Hemoglobin Philly (β35 Tyrosine→Phenylalanine): Studies in the Molecular Pathology of Hemoglobin

RONALD F. RIEDER, FRANK A. OSKI, and J. B. CLEGG

From the Department of Medicine, State University of New York, Downstate Medical Center, Brooklyn, New York 11203; Department of Pediatrics, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104; Nuffield Unit of Medical Genetics, Department of Medicine, University of Liverpool, Liverpool, Great Britain

ABSTRACT An abnormal unstable hemoglobin, hemoglobin Philly, was found in three members of a family, each of whom had evidence of a chronic hemolytic state. The presence of the mutant protein was suggested by the rapid appearance of inclusion bodies upon incubation of erythrocytes with brilliant cresyl blue and by the increased heat precipitability of the hemoglobin. However, no abnormal hemoglobin could be demonstrated by electrophoresis or column chromatography. Sulfhydryl titration of the hemolysates with *p*-mercuribenzoate indicated that there was an average of four reactive sulfhydryl groups per hemoglobin molecule instead of the usual two. The total number of hemoglobin sulfhydryl groups was normal; six groups were measured when denatured globin was reacted with 5,5′-dithiobis[2-nitrobenzoic acid]. This indicated that the increased sulfhydryl reactivity was due to an increased availability to *p*-mercuribenzoate of the usually unreactive hemoglobin cysteines at β112 and α104. After treatment for ½ hr with 4–5 moles of *p*-mercuribenzoate per mole of hemoglobin, electrophoresis revealed that 30–35% of the hemoglobin had been dissociated into α- and β-chains. Normal hemolysates revealed negligible splitting after 72 hr of similar treatment. The α- and β-chains of hemoglobin Philly were separated from the unsplit hemoglobin A by carboxymethyl cellulose chromatography. Fingerprint and amino acid analyses revealed that tyrosine β35 was replaced by phenylalanine. In hemoglobin Philly there is loss of the normal hydrogen bond between the tyrosine hydroxyl group and the carboxyl group of aspartic acid α126 at the $\alpha_1\beta_1$ contact. This shifts the equilibrium from hemoglobin tetramers toward monomers, exposing the β112 and α104 cysteines. In the cell, precipitation of the unstable monomers may contribute to erythrocyte destruction.

Received for publication 24 January 1969.

INTRODUCTION

The severity of the clinical manifestations of the different abnormal hemoglobins is varied. Many of these mutant proteins produce no discernible effect on the patient, while some cause a mild compensated hemolysis, and others are associated with severe hemolytic disease (1). The detection of an abnormal hemoglobin in many patients presenting with clinical states within this spectrum has frequently been straightforward. Since the demonstration of hemoglobin S (2), the widespread use of electrophoresis has resulted in the discovery of over 100 mutant forms of human hemoglobin (3).

Recently it has become evident that a proportion of patients with hereditary nonspherocytic hemolytic disease of the Heinz body type possess an abnormal unstable hemoglobin (4). In several instances the electrophoretic or chromatographic separation of a variant protein from hemoglobin A has been difficult or only partially successful (3). Where complete chemical analyses have been performed, several of the mutations have been shown to consist of amino acid substitutions involving no change in charge or occurring in the interior of the hemoglobin molecule, so that surface charge and electrophoretic mobility are unaffected (3).

The present report describes a hemoglobinopathy which was discovered in three members of a family, each of whom had evidence of a chronic hemolytic state. The demonstration of a mutant hemoglobin was hampered by the inability to separate a variant protein from hemoglobin A by any of the usual methods of electrophoresis or chromatography. Proof of the presence of hemoglobin Philly, and its isolation and purification, depended upon a unique property of this new abnormal hemoglobin.

The properties of hemoglobin Philly have provided an explanation for the pathophysiologic state of the affected

patients. The structural defect of the abnormal hemoglobin has provided information on the importance of one interchain bond in the maintenance of the structural integrity of the normal hemoglobin molecule.

METHODS

Handling of blood samples. Routine hematologic examinations by standard methods (5) and enzyme assays were performed on heparinized freshly drawn venous blood specimens. Other studies were done on samples anticoagulated and mailed packed in ice. Transit time was approximately 1 day.

Preparation of hemolysates. Erythrocytes were washed three to five times with 0.9% NaCl. For studies of hemoglobin, hemolysis was effected by shaking the cells with an equal volume of distilled water and ½ volume of carbon tetrachloride or by stirring with 4 volumes of distilled water followed by the addition of adequate 4 N NaCl to restore the sodium concentration to 0.15 N. Stroma was removed by centrifugation.

Erythrocyte enzyme assays. Hemolysates were prepared by diluting the washed red cell suspension 1:10 in distilled water. Hexokinase, phosphoglucose isomerase, phosphofructokinase, aldolase, triose phosphate isomerase, phosphoglycerate mutase, enolase, and lactic dehydrogenase were assayed by previously described methods (6) with triethanolamine buffer, pH 8.0, at a final concentration of 0.1 moles/liter. Phosphoglycerate kinase was assayed as described by Loder and De Gruchy (7), glyceraldehyde-3-phosphate dehydrogenase as described by Schrier (8), and glucose-6-phosphate dehydrogenase by the method of Zinkham and Lenhard (9). Pyruvate kinase was measured by the procedure of Rose and Warms (10) with phosphoenolpyruvate in a final concentration of 2.0 mmoles/liter and adenosine diphosphate (ADP) at 1.5 mmoles/liter. Glutathione reductase was assayed according to the method of Horn (11).

Effect of redox dye on erythrocytes and hemoglobin. One part of a 1% solution of brilliant cresyl blue, in 0.9% sodium chloride was added to two parts of heparinized blood. This mixture was incubated at 37°C, and blood films were prepared at intervals over the next 24 hr.

Other studies. Methemoglobin was measured by the method of Evelyn and Malloy (12) on fresh hemolysates and after 24 hr of incubation at 37°C. The heat stability of hemoglobin was tested, with and without the addition of NaCN, according to the procedure of Grimes, Meisler, and Dacie (13). The technique of Betke, Marti, and Schlicht was used to assay alkali-resistant hemoglobin (14). The ferrohemoglobin solubility test was performed according to Itano (15).

Ultracentrifuge studies. Sedimentation analyses were made with a Spinco model E ultracentrifuge at 52,000 rpm and 4°C. Measurements were made on Schlieren photographs obtained using a No. 29 Wratten filter and 1 N spectroscopic plates.

Electrophoresis. Standard methods were employed for hemoglobin electrophoresis on starch gel (16), agar gel (17), and starch block (18). The method of Chernoff and Pettit was utilized for the electrophoresis of globin polypeptide chains on starch gel in urea-containing buffers (19).

Column chromatography. Column chromatography of hemoglobin solutions on carboxymethyl cellulose (CMC) and on Amberlite CG-50 was performed according to previously described methods (20, 21).

Sulfhydryl determinations. The reactive sulfhydryl groups of hemoglobin were measured spectrophotometrically by titration with *p-mercuribenzoate* (PMB) according to the method of Boyer (22) as modified by Benesch and Benesch (23). Total sulfhydryl groups in denatured globin preparations were measured by the method of Ellman (24) using 5,5′-dithiobis[2-nitrobenzoic acid] (DTNB), as described by Guidotti (25).

Preparation of α- and β-chains of hemoglobin. The heme-bearing PMB derivatives of the chains of hemoglobin A were prepared by reacting the protein with a twelvefold molar excess of PMB at pH 6.2, according to the method of Bucci and Fronticelli (26) as modified by Rosemeyer and Huehns (27). Isolation of the α-PMB and β-PMB chains was then achieved by CMC chromatography or starch-block electrophoresis.

Fingerprint analysis. Heme was removed from the PMB derivatives of hemoglobin α- and β-chains by the acid-acetone method (28). PMB was removed from the globin by dissolving the lyophilized protein in nitrogen-saturated 8 M urea containing 0.3 M 2-mercaptoethanol and allowing the mixture to stand at room temperature for 3 hr under nitrogen. The protein was subsequently separated from the low molecular weight materials by passage through a Sephadex G25 column equilibrated with 0.5% formic acid. Regeneration of sulfhydryl groups was checked using DTNB. Aminoethylation and trypsin digestion were carried out as previously described (29). High voltage paper electrophoresis of the tryptic digests was done with pyridine–acetic acid–water buffers at pH 4.7 and 6.4. The chromatography step of the two-dimensional fingerprints was performed using solvent systems composed of pyridine–isoamyl alcohol–water, 35:35:37, or butanol–acetic acid–pyridine-water, 15:3:10:12. Automatic amino acid analyses were done on material eluted from the fingerprints and hydrolysed in 6 N HCl containing 1 mg of phenol per ml in order to prevent loss of tyrosine.

Materials. Sigma Chemical Co., St. Louis, Mo., provided PMB sodium salt, cystamine (2,2′-dithiobis[ethylamine dihydrochloride], DTNB, and iodoacetamide. Iodoacetamide-1-^{14}C, 0.05 mc in 4.2 mg, was obtained from New England Nuclear Corp., Boston, Mass.

RESULTS

Clinical data and case report. The propositus (II-1) (Table I), a 9 yr old girl of Italian and German-French ancestry, was originally evaluated at 8 yr of age because of the finding of persistant reticulocytosis and splenomegaly. There is no history of jaundice or dark urine. At 6 yr of age the child was treated for several months with iron because of anemia, but no data about this episode are available. During the present investigation the patient has had intermittent splenomegaly, the spleen edge being palpable at ½–4 cm below the left costal margin. Hepatomegaly has not been observed. There is no family history of anemia, jaundice, or dark urine. No other member of the immediate family has splenomegaly.

