Preface

Hemoglobin and Hemoglobinologists

This volume, *Hemoglobin Disorders: Molecular Methods and Protocols*, will be introduced with a review of the great milestones in the field, and the scientists responsible for those achievements. The history of hemoglobin can be divided into three periods: the Classical period, the Modern period, and the Post-Modern period.

I am inclined to include as the four major members of the classical period Francis Roughton, Quentin Gibson, Jeffries Wyman, and Linus Pauling, not only because of their achievements, but also because of the superb scientists they trained and/or influenced.

Francis John Worsely Roughton (1899–1972) **(Fig. 1)**, in his laboratory at Trinity College in Cambridge, England, made the first measurements of the rapid reaction of oxygen with hemoglobin at the millisecond scale, at first by flow-mixing methods and later by flash photolysis. He not only opened an era of molecular research of hemoglobin, but also invented the methodology for fast reactions through the use of laser technology, which was later improved by others so that even faster reactions could be detected. Another contribution of Roughton was the education of Quentin H. Gibson **(Fig. 2)**, his favorite student, who, in his laboratory in Sheffield, continued to expand the horizon of ligand binding to hemoglobin, defining the oxygen binding constants for each of the hemes of hemoglobin. Though this did not, as expected, solve the underlying mechanism of ligand cooperativity as discussed below, it was nonetheless an important milestone.

Roughton would later have a surprising influence in the Italian hemoglobin group because he trained Luigi Rossi-Bernardi, and because Quentin Gibson introduced Jeffries Wyman to Eraldo Antonini, the hemoglobin man in Rome in that period ***(1)***. In a meeting in Bellagio, Lake Como, Luigi regaled us with stories about this highly talented and very eccentric investigator. It was fortunate to science that eccentricity was perfectly acceptable in England, unlike in other places in the world.

Finally, Quentin continued his highly productive career after emigrating to the United States in the early 1960s, working independently first in Britton Chance's lab in Philadelphia and then at Cornell, where he trained John Olson, who brilliantly carried on the torch and is an author in this book. I consider Quentin my mentor, along with Helen Ranney. It was most exciting to solve the molecular basis of the hemoglobin/haptoglobin reaction together.

Fig. 1. Francis John Worsely Roughton

Fig. 2. Quentin H. Gibson

Quentin Gibson, who is an MD, was famous for having a lathe in the middle of the lab, useful for tinkering with homemade instrumentation. This wonderful "tinkering" habit of British scientists came in handy during World War II, to the benefit of the world. Gibson is still scientifically active, and has contributed widely to the hemoglobin ligand binding field (*see* Chapter 5). He also wrote his recollections of the life and work of Francis J. Roughton in 1973, after Roughton's death at the age of 73 years ***(2)***.

Jeffries Wyman (1901–1995) **(Fig. 3)** was a Boston Brahmin and a remarkable American biophysicist whose grandfather was one of the founders of the National Academy of Science. A Harvard man, he developed an interest in proteins and in 1937 wrote his first hemoglobin paper on the pH titration curves, or oxy-deoxy-hemoglobin ***(3)***. He exhibited a unique understanding of thermodynamics in his analysis of linked function reciprocal relations (1948). He later came back to this subject with a landmark book, *Binding and Linkage: Functional*

Chemistry of Biological Macromolecules, coauthored with Stanley J. Gill, who derived great comfort from this enterprise in the last years of his life.

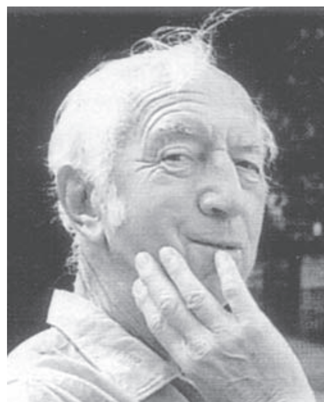

Fig. 3. Jeffries Wyman

According to Edsall ***(4)***, Wyman, while visiting colleagues in Japan in 1950, had an insight based on the work of Felix Haurowitz, a New York scientist who used to walk about with a vial of growing hemoglobin crystals in his vest pocket, so as to maintain the solution close to 37°C. Haurowitz did a remarkable and simple experiment: he reduced a crystal of oxyhemoglobin with dithionite and observed its breakage and dissolution. He concluded that these two ligand states of hemoglobin had different crystal habits. Wyman, in turn, concluded that the result was the consequence of hemoglobin in two different conformational states: in oxy (met in reality) and deoxy, a remarkable anticipation of Perutz's work.

