potential caused by ACh in the M-cells. The hypothesis that a hydrogen-ion gradient established across the membrane is per se the cause of the potential difference cannot be ruled out on the basis of our results. Another interpretation would be that local changes of *p*H trigger indirectly the membrane depolarization by altering the microenvironment and consequently the properties of the macromolecular structures which support the membrane potential.

Several physiological implications of these results can be mentioned: (i) Diffusion barriers are interposed between the external medium and the membrane receptors and might play an important role in the response of cell membranes to their regulatory ligands. (ii) Several sites of action might be involved in the response of a cholinergic membrane to ACh, and the active site of AChE might be one. In this respect the effect of ACh on the M-cells presents several analogies with the response of various conductive membranes to ACh (*8*). (iii) In spite of the rather drastic treatment to which the cells are subjected, they still respond to ACh, and thus some of their constitutive macromolecules are still functional. Studies with alternative treatments and other specific ligands may provide pertinent insight into the molecular mechanisms which control the electrical parameters of these membranes.

T. PODLESKI
J. P. CHANGEUX*
Departments of Biochemistry and *Neurology, College of Physicians and Surgeons, Columbia University, New York 10032*

References and Notes

1. E. Schoffeniel, and D. Nachmansohn, *Biochim. Biophys. Acta* **26**, 1 (1957); H. B. Higman, T. R. Podleski, E. Bartels, *ibid.* **75**, 187 (1964).
2. D. Nachmansohn, *Chemical and Molecular Basis of Nerve Activity* (Academic Press, New York, 1959).
3. Composition of eel's physiological saline solution: 160 mmole of NaCl, 5 mmole of KCl, 0.3 mmole of $NaH_2PO_4 \cdot H_2O$, 1.2 mmole of Na_2HPO_4, 2 mmole of $MgCl_2 \cdot 6H_2O$, 2 mmole of $CaCl_2$, and 10 mmole of glucose per liter, *p*H 6.9.
4. D. Nachmansohn, and M. A. Rothenberg, *J. Biol. Chem.* **158**, 653 (1945).
5. I. B. Wilson, and S. Ginsburg, *Biochim. Biophys. Acta* **18**, 168 (1955).
6. R. Goldman, H. Silman, S. Caplan, O. Kedem, E. Katchalski, *Science* **150**, 758 (1965).
7. H. I. Silman and A. Karlin, in preparation.
8. W.-D. Dettbarn, *Biochim. Biophys. Acta* **32**, 381 (1959); ——— and F. A. Davis, *ibid.* **66**, 397 (1963); C. H. Armett and J. M. Ritchie, *J. Physiol.* **152**, 141 (1960); *ibid.* **155**, 372 (1961); P. Rosenberg and T. R. Podleski, *Biochim. Biophys. Acta* **75**, 104 (1963); W.-D. Dettbarn and E. Bartels, personal communication.
9. We thank Prof. D. Nachmansohn for his interest and encouragement throughout this study. Supported, in part, by grant NSF-GB-4844. and by grant NB-03304.
* On leave from the Pasteur Institute, Paris, France.

26 June 1967

Hemoglobin Gun Hill: Deletion of Five Amino Acid Residues and Impaired Heme-Globin Binding

Abstract. *Hemoglobin Gun Hill, a new variant of adult hemoglobin, was found in a Caucasian and one of his three daughters. The abnormal hemoglobin had only half of the expected number of heme groups. Five amino acid residues appeared to be missing from the β-globin chains. These residues occur in linear sequence in normal β-chains in a region involved in heme-globin binding. A deletion of five amino acids in the β-chains of hemoglobin Gun Hill is postulated. The most likely mechanism for the origin of such a hemoglobin variant would appear to be unequal crossing-over during meiosis.*

The human hemoglobins (Hb) are conjugated proteins which contain four globin chains, each with an attached heme group. There is no evidence to suggest that any of the described abnormal human hemoglobins deviate from this gross structure. With the exception of Hb H (β_4) and Hb Barts (γ_4) the hemoglobins contain two unlike pairs of globin chains. The normal prototypes are Hb A $\alpha_2 \beta_2$, Hb A_2 $\alpha_2 \delta_2$, Hb F $\alpha_2 \gamma_2$.

We now report a newly found hemoglobin, designated hemoglobin Gun Hill (Hb GH), which lacks the normal complement of heme groups. There appears to have been a deletion of five amino acids in a region of each β-globin chain which functions in heme-globin binding.

