. Affinity of unfractionated hemolysates from carriers of Malmö was higher than that of unfractionated hemolysates containing hemoglobin Chesapeake. Comparison was difficult, because the behavior of Chesapeake hemolysates could not be described by a straight line when plotted according to Hill's equation (Fig. 9). The latter samples acted almost as if only Chesapeake combined with oxygen at low partial pressures, and hemoglobin A only began to combine when the Chesapeake was saturated. Absence of such behavior in Malmö hemolysates may of course reflect the poor discrimination of techniques utilized,[4] but two other possibilities may be advanced.

(*a*) Benesch, Benesch, and Tyuma (*23*) pointed out that the disproportionate effect of methemoglobin $\binom{\alpha^+\beta^+}{\alpha^+\beta^+}$ on oxygen affinity may reflect the formation of hybrid molecules, each of which has abnormal affinity:

$$\binom{\alpha^+\beta^+}{\alpha^+\beta^+} + \binom{\alpha\beta}{\alpha\beta} \rightleftharpoons 2\alpha^+\beta^+ + 2\alpha\beta \rightleftharpoons 2\binom{\alpha^+\beta^+}{\alpha\beta}.$$

[4] In a single experiment on partially purified Malmö, *n* was 1.87 rather than the 1.58 observed with hemolysates: two curves, which do not differ as much as those in Chesapeake hemolysates, may have been superimposed because of the low resolution of our methods. Techniques usually used in studies of whole blood have an even lower resolving power, and it seems probable that the *blood* of carriers of Chesapeake also has a biphasic dissociation curve.

TABLE III

Amino Acid Composition of Hemoglobin β-Chains

Amino acid	Observed*	Hemoglobin Malmö-β		Hemoglobin A-β
		Nearest integer	Calculated from sum tryptic peptides‡	Nearest integer§
Lysine	10.32	10	11	11
Histidine‖	7.80	**8**	**8**	**9**
Arginine	2.84	3	3	3
Aspartic acid	13.23	13	13	13
Threonine	7.16¶	7	7	7
Serine	5.24¶	5	5	5
Glutamic acid‖	12.34	12	12	**11**
Proline	6.79	7	7	7
Glycine	13.20	13	13	13
Alanine	15.31	15	15	15
Valine	17.56**	18	18	18
Methionine	0.89	1	1	1
Leucine	18.05	18	18	18
Tyrosine	2.96	3	3	3
Phenylalanine	7.93	8	8	8
Cysteine‡‡			2	2
Tryptophan‡‡			2	2
Sum			146	146

* Except as noted based on the mean of two analyses of 22 hr and three analyses of 72 hr hydrolysis with 5.7 N HCl in vacuum at 120°C. Calculations are based on best fit to 142 residues.

‡ See Table II.

§ As summarized by Dayhoff (18).

‖ Integral values associated with consistent differences between Malmö-β and A-β are shown in bold face.

¶ Extrapolated to zero time.

** Mean of 72-hr hydrolyses only.

‡‡ Not analyzed in whole chain.

Bellingham and Huehns suggested that the same thing may happen in the red cells of carriers of abnormal hemoglobins (24). Some mutant polypeptide chains may initially be assembled asymmetrically as mixed dimers $\begin{pmatrix}\alpha\beta^{\text{Malmö}}\\ \alpha\beta\end{pmatrix}$, rather than as symmetrical molecules $\begin{pmatrix}\alpha\beta^{\text{Malmö}}\\ \alpha\beta^{\text{Malmö}}\end{pmatrix}$. Since about 50% of the β-chains in the Malmö hemolysate are abnormal, every hemoglobin molecule could be abnormal if only hybrids existed, yielding a monophasic dissociation curve. The situation is different in Chesapeake where, as with most α-variants (25), only 22.5% of the α-chains in hemolysate from carriers are abnormal. Thus, only about 45% of the molecules could exist as hybrids. Bunn has shown in vitro that the liganded form of pure Chesapeake does not dissociate into subunits as readily as the liganded form of hemoglobin A (26). If asymmetric molecules $\begin{pmatrix}\alpha^{\text{Ch}}\beta\\ \alpha\ \ \beta\end{pmatrix}$ are formed during synthesis, resistance to dissociation might exaggerate the potential difference in concentration of hybrids, and thereby explain the biphasic shape of the curves obtained when hemolysates were studied. This alternative seems improbable because of studies on hemoglobin Hiroshima, a β-chain variant with oxygen affinity similar to that of Chesapeake. It comprises about 50% of hemolysates, but these samples yield biphasic curves (27).