Wyman's wanderlust took him to the four corners of the world. After the death of his first wife, he left Harvard and the United States for Paris, where he was the first Cultural Attache to the American Embassy. After that, it was an International Organization job in Egypt, and then escapes to the Congo, Alaska, Papua, New Guinea, and so forth. But his most important visit by far was to Rome, where he became part of the hemoglobin team lead by Eraldo Antonini ***(1)***. Italy became his home for most of his life, in spite of the fact that he never obtained a permanent status, and needed to go to Switzerland every year to renew his visa. He never learned to speak Italian.

The Rome group, integrated by Maurizio Brunori, Emilia Chiancone, and others, became a strong presence in the field, concentrating on the biophysical and biochemical aspects of hemoglobin. Another participant in this interactive hemoglobin world was Quentin Gibson, who collaborated with Eraldo early on and had to carry instrumentation and glass artifacts through the corridors and yards of the University of Rome, because it was unseemly for an Italian professor to do so. Maurizio, of course, became the leader of this highly productive group after the untimely death of Eraldo at the age of 52, keeping the high standards set by its founder. During my first visit to Rome, Maurizio introduced me to Wyman, and like everybody else, I was in awe of the magnetic field of his mind and his ability to contribute brilliantly to any problem that might be presented to him.

Finally, the emergence of the Jacob-Monod-Changeux allosteric model fit Wyman's insight into the workings of hemoglobin and rapidly adopted its

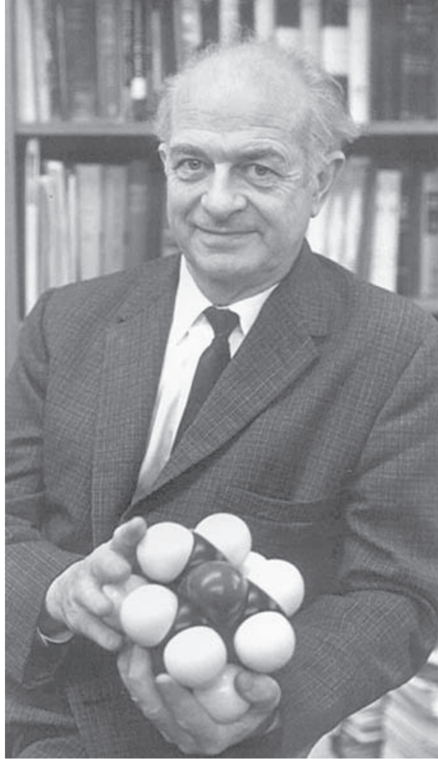

Fig. 4. Linus Pauling

nomenclature. On the other the hand, Eraldo Antonini resisted this concept, postulating an alternative dimer-based model. The demonstration that haptoglobin binds exclusively hemoglobin dimers—and does not bind deoxyhemoglobin ***(5,6)*** because it does not dimerize—made this proposal untenable.

The final member of the Classical period was Linus Pauling (1901–1994) **(Fig. 4)**, a double Nobel Prize winner, and another eccentric and brilliant scientist. He should be considered a luminary in the field of hemoglobin for two reasons. First, he proposed (and demonstrated) that a hemoglobin abnormality had to be the reason for the sickling of red cells in sickle cell anemia. This disease had been discovered by Dr. James B. Herrick, a cardiologist, in Chicago in 1910 ***(7)***. The concept of sickle cell anemia as a "molecular disease" opened a new chapter in medicine ***(8)***. Second, Pauling discovered the differential magnetic susceptibility of oxy- and deoxy-hemoglobin, which is the basis of advanced methods of nuclear magnetic resonance imaging, allowing detection of deoxy-hemoglobin in tissues ***(9)*** and recently applied to the study of sickle transgenic mice ***(10)***.