Table I shows the clinical hematologic data of the patient and her family. The propositus, the father (I-1), and a brother (II-2) have elevated reticulocyte counts. Blood samples obtained from all three persons showed the formation of multiple intraerythrocytic inclusion bodies when incubated with the redox dye, brilliant cresyl blue

TABLE I
Clinical Hematologic Data

Subjects	Hemoglobin	Reticulocytes	Methemoglobin		Hemoglobin heat stability		Autohemolysis		BCB preparation
			Uninc-ubated*	Incu-bated*	Control‡	NaCN‡	Control§	Glucose§	RBC inclu-sions
	g/100 ml	%	%		*% precipitated*		%		
I-1	14.6	2.2–3.6			36.4	3.9			+
I-2	11.0	0.7							0
II-1	12.6–13.6	5.1–8.1	3.0	15.4	26.3	8.5	38.4	2.3	+
II-2	14.0	2.9							+
II-3	11.0	1.1							0

BCB, brilliant cresyl blue.
* Normal control 1.5% before incubation and 1.2% after incubation.
‡ Normal control 6.4% precipitated, with NaCN 5.9% precipitated.
§ Normal control less than 1.2% hemolysis.

(BCB). Precipitates were visible in the cells at 1 hr and were prominent by 2 hr. Inclusions were present in 99% of the cells. The mother (I-2) and a sister (II-3) have normal reticulocyte counts, and their red cells did not form inclusion bodies upon exposure to BCB. Red cell morphology in all family members is normal. Upon incubation, at 37°C for 24 hr, the blood of the patient II-1 showed increased formation of methemoglobin (Table I). Autohemolysis was increased and was corrected by the addition of glucose to a concentration of 30 mmoles/liter. As shown in Table I, hemolysates from both the father and the propositus exhibited increased hemoglobin instability when incubated at 50°C. However, 12–24 hr of heating was necessary to cause hemoglobin precipitation, and this was prevented by the previous addition of NaCN.

The red blood cell survival of patient II-1 was measured by use of ^{51}Cr. The erythrocyte half-life was reduced to 12 days. The osmotic fragility of the patient's red cells was normal. Upon incubation at 37°C for 24 hr the osmotic fragility increased abnormally.

Determinations of hemoglobin F (1.1%), hemoglobin A_2 (3.7%), and the ferrohemoglobin solubility (4.7 g/liter) gave normal results.

Erythrocyte-enzymes and intermediary metabolism. In an attempt to determine the cause of the increased hemolysis, a variety of red cell enzymes and metabolic intermediates was measured in the blood of patient II-1. The following determinations gave normal values: red cell glutathione, glutathione stability, glutathione peroxidase, triphosphopyridine nucleotide (TPNH) and diphosphopyridine nucleotide (DPNH) glutathione reductases, catalase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, hexokinase, glucose-phosphate isomerase, phosphofructokinase, aldolase, glyceraldehyde phosphate dehydrogenase, triose phosphate isomerase, phosphoglycerate kinase, phosphoglycerate mutase, enolase, pyruvate kinase, lactic dehydrogenase, adenosine triphosphate (ATP), ATPase, acetylcholinesterase, erythrocyte glucose consumption, and lactate production. The hexose monophosphate shunt pathway, as reflected in $^{14}CO_2$ production, was normal and responded normally to methylene blue stimulation.

Hemoglobin electrophoresis. Because of the production of intraerythrocytic inclusion bodies upon BCB treatment, and because of the demonstration of decreased heat stability of the hemoglobin in the presence of a normal erythrocyte enzyme complement, an attempt was made to demonstrate the existence of an abnormal hemoglobin. Conventional vertical starch-gel electrophoresis at pH 8.6 and pH 7.0 of hemolysates obtained from the propositus and her family revealed no abnormal hemoglobin band. When the gel was overloaded with hemoglobin from patient II-1, a benzidine-positive band was noted in the position of free α-chains (Fig. 1), suggesting the presence, in the hemolysate, of an unstable β-chain mutant (30). On agar-gel electrophoresis at pH 6.0, a minor abnormal band was noted in a position anodal to hemoglobin A (Fig. 2). This appeared to be precipitated, denatured hemoglobin rather than a true electrophoretic band. No further migration of this band occurred when the current was passed at right angles to the original direction of flow. However, another streak of denatured hemoglobin was subsequently noted to remain behind the then sideways-moving hemoglobin A, a finding suggesting continuing denaturation of an unstable component having a true mobility similar to hemoglobin A. Starch-gel electrophoresis in 8 M urea, of globin prepared from the propositus' hemolysate, did not

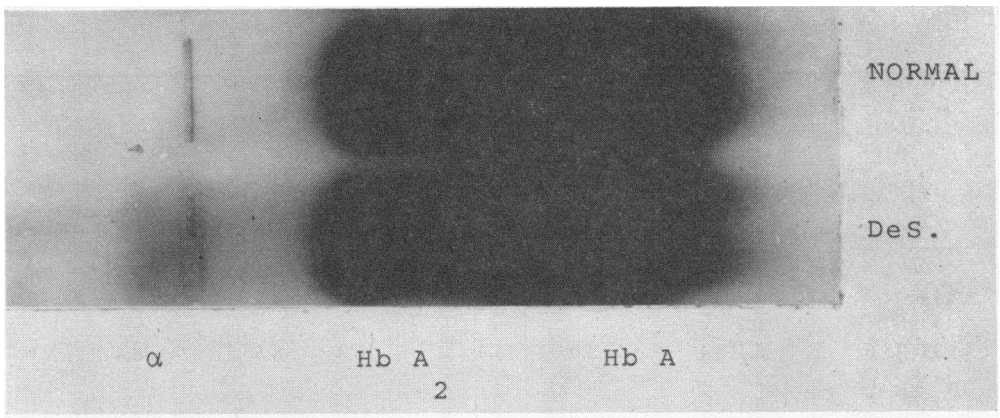

FIGURE 1 Hemoglobin electrophoresis on starch gel at pH 8.6. The gel was overloaded and stained with benzidine to show the presence of free α-chains in the hemolysate from patient II-1 (lower sample). The anode is to the right of the figure.

reveal an abnormally migrating α- or β-globin component.

Column chromatography. Because of suggestive evidence of the presence of an unstable hemoglobin with an electrophoretic mobility identical with hemoglobin A, an attempt was made to demonstrate an abnormal hemoglobin by column chromatography. No new band was present when hemolysates from patient II-1 and subject II-2 were chromatographed on CMC, Amberlite CG-50 in developers 2,4, and 6, or on Sephadex G200.

Absorption spectra. Complete visible and ultraviolet absorption spectra were obtained on deoxy- and carbonmonoxyhemoglobin solutions obtained from the propositus, with a Cary model 14 recording spectrophotometer. These spectra were identical with a normal control and the OD 280: OD 540 ratio was normal, a fact indicating the presence of the usual heme: globin ratio.

Titration of reactive sulfhydryl groups in hemoglobin. Because of a recent suggestion (31) that a blockade of the sulfhydryl (— SH) groups of globin plays a role in the precipitation of unstable hemoglobins and in the induction of hemolysis, the reactive sulfhydryl groups were measured in hemolysates obtained from the propositus, the brother, and the father. Hemoglobin A has three pairs of cysteine residues. Each α-chain has one cysteine at position 104, while the β-chains have residues at positions 93 and 112 (32). The β93 cysteines are situated at the surface of the hemoglobin tetramer, while the β112 and α104 cysteines are in the interior of the molecule at the $\alpha_1\beta_1$ contact (33). Ordinarily, under mild conditions, only the cysteines at β93 in the native hemoglobin molecule are available for reaction with sulfhydryl reagents (34).

The addition of PMB to hemoglobin solutions results in the attachment of this reagent to the reactive — SH groups on the protein. When PMB reacts with sulfhydryl residues there is a specific increase in the optical absorption at 250 mμ (22). Stepwise addition of PMB results in a continued increase in optical density until the free cysteinyl residues in the mixture are exhausted. Knowing the quantity of PMB added, one can calculate the number of —SH groups that have reacted (22, 23). This technique was used to compare the number of reactive sulfhydryl residues in the hemolysates from patients II-1 and I-1 with a normal hemolysate. A hemoglobin A solution with sulfhydryl groups blocked by iodoacetamide was used as the blank cuvette solution.

In the normal hemolysate 2 mmoles of PMB reacted with each millimole of hemoglobin, a finding indicating that there was an average of two reactive cysteinyl residues for each hemoglobin molecule (Fig. 3). When hemolysates from the propositus and the father were tested, the optical density increased until 4 mmoles of PMB had been added per mmole of hemoglobin. This result indicated that on the average there were four reactive cysteinyl groups per hemoglobin molecule. A similar result was obtained when the hemoglobin of subject II-2 was tested.

Determination of total sulfhydryl residues. In order to decide whether the above results indicated the presence of additional globin cysteine residues or simply mirrored an increased reactivity of those residues normally present at β112 and α104, the total number of — SH groups in the hemolysate was measured. Globin was prepared from the hemolysate of subject II-1 and from a normal hemolysate. The protein was dissolved in sodium phosphate buffer at pH 7.15 containing 11% sodium dodecyl sulfate (SDS). SDS denatures the polypeptide chains making all cysteinyl residues reactive. The total sulfhydryl groups were determined by reaction with DTNB and measurement of the optical density at 415 mμ (24, 25). Both hemolysates gave results close

to the expected six sulfhydryl groups per tetramer. The value obtained for normal globin was 6.8; globin from the propositus gave a value of 6.3. These results suggested that the additional reactive sulfhydryl groups found in the hemolysate from I-1, II-1, and II-2 were derived from the normally unreactive cysteine residues at β112 and α104. The mutation in the abnormal hemoglobin did not appear to involve a new cysteine insertion.