(*b*) An alternative explanation for the functional differences between Chesapeake and Malmö stems from Perutz's recent interpretation of the stereochemistry of oxygenation (28). He suggests that when hemoglobin A is oxygenated, the process begins in the α-chain, because the "heme-pocket" of the β-chain cannot accommodate an oxygen molecule without prior structural rearrangement. In an abnormal hemoglobin with increased oxygen affinity, structural abnormalities might either exaggerate or minimize the difference in order of oxygenation of subunits, depending upon whether the site of mutation was in the α- or β-chain. In a

Malmö β-11									
LAP-30'	1.00	1.00	0.91	0.44	0.45	0.30	0.23	0.35	ND
LAP-70'	1.00	1.00	0.96	0.77	0.70	0.73	0.54	0.80	ND
	Leu	- GLN -	Val	(Asp	Pro	Glu	Asn	Phe	) Arg
Untreated	0.97	1.02	1.04	1.01	0.92	1.02	1.01	1.01	1.00
Edman-1	⟶	0.99	0.99	1.01	0.91	0.99	1.01	1.01	1.00
Edman-2		0.32⃗	0.94	0.99	0.92	0.98	0.99	1.04	1.00
Edman-3		0.28	0.45⃗	0.95	0.78	0.97	0.95	0.98	1.00

A-β-11	Leu	- HIS	- Val	- Asp	- Pro	- Glu	- Asn	- Phe	- Arg
Position	96	97	98	99	100	101	102	103	104

FIGURE 6 Sequence analysis of tryptic peptide β11 (residues β-96–104) (18) from hemoglobin Malmö isolated by successive Sephadex G-25 (Pharmacia Fine Chemicals, Inc.) gel filtration, preparative paper pH 4.7 electrophoresis, and descending paper chromatography (17). Numbers *above* Malmö sequence refer to proportions of amino acids released after digestion of ~0.1 μmoles peptide with 6 U of LAP (Miles-Seravac, Miles Laboratories, Inc., Kankakee, Ill.; 122 U/mg) (17) at room temperature for both 30 and 70 min. Numbers *below* the Malmö sequence denote proportions of amino acids found in either untreated peptide or the remainders of three successive Edman degradations (19) after non-vacuum hydrolysis in 5.7 N HCl at 118°C for 22 hr. In all cases, concentrations of amino acids were reduced to proportions shown by taking the amount of leucine as unity in LAP digests and arginine as unity in acid hydrolysates. Arginine assay was not done (ND) in LAP digests. At least 7 nmoles of either leucine or arginine were present in every analysis. The numerical assignments to particular positions are somewhat arbitrary since not only were the amides, asparagine and glutamine, unresolved from one another during analysis of LAP digests, but also distinctions between acids and their corresponding amides, e.g. glutamic acid and glutamine, are lost during acid hydrolysis. The LAP digestion and the subtractive Edman analyses together prove that Malmö contains β-97 glutamine rather than β-97 histidine found in A-β. The positions of residues remaining within brackets are assigned solely through homology with the known A-β sequence (18).

Chesapeake hemolysate, the order of oxygenation might be $\alpha^{Ches} > \alpha^{A} > \beta^{A}$, yielding a biphasic curve, while in Malmö it might be $\alpha^{A} = \beta^{Malmö} > \beta^{A}$, yielding a curve similar in appearance to that of hemoglobin A. Only one other high-affinity α-chain variant is known, hemoglobin J Cape Town. The dissociation curve of hemolysates containing it is not biphasic (29), but its affinity is not as high as that of Chesapeake (30), and the biphasic character may not have been detected.