The Modern period was inaugurated with the discovery by Max Ferdinand Perutz (1914–2002) ***(11)*** **(Fig. 5)** that the isomorphic replacement method was applicable to large molecules, and that binding of mercury to the Cys93 did not distort the molecule. This solved the phase problem and aided in the description of the tridimensional structure of the hemoglobin molecule. This effort was stimulated by conversations with Felix Haurowitz and realized with the help of a small grant from the Rockefeller Foundation, obtained through the good offices of Sir Lawrence Bragg, inventor of crystallography.

Fig. 5. Max Ferdinand Perutz

As a young investigator, I met Max Perutz in Cambridge shortly after he had published his milestone work, and true to his modesty and bonhomie, he told me that he was happy to have finally published because doing so guaranteed him lab space at the Cavendish, which he was previously at risk of losing for lack of publications. This publication's followup, on deoxyhemoglobin, allowed us to understand the molecular basis of cooperativity and was recognized with the Nobel Prize, which Perutz shared with Kendrew, who had worked on the less complex problem of the crystallography of myoglobin. After the Nobel Prize, he worked even harder. I saw him in Cambridge in the last few months before his death, and we discussed the need to understand why HbC has a highly increased tendency to crystallize. It was a fruitful exchange. We also talked about his leaving Vienna in 1936 to work with J. D. Bernal in crystallography. At the beginning of the war, Britain interned all immigrants born in enemy countries in mild detention camps, even if they were of Jewish origin. Fortunately, the authorities put him to work on plans to construct gigantic ice surfaces that could serve as airplane landing sites in the North Sea. For this plan they needed a crystallographer's knowledge to help them strengthen the crystallized water, at which Max eventually succeeded through the use of wood pulp, although too late to be useful.

Vernon M. Ingram clearly deserves a place in the Modern period. Also in Cambridge, using a sickle cell anemia patient's blood samples left behind by a colleague, he purified the hemoglobin, and ran a trypsin digestion and a combination of electrophoresis and paper chromatography (to be known as "fingerprinting") on the sample, revealing that the mutation in sickle hemoglobin was limited to a single amino acid change: glutamic acid replaced by valine. This proved a momentous finding and the launching of a technique that was used

widely for decades in the analysis of proteins. Vernon wrote a recollection of this discovery later ***(12)***.

Another feature of the Modern period is the highly compatible, yet unlikely pairing of Reinhold and Ruth Benesh, called by many R2B2. Reinhold was a Polish immigrant who went to England to study chemistry, and survived by performing stand-up comedy in English vaudeville theaters. He eventually emigrated to the United States, met Ruth, and formed a powerful scientific team. Preparing for a lecture to students of medicine, he realized that 2,3-DPG existed in the red cell in almost identical quantities as hemoglobin. The next day they mixed 2,3-DPG and hemoglobin and observed a right shift of the oxygen equilibrium curve. A new allosteric effector had been found. This finding had tremendous scientific and medical impact. To date, a PubMed search for 2,3-DPG yields 1793 results. Reinhold also contributed to, among other things, the definition of the contact sites in α-chains that contribute to the stabilization of the sickle polymer.

The Post-Modern period, being contemporary, cannot be judged in the same way as the two previous periods. But important accomplishments need to be recognized in the field of hemoglobins. First, George Stamatoyannopoulos merits special mention. Not only has he and his laboratory contributed enormously to the field and trained a slew of young scientists, but he has become the "cheerleader" of research in hemoglobin molecular biology. His "Switching Meetings," at first in collaboration with Art Nienhuis and George Dover, have become, with time, a classic "George's show." Everybody waits for George's phone call: "What have you done lately?" Stamatoyannopoulos has also been a constant and successful lobbyist to NIH for more money for globin research and for greater opportunities for young investigators to join the field.

The explosion of molecular biology is one of the most important events characterizing this period. Too many important participants are worth mentioning, so I will limit the list to a few that contributed to the field up to 1990 (recent work is outlined in "late-breaking news"):

A. W. Nienhuis
F. G. Grosveld
T. J. Ley
L. I. Zon
T. M. Towns (*see* Chapter 13)
J. B. Ligrel
G. Felsenfeld
D. R. Higgs
S. A. Liebhaber
T. Papayannoupolous
S. H. Orkin
Y. W. Kan
S. L. Thein
J. M. Old (*see* Chapters 7 and 8)
T. M. Ryan (*see* Chapter 13)
A. N. Schechter
E. J. Benz
J. B. Clegg
S. M. Weissman
B. G. Forget

H. H. Kazazian
R. Krishnamoorthy (*see* Chapter 12)
A. Bank
N. P. Anagnou
J. L. Sleighton
K. Adachi (*see* Chapter 14)
S. A. Acharya (*see* Chapter 11)
N. J. Proudfoot
D. Labie (*see* Chapter 12)
J. D. Engel
C. Driscoll
W. G. Wood
R. C. Hardison
and many others.