The propositus was a 41-year-old man of remote German and English ancestry who had had mild jaundice from early adolescence. He had splenomegaly and signs of a compensated hemolytic state. An 8-year-old daughter also had compensated hemolysis, but lacked splenomegaly. Both the father and daughter were heterozygous for the abnormal hemoglobin. Two other daughters did not have the abnormal hemoglobin and showed no signs of hematologic disease.

Starch-gel electrophoresis (*1*) revealed two abnormal components in addition to hemoglobin A in the hemolyzates of the affected individuals (Fig. 1). The major abnormal band migrated in the position of Hb A_2; a minor abnormal component occupied a slightly more anodal position. The two abnormal hemoglobins comprised about one-third of the total hemoglobin, an estimate based upon the heme absorption of the components eluted after electrophoretic separation on starch granules (*2*), corrected for the abnormal ratio of heme to globin in Hb GH.

Our report concerns studies of the major abnormal component, which had been separated from Hb A_2, and the minor component by carboxymethylcellulose chromatography (*1*), followed by electrophoresis on starch granules.

Half of the globin chains in Hb GH lack heme groups. The concentrations of the cyanmethemoglobin derivatives of Hb GH and Hb A were determined at 540 mμ [molar extinction (ε) being 4.37×10^4] and confirmed by measurement of the pyridine hemochromogen (*3*). The protein concentrations of these solutions were calculated from values of protein nitrogen obtained by the Kjeldahl method (*4*). In Hb A, the two methods for the estimation of protein concentration gave comparable results; the value based upon the Kjeldahl nitrogen determination averaged 103 percent (range 92 to 110 percent) of the concentration obtained by the spectrophotometric method. In Hb GH, the Kjeldahl method yielded values twice those obtained when concentration was estimated from the optical absorption of the heme groups, with an average of 205 percent (range 198 to 220 percent).

The absorption spectrum of Hb GH reflected the abnormal ratio of heme to globin. A greater optical absorption in the ultraviolet region relative to the absorption at the visible wavelengths was observed in Hb GH when compared to Hb A. The shapes of both the oxyhemoglobin and the cyanmethemoglobin absorption spectra of Hb GH in the region 350 to 700 mμ were identical to those of Hb A.

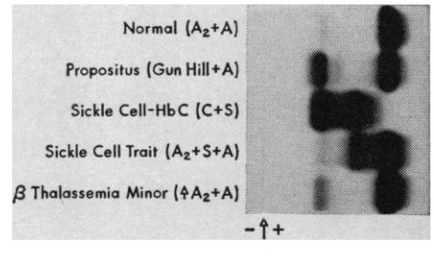

Fig. 1. Starch-gel electrophoresis in tris, boric acid, EDTA buffer at *p*H 8.6, stained with benzidine. Two abnormal components are evident in the hemolyzate of the propositus: a major component in the position of Hb A_2, and a minor component with slightly more anodal migration. Hemoglobin A_2, present in the major abnormal band, could be separated from the Hb GH component by carboxymethylcellulose chromatography.