Differences in oxygen affinity and heme-heme interaction. As noted above the degree of increased oxygen affinity, and decreased heme-heme interaction of Malmö is probably different from that of Chesapeake. Normally, there seem to be more intramolecular bonds in deoxy- than in oxyhemoglobin. Heme-heme interaction is thought to result from the energy released during rupture of these bonds as the molecule is forced to change structure during oxygenation (28). If extra bonds are present in the oxy configuration of an abnormal hemoglobin, interaction is decreased, and oxygen affinity may be increased. It seems likely that altered bonds do exist in the oxy form of Chesapeake (31), perhaps at the interface between the α- and β-chains. Residue FG-4 participates in that interface, which must be forced apart before the molecule can "click" from the deoxy to the oxy form (28).

In three respects, this interface may be less abnormal in Malmö than in Chesapeake. First, residue FG-4-β (Malmö) makes fewer contacts with the α-chain than does FG-4-α (Chesapeake) with its opposite subunit (32). Second, when subunits rotate during the change in structure produced by oxygenation, the FG region of the β-chain undergoes a lesser shift in position than does FG-α (28). Third, at physiologic pH, the amino acids involved in the Malmö mutation (histidine → glutamine) differ less in electric charge and volume than do the amino acids involved in the Chesapeake substitution (arginine → leucine).

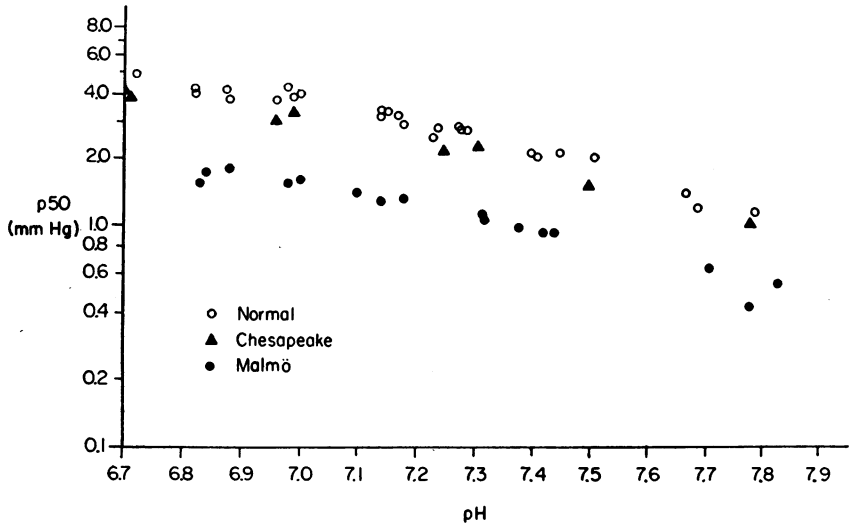

FIGURE 7 Oxygen affinity of dilute hemolysates (approximately 0.1 g/100 ml). The oxygen tension required for half-saturation (p50) at 10°C in 0.1 M phosphate buffer is plotted against pH. Malmö and Chesapeake hemolysates exhibit a normal Bohr effect.

Degree of polycythemia in carriers. Although oxygen affinity of the whole blood of carriers of Malmö and Chesapeake could not be compared, studies of hemolysates suggest that the dissociation curve is further to the left in carriers of Malmö (probably because its concentration is twice as high as that of Chesapeake). If that be true, the carriers of Malmö should be more polycythemic, and that was the case (Fig. 2). On the other hand, it is a truism that red cell mass is determined by a multiplicity of factors: the proportion of Malmö β-chains was remarkably constant in carriers, while hematocrit values varied over a considerable range.