Electrophoretic demonstration of hemoglobin Philly. The finding of additional reactive sulfhydryl groups in the hemolysates from affected family members strongly indicated the presence of an abnormal hemoglobin and suggested a method of separating the mutant protein (hemoglobin Philly) from hemoglobin A. Cystamine combines with the two reactive cysteines of hemoglobin to place one additional positive charge on each β-chain (35). This increased positive charge retards the migration of hemoglobin A towards the anode during electrophoresis at pH 8.6. It was postulated that hemoglobin Philly might react with more than two cystamine molecules and thus be retarded in its migration even more than hemoglobin A, thereby affording a separation of the two proteins. However, the electrophoretic migrations of the hemoglobin in a normal hemolysate and the hemoglobin from patient II-1 were equally decreased after exposure to excess cystamine, and no separation of hemoglobin Philly from hemoglobin A occurred. These results suggested that the additional reactive sulfhydryl groups in hemoglobin Philly, while available to PMB, were not available to cystamine. An attempt was next made to use the extra negative charges placed on hemo-

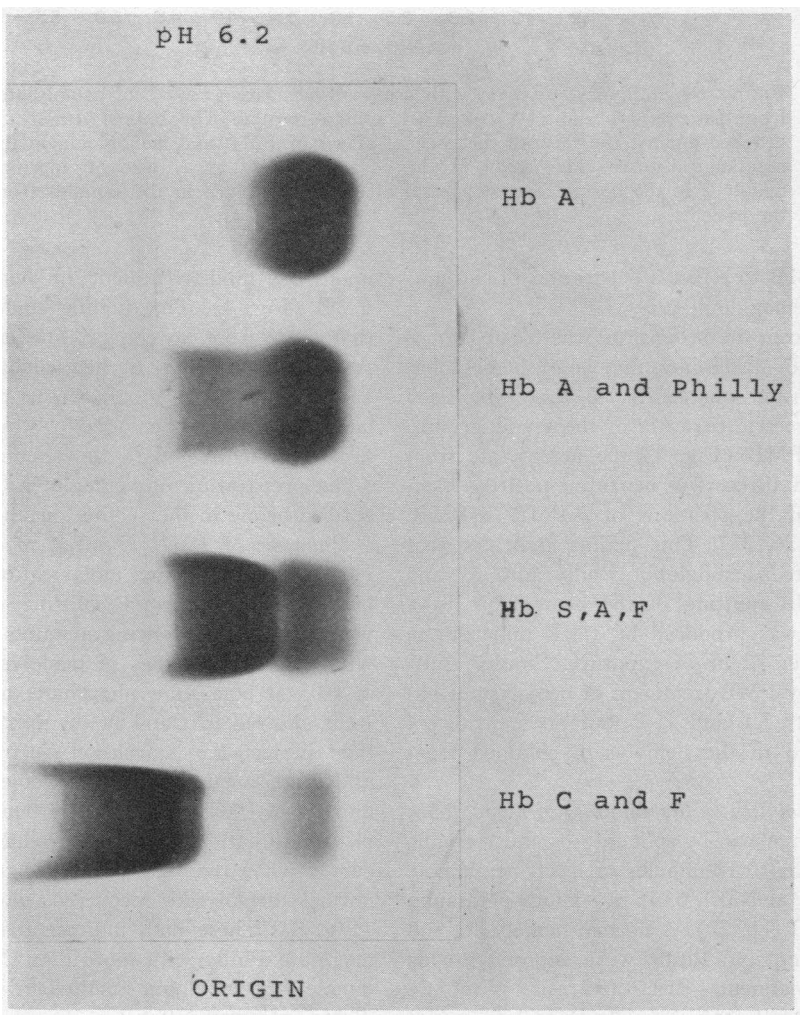

FIGURE 2 Agar-gel electrophoresis at pH 6.2. A band of denatured hemoglobin remains behind the main hemoglobin band in the hemolysate from patient II-1 (second from the top). Bromthymol blue stain. The anode is to the left of the figure.

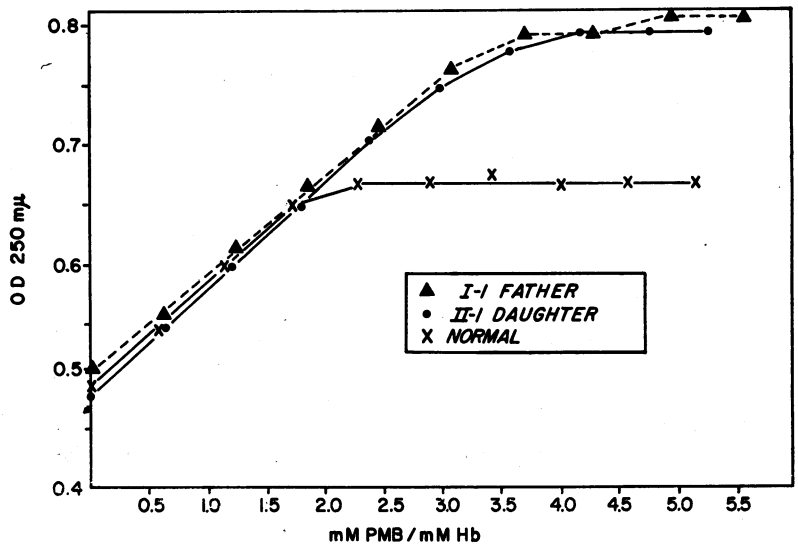

FIGURE 3 Sulfhydryl titration with *p*-mercuribenzoate (PMB) of hemolysates from patients I-1 and II-1 compared with a normal. The optical density is graphed against the ratio of the concentrations of PMB and hemoglobin in the reaction mixtures. The break in the curve occurred at a ratio of approximately 2 in the normal and at a ratio of approximately 4 in the samples from the patients.

globin Philly by PMB to effect a separation of the mutant protein from hemoglobin A.

The hemolysate from the propositus was treated for 2 hr with 4 mmoles of PMB per mmole of hemoglobin in 0.05 M potassium phosphate, pH 7.0, the buffer used in the spectrophotometric titration of the —SH groups. After exposure to PMB (Fig. 4) the hemolysate from patient II-1 showed three new benzidine-positive electrophoretic bands in the positions of β-PMB, α-PMB, and free α-chains (26, 27). This finding indicated that PMB had dissociated hemoglobin Philly into α- and β-chain subunits. No splitting of the hemoglobin in a normal hemolysate was produced by PMB under these conditions, even after 72 hr of exposure. Similar splitting was produced by PMB treatment of hemolysates obtained from subjects I-1 and II-2, but no splitting of hemoglobin occurred in the hemolysate obtained from subject I-2 (Fig. 5).

Electrophoretic mobility of the subunits of hemoglobin Philly. Hemoglobin A can be split into α- and β-chains by exposure for 12 hr to 12 mmoles of PMB per mmole of hemoglobin in 0.2 M NaCl, 0.075 M sodium–potassium phosphate, at pH 6.0 (26, 27). A normal hemolysate and one containing hemoglobin Philly were exposed to this more strenuous treatment. Electrophoresis (Fig. 6) showed that both hemolysates were resolved into monomers, and that the migrations of corresponding subunits were identical. The mobilities of the separated chains were the same as those of the subunits formed upon the mild treatment of hemoglobin Philly with PMB (Fig. 4). This finding confirmed the observation that there were no charge differences between the corresponding subunits of hemoglobin A and hemoglobin Philly. In addition, globin chains prepared from the isolated subunits of hemoglobin Philly had mobilities on urea gel identical with the subunits of hemoglobin A. (The preparation of hemoglobin Philly subunits is described below in the section on fingerprint analysis.)

Quantity of PMB required to dissociate hemoglobin Philly. The minimum molar ratio of PMB to hemoglobin required to obtain splitting of hemoglobin Philly was determined. Varying amounts of PMB were mixed with constant volumes of hemolysate from patient II-1 in 0.05 M potassium phosphate, pH 7.0. The mixtures were allowed to stand in the refrigerator for 1 hr and then subjected to starch-gel electrophoresis. There was a definite appearance of monomers at the ratio of 3 mmoles of PMB per mmole of hemoglobin (Fig. 7). By visual estimation maximum production of monomers occurred when the ratio reached 4 to 5. No splitting of a normal hemolysate occurred under these conditions (Fig. 8). These data indicate that the titration of hemoglobin Philly with more than 2 mmoles of PMB per mmole of hemoglobin resulted in splitting of the hemoglobin into monomers.

Ultracentrifuge studies. Sedimentation velocity experiments were done on the hemolysate from patient II-1. The hemoglobin concentration was varied from 2

to 12 mg/ml in 0.01 M potassium phosphate buffer, pH 7.0. A single, monodisperse boundary was observed with an $s^\circ_{20,w}$ value of 4.55S. This is the expected value for the hemoglobin tetramer. Thus evidence for the presence of a significant amount of free monomers in the untreated hemolysate could not be established by this technique.

Reaction of hemoglobin Philly with iodoacetamide. The observation that cystamine was unable to combine with all the PMB-reactive — SH groups of hemoglobin Philly prompted an investigation of the reactivity of the abnormal hemoglobin to another sulfhydryl reagent, iodoacetamide. When hemoglobin A is treated with a molar excess of iodoacetamide the alkylating reagent attaches only to the cysteines at β93 (34). All the reactive cysteines are blocked, and subsequent combination with PMB is prevented. This was not the case with the abnormal hemolysate. The PMB titration of a hemoglobin solution from patient II-1, previously treated with 10 mmoles of iodoacetamide per mmole of hemoglobin, indicated that approximately 2 mmoles of free —SH group remained to react with PMB after iodoacetamide treatment (Fig. 9).