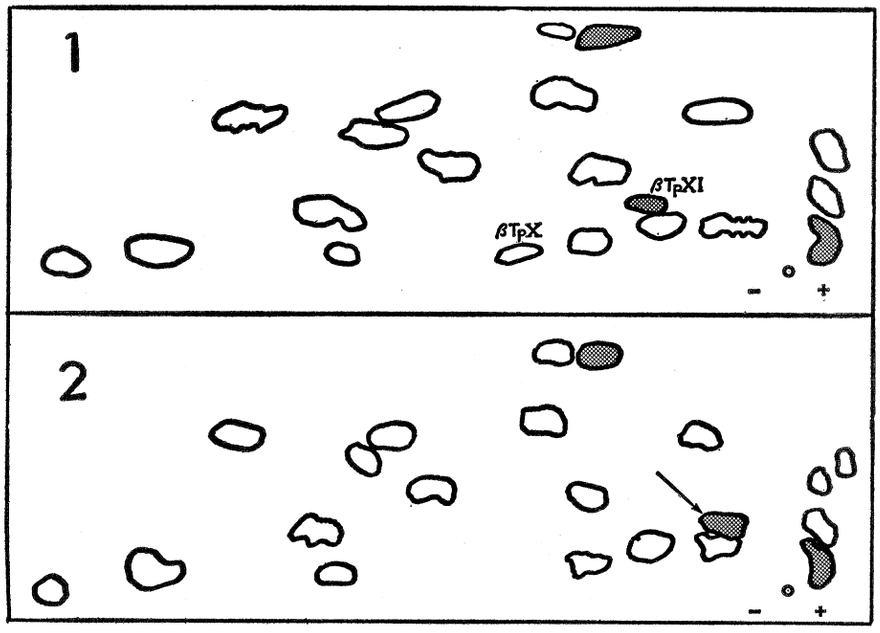

Fig. 2. Tracing of ninhydrin-stained peptide maps, *p*H 4.7, of tryptic digests of aminoethylated β-globin. The cross-hatched peptides showed a positive stain for arginine. On map 1, prepared from hemoglobin A, peptides βTp X and βTp XI are indicated. In hemoglobin Gun Hill (map 2) βTp X and βTp XI were missing, but a new arginine-positive peptide, indicated by an arrow, was present. The arginine-positive peptide in the lower right corner of each map is βTp III; its position in Hb GH is characteristic of a β- rather than a δ-chain peptide.

Studies following hybridization of Hb GH with Hb L, an α-chain abnormality with an electrophoretic migration similar to Hb S, indicated that the abnormality in Hb GH resided in the β-globin chains. An equal mixture of the two hemoglobins (at a concentration of 2 g per 100 ml) was acidified with 0.15 volume of 2*M* acetate buffer *p*H 4.7, then neutralized after 4 hours with K_2HPO_4 (*5*). Electrophoresis on starch granules revealed the presence of four hemoglobin components, with the migration characteristics expected for the original hemoglobins and the new hybrid hemoglobins which would result from an exchange of subunits between $\alpha_2\ \beta_2^{GH}$ and $\alpha_2^{L}\ \beta_2$. The order of their electrophoretic migration from cathode to anode is: (i) $\alpha_2^{L}\ \beta_2^{GH}$, (ii) $\alpha_2\ \beta_2^{GH}$, (iii) $\alpha_2^{L}\ \beta_2$, (iv) $\alpha_2\ \beta_2$. The α- and β-chains of globin prepared from each of these components were separated on starch gel in a urea-barbital buffer (*6*). Components (i) and (iii) had the α-chains of Hb L. Positive identification of the β-chains of Hb GH is not possible on urea gels since they migrate in the position of normal β-globin. (The characteristic electrophoretic migration of Hb GH is attributed to the deficiency of heme groups; the abnormality in the globin chains is not detected electrophoretically.) The presence of the Hb GH β-chains in components (i) and (ii) was supported by the electrophoretic migration of these hemoglobins. The appearance of new hemoglobin species following acid recombination with an α-chain abnormality demonstrated that the β-chains of Hb GH are abnormal (*7*).

The four hemoglobin products of acid recombination were eluted after electrophoretic separation on starch granules. A comparison of the protein content determined by the Kjeldahl method with the heme absorption of the cyanmethemoglobin derivatives at 540 mμ indicated that both the $\alpha_2^{L}\ \beta_2^{GH}$ and $\alpha_2\ \beta_2^{GH}$ components lacked half of the expected number of heme groups, while both the $\alpha_2^{L}\ \beta_2$ and $\alpha_2\ \beta_2$ hemoglobins exhibited the usual ratio of heme-to-globin. Thus the abnormal β-chains of Hb GH lack heme; the only heme groups of Hb GH are those of the α-chains.

Several other lines of evidence showed that the β-chains of Hb GH are abnormal. (i) A split Hb A_2 characteristic of α-chain abnormalities was not detected. (ii) Free α-chains were present in the chromatographically separated abnormal components. The α-chains, eluted from starch blocks, combined normally with Hb H (β_4) to produce a component with the electrophoretic properties of Hb A (*8*). The presence of free α-chains suggests either a reduced rate of synthesis or an increased rate of degradation of abnormal β-chains. (iii) Peptide mapping of tryptic digests of both the α- and β-chains revealed abnormalities only in the β-chain peptides.