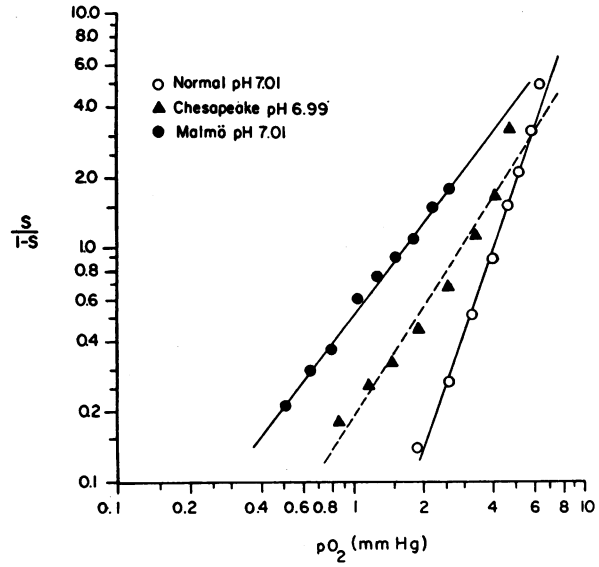

FIGURE 8 Oxygen affinity of unfractionated hemolysates containing hemoglobin A and Malmö or Chesapeake, and normal hemolysates, plotted according to the logarithmic form of Hill's equation (20): $\log S/1 - S = \log K + n \log pO_2$, where S is per cent saturation, K is a constant, and n is a measure of heme-heme interaction. Straight lines were fit to data by the method of least squares. Both Malmö and Chesapeake have a decreased value of n; behavior of the latter hemolysate cannot be described by a straight line.

Diagnostic and therapeutic caveats. Not all heritable polycythemias are produced by abnormal oxygen affinity of the blood. We have studied the dissociation curves of blood and hemolysate from four other families with heritable polycythemia, including a member of the family originally reported by Nadler and Cohn (33). No abnormalities were found, suggesting that polycythemia in these patients may have been produced by an abnormality of the sensor which initiates erythropoietin production, an increased or autonomous response of the erythropoietin producing organ, or synthesis of an abnormal erythropoietin molecule.

It has been suggested that a "normal" red cell mass should be redefined in terms of oxygen release to tissues, rather than the volume of circulating red cells. That might be a reasonable approach, if it were not fairly clear that the increased viscosity of the blood which follows an increase in red cell mass may accelerate or precipitate vascular occlusions. Stamatoyannopoulos and his coworkers have recently suggested that polycythemia due to a hemoglobinopathy "should probably not be modified by phlebotomy or marrow suppressants since oxygen transport would be unfavorably

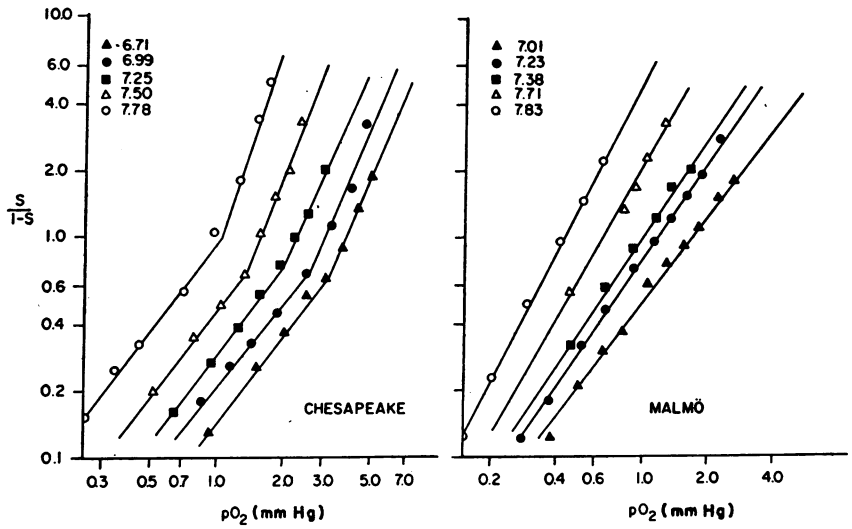

FIGURE 9 Comparison of oxygen affinity of unfractionated hemolysates containing hemoglobin A and Chesapeake or Malmö, plotted as in Fig. 8. The Chesapeake hemolysates yield biphasic lines with slopes of approximately 1.30 and 2.76; the average slope for Malmö in 18 experiments was 1.58.

affected" (7). Therapeutically the situation seems entirely analogous to treatment of polycythemia secondary to chronic lung disease, viz., there are no clear guides to therapy, and each instance must be decided on its merits. Radiation or radiomimetic agents are surely not indicated, but in persons with hematocrit values distinctly outside the normal range, cautious trials of phlebotomy may avert catastrophic vascular accidents.

Note added in proof. After submission of this manuscript, some properties of hemoglobin Malmö were described in: Morimoto, H., H. Lehmann, and M. F. Perutz. 1971. Molecular pathology of human haemoglobin: stereochemical interpretation of abnormal oxygen affinities. *Nature (London)*. **232**: 408.

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