In another experiment aliquots of an iodoacetamide-treated hemolysate containing hemoglobin Philly were exposed to varying amounts of PMB and the reaction mixtures subjected to starch-gel electrophoresis. Fig. 10 shows that the addition of PMB to iodoacetamide-treated hemoglobin Philly resulted in enhanced dissociation of the abnormal hemoglobin into α and β subunits. The appearance of monomers was apparent at a PMB: hemoglobin ratio of only 0.5 and appeared to reach a maximum at a ratio of 2. Iodoacetamide alone did not produce the appearance of dissociated subunits. No splitting occurred when iodoacetamide was added after the hemolysate had been treated with 2 mmoles of PMB per mmole of hemoglobin.

The hemolysate from patient II-1 was reacted with a 10 mM excess of iodoacetamide to which had been added a tracer amount of iodoacetamide-1-^{14}C. After standing 5 hr at room temperature the mixture was dialysed to remove unreacted reagent. PMB, 4 mmoles per

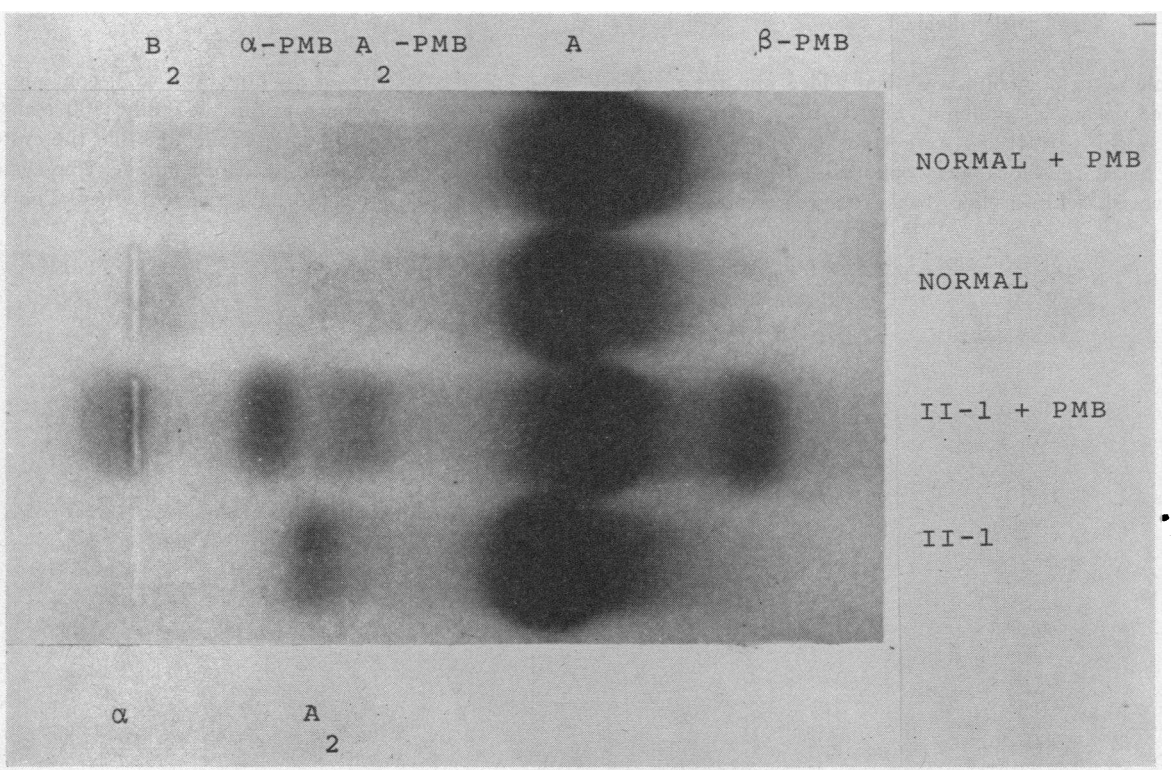

FIGURE 4 Starch-gel electrophoresis at pH 8.6 of hemolysates treated for 2 hr with *p*-mercuribenzoate, 4 mmoles/mmole of hemoglobin, in 0.05 M potassium phosphate at pH 7.0. The treated sample from patient II-1 has bands in the positions of β-PMB, α-PMB, and free α-chains. There was a slight increase in the mobility of hemoglobin A and A_2 upon addition of the negatively charged PMB. B_2 refers to a mutant hemoglobin A_2 in the normal sample. This accounts for the decreased intensity of staining of the minor hemoglobin band in the control specimen. Benzidine stain.

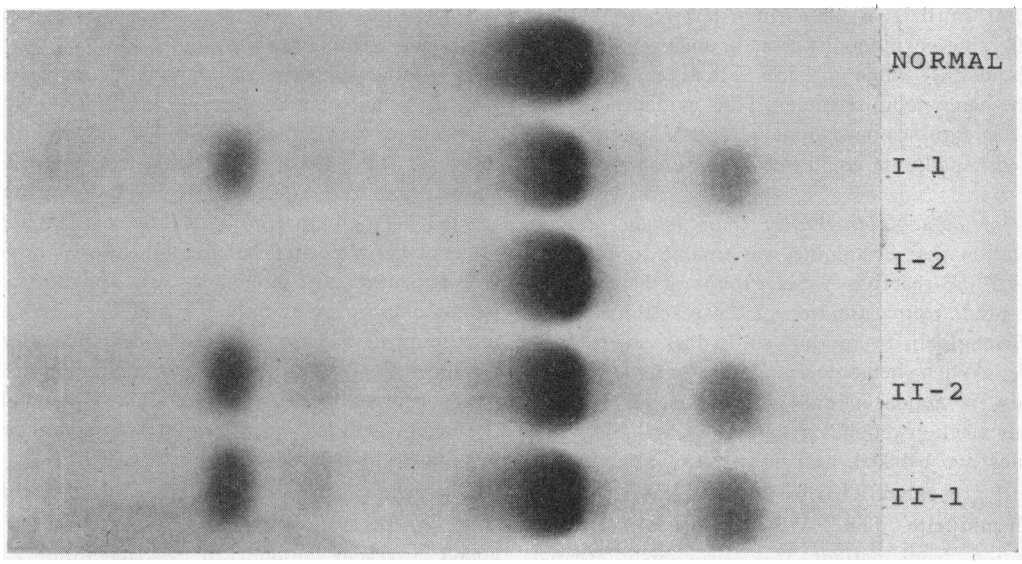

FIGURE 5 Starch-gel electrophoresis of PMB-treated hemolysates obtained from members of the propositus' family. The conditions were the same as in Fig. 4. The specimens from the propositus, II-1, the father, I-1, and the brother, II-2, showed the presence of α and β subunits. No dissociation was present in the samples from the mother, I-2, and from an unrelated normal individual.

mmole of hemoglobin, was then added and the mixture placed on a CMC column. The β-Philly, hemoglobin A, and α-Philly fractions were collected and further purified by starch-block electrophoresis. Radioactivity was measured with a low background gas-flow counter. The specific activity of β-Philly was 4270 cpm/mg, hemoglobin A, 2450 cpm/mg, and α-Philly, 180 cpm/mg. The absence of α-chain counts indicated that the cysteine at α104 was not available to iodoacetamide. The cysteine at β112 did not appear to have reacted either. The ratio

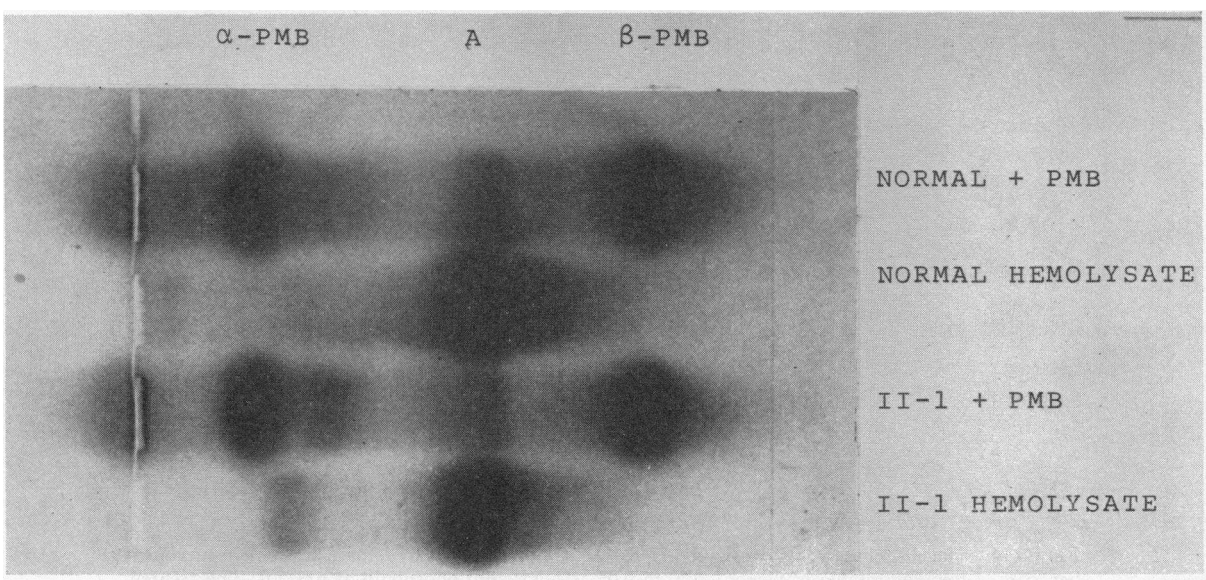

FIGURE 6 Starch-gel electrophoresis of PMB-treated hemoglobin. The hemolysates from a normal person and patient II-1 were almost completely dissociated into subunits by exposure for 12 hr to 12 mmoles of PMB per mmole of hemoglobin, in 0.2 N NaCl, 0.075 M potassium–sodium phosphate, pH 6.0. The electrophoretic mobilities of the α- and β-chains of the patient's hemoglobin were the same as the corresponding subunits from the normal individual.

of the specific activity of β-Philly to the specific activity of hemoglobin A was 2:1. A ratio of 4:1 would have been expected if both β-Philly —SH groups had reacted with the radioactive iodoacetamide. These results are consistent with a limitation, in hemoglobin Philly as in hemoglobin A, of the attachment of iodoacetamide to only the surface —SH groups at β93.