Globin was prepared in acid acetone at − 20°C. Separation of the α- and

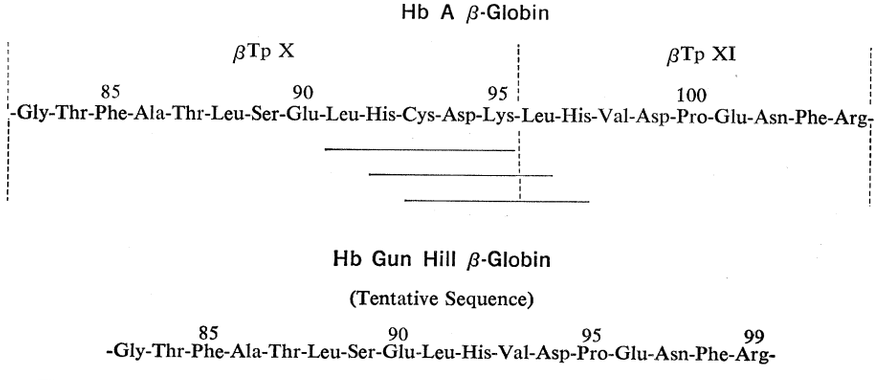

Fig. 3. The amino acid sequences of the normal tryptic peptides βTp X and βTp XI, and of that postulated for the corresponding region in hemoglobin Gun Hill. The numbers indicate the position of the residues from the NH_2-terminal end of the globin chain. The three possible regions of linear deletion are indicated by the horizontal rules.

Table 1. Mole ratios of amino acids in the abnormal peptide of hemoglobin Gun Hill (the nearest whole number is given in parentheses), compared with the expected ratios in the normal βTp X and βTp XI sequence (*20*).

Residue	Expected βTp X + XI	Observed Hb GH	Difference
Ala	1	1.1 (1)	
Arg	1	1.1 (1)	
Asp	3	2.0 (2)	−1
Gly	1	1.0 (1)	
Glu	2	1.8 (2)	
His	2	0.9 (1)	−1
Leu	3	1.9 (2)	−1
Lys	1		−1
Phe	2	1.9 (2)	
Pro	1	1.0 (1)	
Ser	1	1.1 (1)	
Thr	2	1.9 (2)	
Val	1	0.7 (1)	
Cys*	1		−1

*See text.

β-chains, aminoethylation and digestion with trypsin were performed by the methods of Clegg, Naughton, and Weatherall (*9*). Peptide mapping was carried out by electrophoresis at 3000 volts in pyridine-acetate buffers (*p*H 4.7 for 65 minutes; *p*H 6.5 for 90 minutes) followed by ascending chromatography in pyridine, isoamyl alcohol and water (35:35:27) (*10*). Maps (at *p*H 4.7) of the β-chain tryptic peptides of Hb GH revealed three abnormalities: absence of tryptic peptides βTp X and βTp XI, and the presence of an abnormal peptide in the neutral zone (Fig. 2). Discrete separation of the abnormal peptide from adjacent normal peptides was achieved by electrophoresis at *p*H 6.5.

Amino acid composition (Beckman-Spinco Model 120B) was determined on hydrolyzates (6*N* HCl for 21 hours) of the eluted abnormal peptide (*9*). The observed mole ratio of amino acids of the abnormal peptide was compared to that expected for the missing peptides βTp X and βTp XI (Table 1). Four residues appeared to be missing, an aspartyl, a histidyl, a leucyl, and a lysyl (*11*).

The results of amino acid analysis of Hb GH β-globin chains after hydrolysis in 6*N* HCl at 105°C for 22 and 72 hours also suggested the absence of 1 mole of each of the four residues. In addition to these four residues the expected cysteine at postion 93 also appeared to be absent from the β-chains of Hb GH. The β93 cysteines account for the only titratable -SH groups in Hb A (*12*). No reactive -SH groups were detectable in Hb GH during titration with *p*-mercuribenzoate by the method of Boyer (*13*). The cysteic acid content of whole β-globin was determined by amino acid analysis after reaction with performic acid (*14*). The β-chains of hemoglobin Gun Hill had one cysteic acid residue compared to two observed in Hb A.

Five amino acid residues appear to be missing from the region of βTp X and βTp XI in Hb GH. Since these residues occur in sequence in the normal β-chain, a linear deletion could account for their absence (Fig. 3). Three possibilities for linear deletion exist, but because of the repetition of the leucyl-histidyl pair (residues 91 and 92; 96 and 97) the resultant new amino acid sequence would be the same with each of the possibilities, that is, the 92nd residue would be histidyl as it is in the normal β-chain, the 93rd residue would be the valyl that normally occupies the 98th position.

Examination of the published models of hemoglobin and myoglobin (*15*) indicates that the postulated deletion involves the final residue of the F-helix and the first four residues of the FG corner (cysteine F9, aspartic acid FG1, lysine FG2, leucine FG3, histidine FG4). The deletion would require formation of a peptide bond between the histidine and valine which normally occupy positions F8 and FG5.