Sir David Weatherall also deserves special mention. He is responsible for major developments in the understanding of thalasssemia in the last 40 years, including some all-encompassing and very readable textbooks on the subject ***(13)***. David Nathan is also a major figure in this field in America, contributing to both the scientific and clinical sides ***(14)***. Clinical advances can be credited to Sergio Piomelli in the general management of this difficult disease ***(14)*** and to G. Lucarelli ***(16)*** for his contribution on bone marrow transplantation of thalassemic patients in Italy and the world.

A major and groundbreaking contribution to sickle cell anemia was the discovery by William Eaton and his group that the polymerization of HbS was a nucleation-driven reaction in its two forms: homogeneous and heterogeneous ***(17)***. In addition, they discovered that the delay time of polymerization was dependent exclusively on the initial concentration of Hb with the potential of modifying the extent of the phenotype ***(18)***.

The next important discovery in the field, credited to Robert Hebbel and associates ***(19)***, was the capacity of young sickle cells to adhere to cultured endothelial cells. This finding was confirmed by Dhananjay K. Kaul in ex vivo and in vivo microcirculatory beds, and was followed by the demonstration that sickle vasocclusion occurred, not predominately in the capillaries as previously thought, but in the small venules, in which the adhesion of young sickle cells preceded obstruction by rigid sickle cells ***(20)***.

The structure of the sickle polymer was resolved by a combination of the following discoveries: (1) the crystallography of sickle hemoglobin ***(21)***, (2) the study of the polymerization tendency of binary mixtures of sickle and other hemoglobin mixtures to define residues in the area of contact of the polymer ***(22)***, and (3) electron microscopy of the polymer and modeling ***(23,24)***.

Another surprise was the linkage of the sickle mutation with several haplotypes of polymorphic sites in the globin gene cluster. This effort arose based on early work by Y. W. Kan and Stuart Orkin, which was followed by genetic epidemiological studies in Jamaica ***(25)***, Africa ***(26)***, and India ***(27,28)***. Besides demonstrating the multicentric origin of the sickle mutation, this effort revealed the linkage between severity and certain haplotypes and the

role of -158 Xmn I polymorphism in the expression of HbF ***(29,30)***, in addition to their power as instruments in anthropological and gene flow studies.

Alpha thalassemia was, after the ameliorating effect of HbF, the first modifier of sickle cell anemia found and most of the credit for this finding belongs to Steve Embury ***(31)***.

The discovery of Locus Control Region (LCR), 5' to the β-like gene cluster, by Dorothy Tuan and Irving London ***(32)*** had unexpected consequences. In addition to the involvement of LCR in the development of appropriate expression of the β-like globins, it made possible the high and tissue-specific expression of transgenes in mouse models as well as in vectors containing anti-hemoglobinopathies for gene therapy. Although much progress has been made owing to the efforts of George Stamatoyannopoulos, Marc Groudine, and F. G. Grosveld, a definitive picture has not yet emerged.

The development of transgenic sickle and thalassemic mice, very useful in the field despite unfriendly NIH committee reviews for many years, is a complicated history with many players, so I refer the interested reader to a recent review ***(33)*** and Chapter 13.

The successful clinical trial, beyond any rational expectation, of hydroxyurea as a specific treatment for sickle cell anemia ***(34)*** is a great landmark in the history of sickle cell in America because it is the only drug approved by the FDA for the treatment of this disease. Investigations leading to this breakthrough involved Paul Heller, Joe DeSimone, George Stamatoyannopoulos, George Dover, and others. The leader of the clinical trial was Sam Character, after years of frustrating rejections by unsympathetic and misguided reviewers, with the competent help of Martin Steinberg ***(35)***.