Determination of the percentage of hemoglobin Philly in hemolysates. Hemolysates containing hemoglobin Philly were treated with various amounts of PMB for ½–24 hr at 4°C in the titration buffer. The reaction products were separated using starch-block electrophoresis or a CMC column equilibrated with 0.01 M sodium phosphate, pH 6.2, and employing a gradient formed by mixing equal volumes of pH 6.7 and pH 8.4 0.01 M sodium phosphate (Fig. 11). The percentage of hemoglobin split by PMB varied but was most often in the range of 30–35% (Table II). The time of exposure to PMB did not appear to greatly affect the proportion of the hemoglobin dissociated into monomers. The hemoglobin A peak from the CMC chromatography of the treated hemolysate from patient II-1 was treated again with varying amounts of PMB. No further splitting was noted when the mixtures were subjected to starch-gel electrophoresis. This finding indicated that the phenomenon of easy dissociation by PMB was restricted to a definite fraction of the hemoglobin in the hemolysate.

Fingerprint analysis of hemoglobin Phily. The α- and β-chains of hemoglobin Philly were prepared free of hemoglobin A. Hemolysates from patients I-1 and II-1 were treated with 5 mmoles of PMB per mmole of hemoglobin and allowed to react for 1 hr. This split he-

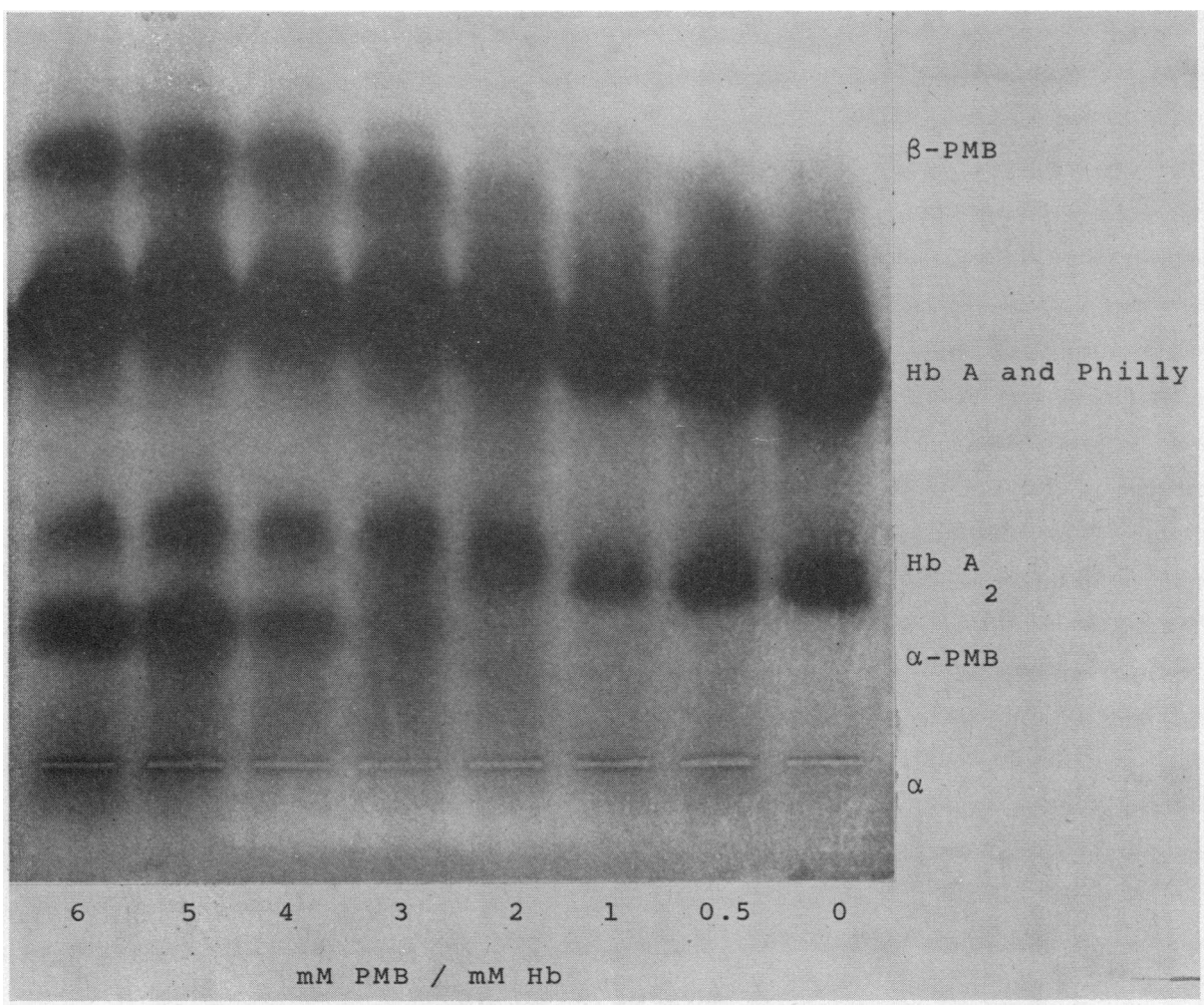

FIGURE 7 Treatment of patient's (II-1) hemolysate at pH 7.0 with varying amounts of PMB. There was evidence of subunit dissociation at concentration ratios of PMB to hemoglobin greater than 2. A slight increase in the mobilities of Hb A and A_2 is also noted. This is probably due to the negative charge of PMB.

moglobin Philly but left hemoglobin A intact. The mixtures were then chromatographed on CMC and the β-PMB, α-PMB, and free α-chain fractions collected. On CMC the unsplit hemoglobin A is eluted between the β-PMB and α-PMB fractions (Fig. 11). Heme was removed by the acid-acetone method (28) and the globin lyophilized. The —SH groups were regenerated by treatment of the protein with 2-mercaptoethanol in 8 M urea. Completeness of removal of PMB was ascertained by sulfhydryl titration using the Ellman reagent (24, 25). The free chains were then treated with ethylenimine and subjected to trypsin digestion (29). Fingerprint analysis of the α-chain of hemoglobin Philly gave patterns identical with that of α-A. The fingerprint of β-Philly showed that peptide trypsin IV (TpIV) had a slightly faster chromatographic mobility than β-A TpIV (Fig. 12). In addition, the ninhydrin stain of the β-Philly peptide was much weaker than that for the corresponding peptide in β-A. Specific stains for tryptophan and arginine were positive on the β-Philly TpIV spot, a finding indicating that these amino acids were present. The addition of urea to the tryptic digest before fingerprinting increased the yield of β-Philly TpIV, as judged by the increased intensity of the ninhydrin and arginine stains. This result suggested that the low yield was due to adsorption of the abnormal peptide to other peptides, perhaps to those in the neutral band. The abnormal spot was cut from a fingerprint stained with 0.02% ninhydrin, eluted, and hydrolysed with 6 N HCl containing phenol. The addition of phenol prevents the loss of any tyro-

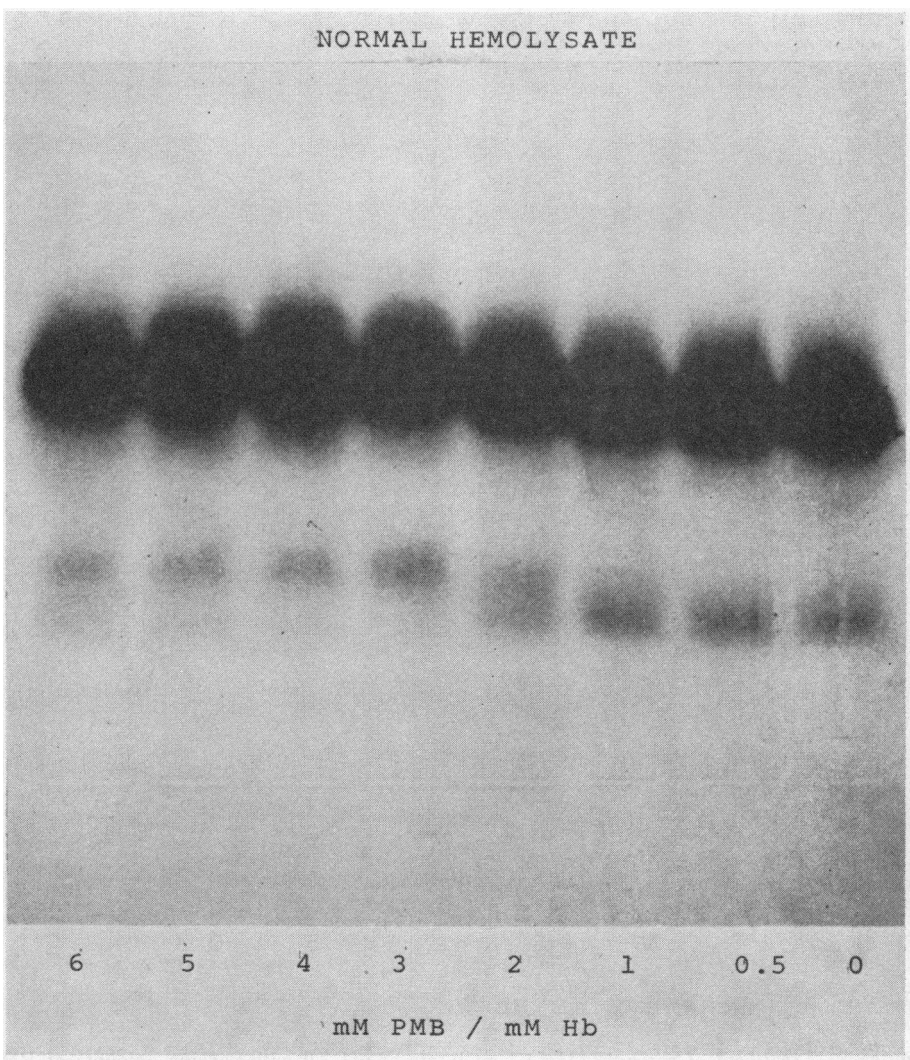

FIGURE 8 Treatment of a normal hemolysate at pH 7.0 with varying amounts of PMB. No subunit dissociation occurred. PMB imparts a slight mobility change to Hb A and Hb A_2.

sine present, and this finding was verified by analysis of β-TpIV prepared from hemoglobin A. The amino acid analysis of β-Philly TpIV revealed that the tyrosine residue was missing, and a phenylalanine residue was present (Table III). The tyrosine in hemoglobin A is at position 35 of the β-chain, counting from the amino terminal end, and occupies position number one in the C helix. (32, 33). Hemoglobin Philly can therefore be designated as β35 (C1) tyrosine → phenylalanine.