The involved portion of the β-chain is important in heme-globin binding (*15*). The imidazole nitrogen of the histidine at F8 is linked to a coordination site of the heme iron. According to Perutz, the lysine FG2 may form a salt bridge with a propionic acid side chain of heme, and leucine FG3 makes van der Waals contact with one of the CH groups of the main ring of heme. The possible alteration in the position of histidine F8, and the absence of the FG2 and FG3 residues probably prevent the β-chain of Hb GH from forming a stable bond with heme.

The loss of five amino acids represents a major alteration in the primary structure of β-globin. The effect upon the tertiary structure cannot be determined from available evidence. However, the F8 and FG5 residues appear to be in close proximity in the three-dimensional models, so that loss of the intervening amino acids may not result in a major distortion of the tertiary structure of the globin chain. Although the defective β-chains are apparently incapable of binding heme groups and result in other alterations of structure and function (*16*), their conformation is sufficiently intact to permit combination with normal α-chains to produce Hb GH.

The occurrence of unequal crossing-over during meiosis has been postulated for the origin of the Hp-2 genetic locus (*17*), for Hb Lepore (*18*) and recently for the deletion of a valine in the β-chain of Hb Freiburg (*19*). The Hb GH β-chain may have arisen by unequal crossing-over between homologous β-chain genetic loci. The possibility that Hb GH arose as the result of a crossover between the linked δ and β genetic loci (as in Hb Lepore) seems excluded by the peptide maps since the position of βTp III is characteristic of a β- rather than a δ-chain peptide.

THOMAS B. BRADLEY, JR.
ROBERT C. WOHL
Department of Medicine, Albert Einstein College of Medicine, Bronx, New York 10461
RONALD F. RIEDER
Department of Hematology, Division of Medicine, Montefiore Hospital and Medical Center, Bronx, New York

References and Notes

1. A. I. Chernoff, N. Pettit, J. Northrop, *Blood* **25**, 646 (1965).
2. H. G. Kunkel, R. Ceppelini, U. Muller-Eberhard, J. Wolf, *J. Clin. Invest.* **36**, 1615 (1957).
3. L. P. Vernon and M. D. Kamen, *J. Biol. Chem.* **211**, 643 (1954).
4. E. A. Kabat and M. M. Mayer, *Experimental Immunochemistry* (Thomas, Springfield, Ill., ed. 2, 1961), pp. 476–483.
5. H. M. Ranney, *Ann. N.Y. Acad. Sci.* **119**, 496 (1964).
6. A. I. Chernoff and N. M. Pettit, Jr., *Blood* **24**, 750 (1964).
7. H. A. Itano and E. A. Robinson, *Nature* **183**, 1799 (1959).
8. E. R. Huehns and E. M. Shooter, *ibid.* **193**, 1083 (1962).
9. J. B. Clegg, M. A. Naughton, D. J. Weatherall, *J. Mol. Biol.* **19**, 91 (1966).
10. C. Baglioni, *Biochim. Biophys. Acta* **48**, 392 (1961); E. R. Huehns, E. M. Shooter, N. Dance, G. H. Beaven, K. V. Shooter, *Nature* **192**, 1057 (1961).
11. Abbreviations for the amino acid residues are Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartic acid; Gly, glycine; Glu, glutamic acid; His, histidine; Leu, leucine; Lys, lysine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Val, valine; Cys, cysteine.
12. J. Goldstein, G. Guidotti, W. Konigsberg, R. J. Hill, *J. Biol. Chem.* **236**, PC77 (1961).
13. P. D. Boyer, *J. Amer. Chem. Soc.* **76**, 4331 (1954).
14. S. Moore, *J. Biol. Chem.* **238**, 235 (1963).
15. M. F. Perutz, *J. Mol. Biol.* **13**, 646 (1965).
16. T. B. Bradley, Jr., and R. F Rieder, *Blood* **28**, 975 (1966) (Abstr.).
17. O. Smithies, G. E. Connell, G. H. Dixon, *Nature* **196**, 232 (1962).
18. C. Baglioni, *Proc. Nat. Acad. Sci. U.S.* **48**, 1880 (1962).
19. R. T. Jones, B. Brimhall, T. H. J. Huisman, E. Kleihauer, K. Betke, *Science* **154**, 1024 (1966).
20. R. T. Jones, *Cold Spring Harbor Symp. Quant. Biol.* **29**, 297 (1964).
21. We thank Dr. H. M. Ranney for interest, suggestions, and the hemoglobin L; and Drs R. L. Nagel, P. M. Gallop, and O. O. Blumenfeld for valuable discussion. Supported by PHS grants HE 09014, AM 09798, and 5 MO 1 FR-50. T.B.B. is a career scientist, Health Research Council of the City of New York (I-354).

16 February 1967