DISCUSSION

A number of patients have now been found with congenital nonspherocytic hemolytic anemia due to unstable mutant hemoglobins (4). These hemoglobins characteristically cause hemolysis even when present in the heterozygous state. Large numbers of Heinz bodies have appeared in the peripheral blood of patients with unstable hemoglobins who have undergone splenectomy (13, 36). In nonsplenectomized patients, inclusion bodies have been readily produced, in vitro, by exposure of erythrocytes to redox dyes (4, 37). Precipitation of hemoglobin upon heating to 50°C has been a characteristic of some of these mutant proteins (4, 13, 30).

All the amino acid substitutions found in the unstable hemoglobins have involved residues in the interior of the molecule (3). Substitutions causing increased ease of denaturation have been observed at positions in contact with the heme groups, at certain general internal positions, and at some of the contacts between subunits.

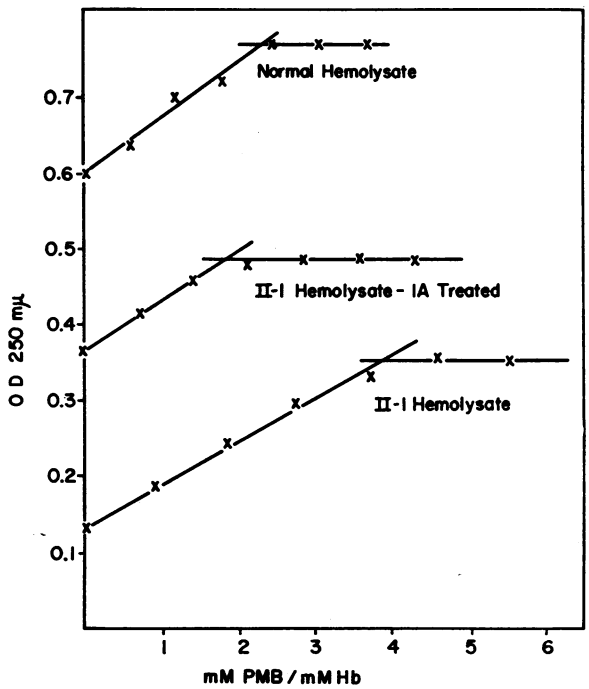

FIGURE 9 Sulfhydryl titration with PMB of an iodoacetamide-treated hemolysate from patient II-1 compared with an untreated hemolysate from patient II-1 and an untreated normal hemolysate. An iodoacetamide-blocked normal hemolysate was the solution in the blank cuvette. Iodoacetamide did not completely block the sulfhydryl groups in the abnormal hemoglobin; two titratable groups remained.

TABLE II

Estimation of Amount of Hemoglobin Philly in Hemolysates

Subject	Amount of PMB added	Reaction time	Method of separation of products	Amount of each fraction found			
				Hb β-PMB	Hb A	Hb α-PMB	Hb α
	millimoles PMB / millimoles Hb	*Hr*		%			
II-1	5	½	starch block	23.0	64.0*	13.0‡	
II-1	10	½	starch block	29.7	52.6*	17.7‡	
I-1	5	1	starch block	13.6	69.6	6.9§	9.9
I-1	5	1	CMC chromatography	16.4	64.9	10.1§	8.6
II-1	5	1	starch block	13.0	76.3*	10.7‡	
II-1	5	4	starch block	14.1	73.5*	12.4‡	
II-1	5	8	starch block	13.6	74.3*	12.1‡	
II-1	5	24	starch block	14.4	71.8*	13.8‡	
II-1	6	1	starch block	16.2	68.5*	15.3‡	
II-1	10	1	starch block	16.6	65.9*	17.5‡	

PMB, *p*-mercuribenzoate; CMC, carboxymethyl cellulose.
* $A+A_2$.
‡ α-PMB + α.
§ A_2 + α-PMB.

For the most part, the positions involved are occupied by nonpolar residues.

As in the present case, a number of these hemoglobin variants have not been separated easily from hemoglobin A by electrophoresis (38, 39). All attempts to separate intact hemoglobin Philly from hemoglobin A have been unsuccessful. The increased tendency of hemoglobin Philly to dissociate in the presence of PMB allowed electrophoretic and chromatographic purification of the subunits of this unstable hemoglobin.

Initially the addition of PMB to hemoglobin A results in the attachment of the reagent to the $\beta 93$ cysteines at the surface of the hemoglobin molecule and is associated with a reduction in the molecular weight of the protein. The hemoglobin tetramer dissociates at the $\alpha_1\beta_2$

TABLE III

Mole Ratios of Amino Acids Found in β-Philly Tp IV *compared with Values Expected in β-A Tp* IV

Amino acid	β-Philly/Tp IV	β-A/Tp IV	Difference
Arg	0.9 (1)	1	
Glu	1.2 (1)	1	
Leu	1.7 (2)	2	
Phe	0.9 (1)		+1
Pro	0.8 (1)	1	
Thr	1.0 (1)	1	
Try*	(1)	1	
Tyr		1	−1
Val	1.7 (2)	2	

* By staining reaction on paper.

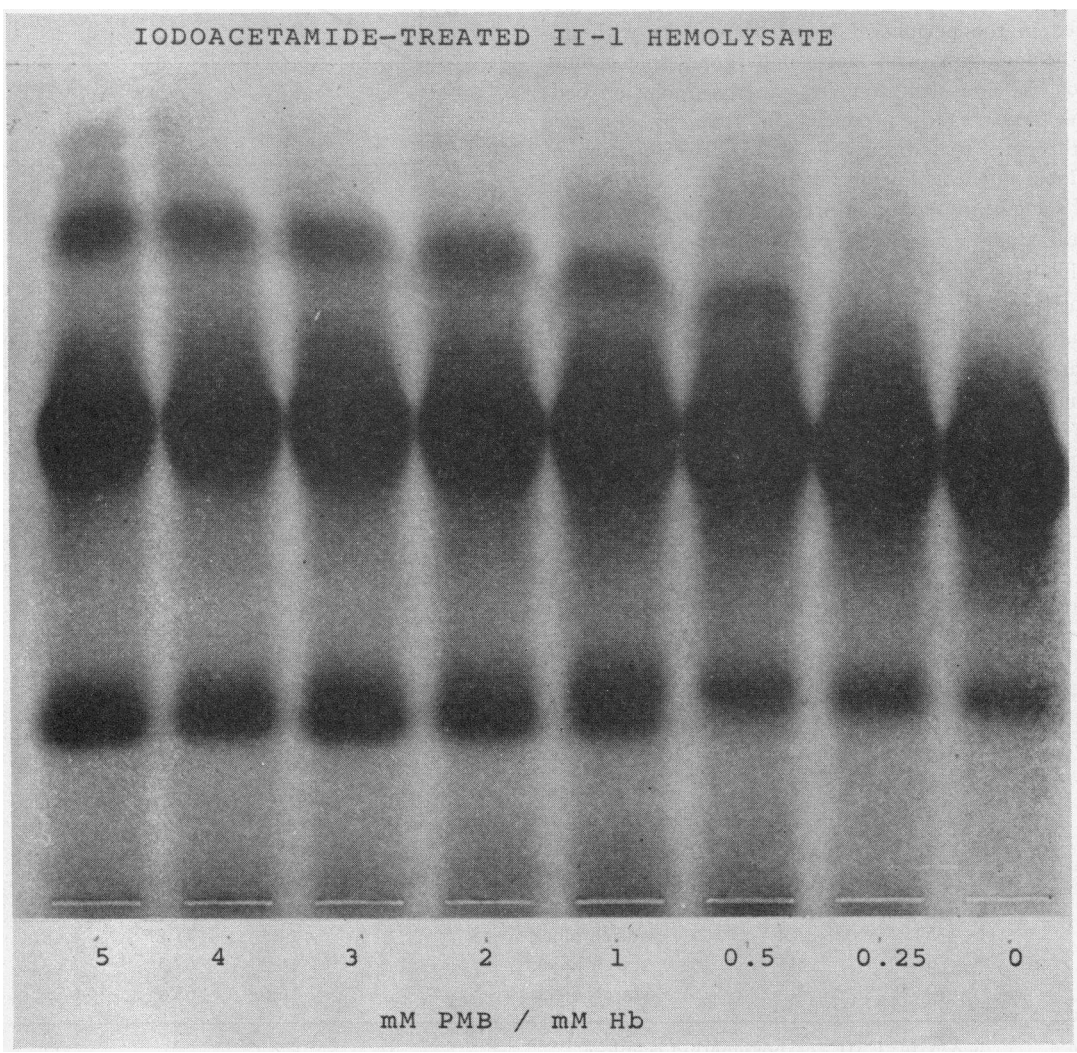

FIGURE 10 Starch-gel electrophoresis of patient's iodoacetamide-reacted hemolysate after treatment with varying amounts of PMB. The enhanced subunit dissociation began at a concentration ratio of PMB to hemoglobin greater than 0.25.

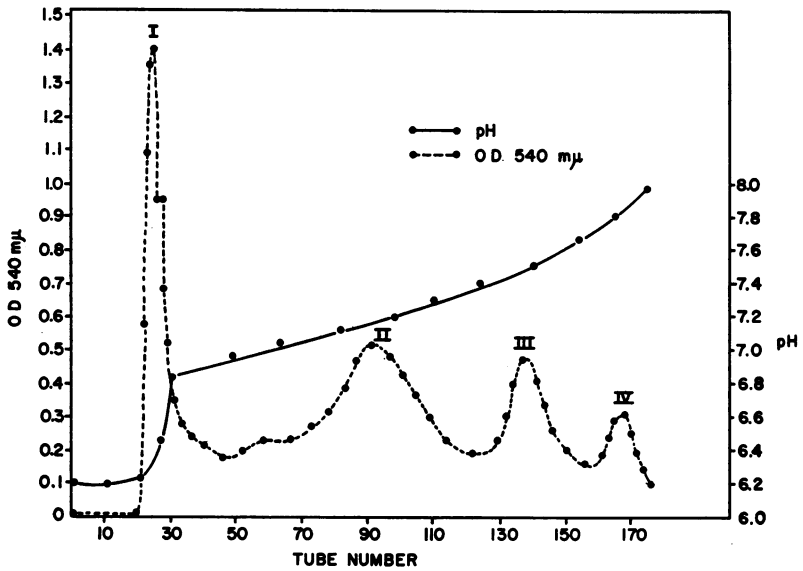

FIGURE 11 Carboxymethyl cellulose column chromatography of a PMB-treated hemolysate from patient I-1. Peak 1 consisted of Philly β-PMB, peak II was hemoglobin A, peak III was predominantly hemoglobin A_2, and α-PMB, and peak IV was made up of free α-chains. The components of the various peaks were verified by starch-gel electrophoresis, fingerprint analyses, and sulfhydryl titration.

contact into $\alpha_1\beta_1$ dimers (27). The sulfhydryls at β112 and α104 remain unavailable to PMB because they lie buried in the $\alpha_1\beta_1$ contact (3, 27, 33). Under conditions of low pH and higher ionic strength, which increase the tendency of dimers to dissociate into monomers, the α104 and β112 cysteines become available for substitution with PMB. This results in complete irreversible dissociation of the tetramer into subunits (27). The present report indicates that a similar mechanism was responsible for the PMB dissociation of hemoglobin Philly. The chief difference would appear to be the mildness of the conditions under which the variant hemoglobin was dissociated.

The initial two equivalents of PMB probably attached to the surface β93 cysteines of hemoglobin Philly, as dissociation into α and β subunits was not detected on starch-gel electrophoresis. Further addition of PMB then resulted in the production of monomers. The spectrophotometric titration of the abnormal hemolysates indicated that approximately 4 mmoles of cysteine reacted per mmole of hemoglobin. Using a figure of 3.6 cysteines reacted per molecule of total hemoglobin in the hemolysate, if 60% of the protein consisted of hemoglobin A, and only two residues of cysteine reacted on each hemoglobin A molecule, it can be calculated that each molecule of hemoglobin Philly had all six sulfhydryls titrated.

After treatment with iodoacetamide the splitting of hemoglobin Philly was achieved upon the addition of less than 2 mmoles of PMB per mmole of hemoglobin. Assuming a 60:40 ratio of hemoglobin A to hemoglobin Philly, if iodoacetamide blocked the β93 cysteines on all the hemoglobin molecules and rendered hemoglobin A unreactive to PMB, it can be calculated that a subsequent titration value of 1.6 PMB residues reflected an attack on all four remaining cysteines in the hemoglobin Philly fraction. This titration was associated with the appearance of α and β monomers on starch-gel electrophoresis. Thus after iodoacetamide treatment, the α104 and β112 cysteines remained reactive to PMB. The results of the experiments using radioactive iodoacetamide and unlabeled PMB support this interpretation. Almost no radioactivity was found on the dissociated α subunits. The β-Philly fraction had a specific activity only twice that of hemoglobin A, a finding suggesting that in hemoglobin Philly as in hemoglobin A, the attachment of the alkylating agent occurred at β93 but not β112.

The unavailability of the α104 and β112 cysteines to iodoacetamide was further indicated by the inability of an excess of the alkylating agent alone to split hemoglobin Philly even after the β93 cysteines had reacted with PMB.

The increased availability of the α104 and β112 cysteines of hemoglobin Philly to PMB can be explained by the amino acid substitution in the mutant protein. The normal dissociation of hemoglobin is thought to occur at the $\alpha_1\beta_2$ contact with formation of $\alpha_1\beta_1$ dimers (27). The equilibrium of the dimer-monomer conversion is strongly

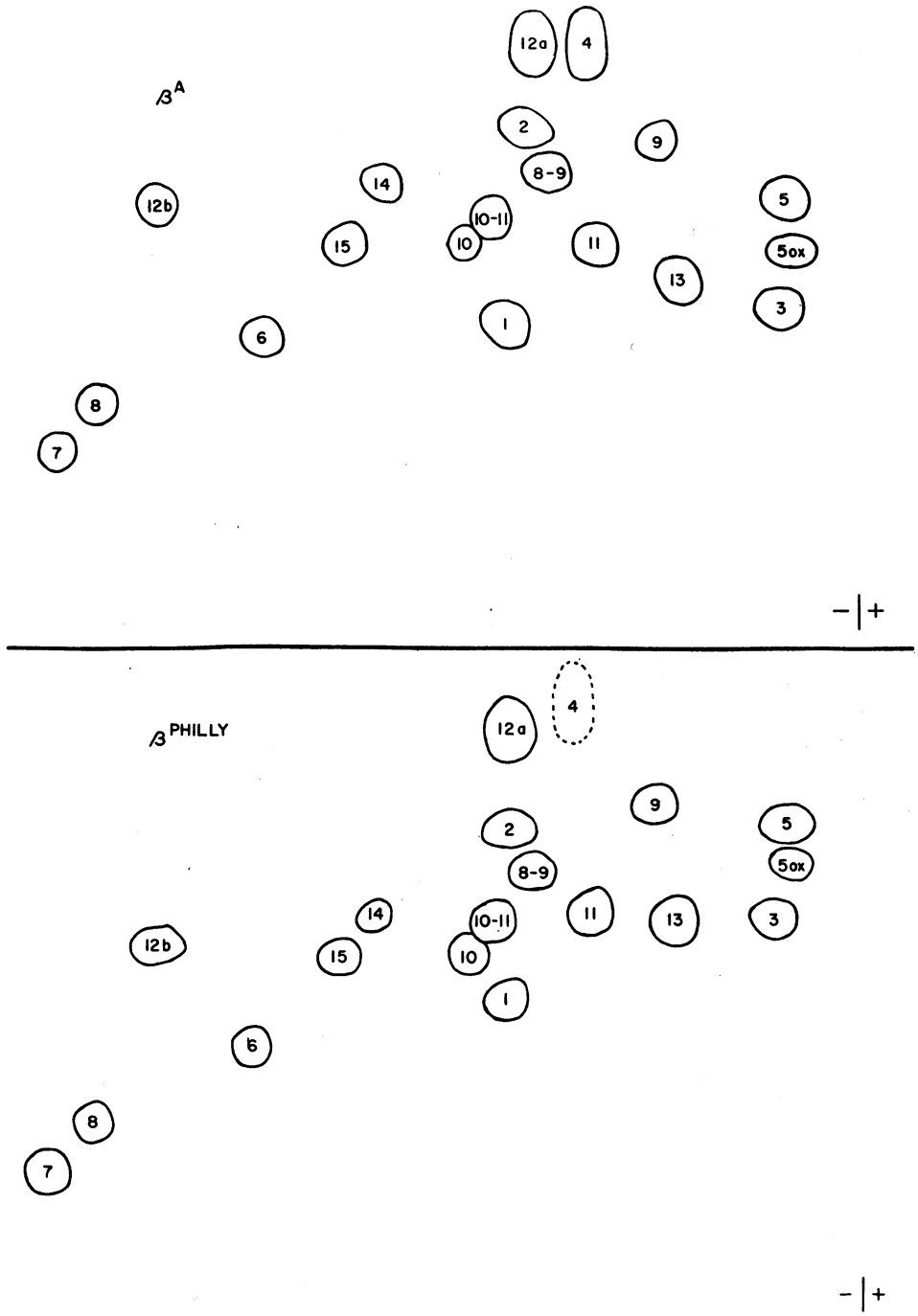

FIGURE 12 Tracings of ninhydrin-stained fingerprints of β-A globin and β-Philly globin. Peptide β-Philly TpIV was invariably very faint and had a slightly faster chromatographic mobility than β-A TpIV. The sample was applied as a thin vertical line (indicated) on a strip of filter paper, and electrophoresis was performed (horizontal direction) at pH 4.7 in pyridine–acetic acid–water buffer. The strip was then sewn to a sheet of filter paper followed by descending chromatography in butanol–acetic acid–water-pyridine (vertical direction).

in favor of dimers. In hemoglobin Philly there is a replacement of tyrosine by phenylalanine at position 35 of the β-chain. Normally this tyrosine is situated at the $\alpha_1\beta_1$ contact, and its hydroxyl group forms a hydrogen bond with the carboxyl group of an aspartic acid residue in the α-chain at position 126 (33). The side chain of phenylalanine cannot form this hydrogen bond. This substitution probably weakens the $\alpha_1\beta_1$ contact and shifts the hemoglobin tetramer-dimer-monomer equilibrium towards formation of monomers.[1] This effect would lead to exposure of the internal sulfhydryls, making them available to PMB. As phenylalanine is an almost perfect isomorphous replacement of tyrosine, it is unlikely that any conformational changes are involved.[1]

There is no explanation at present for the inability of excess iodoacetamide or cystamine to attach to the α104 and β112 cysteines and cause monomer formation.

The shifting of the equilibrium towards monomers is also probably responsible for the decreased life-span of the patient's erythrocytes. Free α- and β-chains are much less stable than hemoglobin A and tend to precipitate out of solution, and this presumably leads to formation of intraerythrocytic inclusions, or Heinz bodies (40). Sequestration by the spleen of red cells with inclusions then occurs (41). In addition, the spleen may remove inclusions from erythrocytes with subsequent circulation of the "pitted" cells (42). If some loss of membrane resulted from this splenic activity these cells would be shorter lived. Attachment of Heinz body inclusions to the red cell membrane by means of mixed disulfide bonds has also been postulated (31), resulting in damage to the cell membrane and ensuing hemolysis (43).

ACKNOWLEDGMENTS

We thank Dr. Robert Kaye for referring the propositus for study.

This work was supported by U. S. Public Health Service Grants AM-12401 and HD-01919 and also received financial support from the World Health Organization. Some of the clinical studies were performed on the Clinical Research Center of the University of Pennsylvania supported by Grant 3MO1FR4006. Dr. Oski is a recipient of U. S. Public Health Service Career Development Award 1-KO3-HD38600.

REFERENCES

1. Heller, P. 1966. Hemoglobinopathic dysfunction of the red cell. *Amer. J. Med.* **41:** 799.
2. Pauling, L., H. A. Itano, S. J. Singer, and I. C. Wells. 1949. Sickle cell anemia, a molecular disease. *Science (Washington).* **110:** 543.
3. Perutz, M. F., and H. Lehmann. 1968. Molecular pathology of human hemoglobin. *Nature (London).* **219:** 902.
4. Rieder, R. F., and T. B. Bradley, Jr. 1968. Hemoglobin Gun Hill: an unstable protein associated with chronic hemolysis. *Blood.* **32:** 355.
5. Cartwright, G. E. 1968. Diagnostic Laboratory Hematology. Grune & Stratton, Inc., New York. 4th edition.
6. Chapman, R. G., M. A. Hennessey, A. M. Waltersdorph, F. M. Huennekens, and B. W. Gabrio. 1962. Erythrocyte metabolism. V. Levels of glycolytic enzymes and regulation of glycolysis. *J. Clin. Invest.* **41:** 1249.
7. Loder, P. B., and G. C. de Gruchy. 1965. Red-cell enzymes and co-enzymes in non-spherocytic congenital haemolytic anaemias. *Brit. J. Haematol.* **11:** 21.
8. Schrier, S. L. 1966. Organization of enzymes in human erythrocyte membranes. *Amer. J. Physiol.* **210:** 139.
9. Zinkham, W. H., and R. E. Lenhard, Jr. 1959. Metabolic abnormalities of erythrocytes from patients with congenital nonspherocytic hemolytic anemia. *J. Pediat.* **55:** 319.
10. Rose, I. A., and J. V. B. Warms. 1966. Control of glycolysis in the human red blood cell. *J. Biol. Chem.* **241:** 4848.
11. Horn, H. D. 1963. Glutathion reductase. *In* Methods of Enzymatic Analysis. M. V. Bergmeyer, editor. Academic Press Inc., New York. 875.
12. Evelyn, K. A., and H. T. Malloy. 1938. Microdetermination of oxyhemoglobin, methemoglobin, and sulfhemoglobin in a single sample of blood. *J. Biol. Chem.* **126:** 655.
13. Grimes, A. J., A. Meisler, and J. V. Dacie. 1964. Congenital Heinz-body anaemia. Further evidence on the cause of Heinz-body production in red cells. *Brit. J. Haematol.* **10:** 281.
14. Betke, K., H. R. Marti, and I. Schlicht. 1959. Estimation of small percentages of fetal hemoglobin. *Nature (London).* **184:** 1877.
15. Itano, H. A. 1953. Solubilities of naturally occurring mixtures of human hemoglobin. *Arch. Biochem. Biophys.* **47:** 148.
16. Smithies, O. 1965. Characterization of genetic variants of blood proteins. *Vox Sang.* **10:** 359.
17. Marder, V. J., and C. L. Conley. 1959. Electrophoresis of hemoglobin on agar gels. Frequency of hemoglobin D in a Negro population. *Johns Hopkins Med. J.* **105:** 77.
18. Kunkel, H. G., R. Ceppellini, V. Müller-Eberhard, and J. Wolf. 1957. Observations on the minor basic hemoglobin component in the blood of normal individuals and patients with thalassemia. *J. Clin. Invest.* **36:** 1615.
19. Chernoff, A. I., and N. M. Pettit, Jr. 1965. The amino acid composition of hemoglobin. III. A qualitative method for identifying abnormalities of the polypeptide chains of hemoglobin. *Blood.* **24:** 750.
20. Chernoff, A. I., N. Pettit, and J. Northrop. 1965. The amino acid composition of hemoglobin. V. The preparation of purified hemoglobin fractions by chromatography on cellulose exchangers and their identification by starch gel electrophoresis using tris-borate-EDTA buffer. *Blood.* **25:** 646.
21. Allen, D. W., W. A. Schroeder, and J. Balog. 1958. Observations on the chromatographic heterogeneity of adult and fetal human hemoglobin: a study of the effects of crystallization and chromatography on the heterogeneity and isoleucine content. *J. Amer. Chem. Soc.* **80:** 1628.
22. Boyer, P. D. 1954. Spectrophotometric study of the reaction of protein sulfhydryl groups with organic mercurials. *J. Amer. Chem. Soc.* **76:** 4331.
23. Benesch, R., and R. E. Benesch. 1962. Determination of -SH groups in proteins. *Methods Biochem. Anal.* **10:** 43.

[1] Perutz, M. F. Personal communication.

24. Ellman, G. L. 1959. Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* **82:** 70.
25. Guidotti, G. 1967. Studies on the chemistry of hemoglobin. 1. The reactive sulfhydryl groups. *J. Biol. Chem.* **242:** 3673.
26. Bucci, E., and C. Fronticelli. 1965. A new method for the preparation of α and β subunits of human hemoglobin. *J. Biol. Chem.* **240:** PC551.
27. Rosemeyer, M. A., and E. R. Huehns. 1967. On the mechanism of the dissociation of haemoglobin. *J. Mol. Biol.* **25:** 253.
28. Rossi-Fanelli, A., E. Antonini, and A. Caputo. 1958. Pure native globin from human haemoglobin: preparation and some physico-chemical properties. *Biochem. Biophys. Acta.* **28:** 221.
29. Clegg, J. B., M. A. Naughton, and D. J. Weatherall. 1966. Abnormal human hemoglobins. Separation and characterization of the α and β chains by chromatography, and the determination of two new variants, Hb Chesapeake and Hb J (Bangkok). *J. Mol. Biol.* **19:** 91.
30. Huehns, E. R., and E. M. Shooter. 1965. Human haemoglobins. *J. Med. Genet.* **2:** 48.
31. Jacob, H. S., M. C. Brain, J. V. Dacie, R. W. Carrell, and H. Lehmann. 1968. Abnormal haem binding and globin SH group blockade in unstable haemoglobins. *Nature (London).* **218:** 1214.
32. Braunitzer, G., K. Hilse, V. Rudloff, and N. Hilschmann. 1964. The hemoglobins. *Advan. Protein Chem.* **19:** 1.
33. Perutz, M. F., H. Muirhead, J. M. Cox, and L. C. G. Goaman. 1968.. Three-dimensional Fourier synthesis of horse oxyhaemoglobin at 2.8 Å resolution: the atomic model. *Nature (London).* **219:** 131.
34. Guidotti, G., and W. Konigsburg. 1964. The characterization of modified human hemoglobin. I. Reaction with iodoacetamide and N-ethylmaleimide. *J. Biol. Chem.* **239:** 1474.
35. Smithies, O. 1965. Disulfide-bond cleavage and formation in proteins. *Science (Washington).* **150:** 1595.
36. Hutchison, H. E., P. H. Pinkerton, P. Waters, A. S. Douglas, H. Lehmann, and D. Beale. 1964. Hereditary Heinz-body anaemia, thrombocytopenia, and haemoglobinopathy (Hb. Köln) in a Glasgow family. *Brit. Med. J.* **2:** 1099.
37. Rieder, R. F., W. H. Zinkham, and N. A. Holtzman. 1965. Hemoglobin Zürich. Clinical, chemical and kinetic studies. *Amer. J. Med.* **39:** 4.
38. Dacie, J. V., N. K. Shinton, P. J. Gaffney, Jr., R. W. Carrell, and H. Lehmann. 1967. Haemoglobin Hammersmith (β42(CD1) Phe $\rightarrow$ Ser). *Nature (London).* **216:** 663.
39. Beretta, A., V., Prato, E. Gallo, and H. Lehmann. 1968. Haemoglobin Torino — α43(CD1) Phenylalanine $\rightarrow$ Valine. *Nature (London).* **217:** 1016.
40. Fessas, P., D. Loukopoulos, and A. Kaltsoya. 1966. Peptide analysis of the inclusions of erythroid cells in β-thalassemia. *Biochim. Biophys. Acta.* **124:** 430.
41. Rifkind, R. A. 1966. Destruction of injured red cells *in vivo*. *Amer. J. Med.* **41:** 711.
42. Crosby, W. H. 1959. Normal functions of the spleen relative to red blood cells: a review. *Blood.* **14:** 399.
43. Jacob, H. S., and J. H. Jandl. 1962. Effects of sulfhydryl inhibition on red blood cells. II. Studies *in vivo*. *J. Clin. Invest.* **41:** 1514.