The pioneering work of Chien Ho (*see* Chapter 15) on NMR of hemoglobin and hemoglobin variants was highly successful and contributed to, among other things, the molecular localization of the Bohr protons.

Other less glamorous but equally important clinical advances can be credited to Helen Ranney, who contributed all of her scientific life to hemoglobin research, with her pioneering work on HbA1c as a noted example. She also contributed by organizing what I believe to be the first hemoglobinopathies-dedicated clinic in America, at Jacobi Hospital, Bronx, NY.

The NIH Natural History initiative, under the leadership of Marilyn Gaston ***(36)***, saved many lives by demonstrating the effectiveness of penicillin prophylaxis in decreasing infections and modality in infants with sickle cell disease. The Herculean effort of Graham Serjeant, who headed an MRC unit in Jamaica dedicated to the care and study of sickle cell anemia patients, must be recognized. He produced considerable and reliable natural history clinical data on sickle cell anemia with much less funding than the NIH effort ***(37)***.

The discovery of desferrioxamine and the use of chelation therapy to increase the life expectancy of patients with thalassemia major and thalassemia intermedia is also a major accomplishment. The compound is a natural product extracted for actinomycetes, and was reported to be an iron chelator useful in the treatment of hemochromatosis by P. Imhof of Ciba Geigy at the joint annual meeting of the 1962 Swiss Medical in Lugano. An annotation in *Lancet* ***(38)*** concludes that "it is unfortunate that in secondary hemochromatosis, usually the result of repeated transfusions in patients with aplastic anemia and other anemias when repeated blood letting is not possible, the drug is apparently less efficacious than in the idiopathic type." Fortunately, this prediction did not come to pass, and the drug is now the mainstay of the treatment of severe thalassemia. The quest for a clearly effective oral form seems to be close at hand.

Another aspect of research in hemoglobinopathies is the effort to characterize hemoglobin mutants, useful in many of the studies referred to above. In this realm, three investigators have been particularly successful. The first is Herman Lehmann ***(39,40)***, who emigrated to Britain early in life, worked in Cambridge, and spent World War II in the British Army in India, in which his training as a hematologist was welcome. He discovered HbS among the "tribals" of India, and contributed profusely to the works on hemoglobin , particularly in the identification and characterization of Hb mutants. He also predicted the duplication of the α-globin loci. The second great figure in this realm was Titus Huisman ***(41)***, who published 661 papers in his life, almost all on hemoglobin. He was a refined analytical biochemist and a highly focused and productive researcher. Finally, the successor in this field today is Henri Wajcman, editor of *Hemoglobin*, who runs a highly efficient reference laboratory in Paris for abnormal hemoglobins that has been enormously useful to all of us. Dr. Wajcman is an expert on unstable hemoglobins.

Finally, in "late-breaking news," the very recent correction of sickle cell anemia ***(42)*** and thalassemia ***(43)*** by transplantation of stem cells transducted with a lentivirus construct containing human globin genes in mice transgenic models is an encouraging event, and bodes well for the future of gene therapy in hemoglobinopathies.

The remarkably successful adventures that have characterized research and clinical endeavors in hemoglobinopathies have been the product of the efforts of an army of highly qualified and imaginative investigators and clinicians, interested in diseases that affect not only Europe and North America, but most of the third world.

In conclusion, the last century has been good to hemoglobin. Maybe because hemoglobin is red, which helped in its isolation, maybe because it is abundant, or maybe because, as the third book of the Torah (and the Old Testa-

ment) says, "the soul of the flesh is the blood," hemoglobin has been an active participant in the development of biochemistry, protein chemistry, molecular biology, human genetics, and molecular medicine. It is also apparent that behind it all there was a real network of investigators, sometimes interacting competitively, some times cooperatively, but always in contact. The network has indeed produced a cascade of findings and valuable and unforgettable human interactions. Maybe the lure of this unique and beautiful molecule attracted brilliant, eccentric, imaginative, and one-of-a-kind investigators who blazed a brilliant trail of successes.

Ronald L. Nagel, MD

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2

Analysis of Hemoglobins and Globin Chains by High-Performance Liquid Chromatography

Henri Wajcman

1. Introduction

In recent years, high-performance liquid chromatography (HPLC) has become a reference method for the study of hemoglobin (Hb) abnormalities. This technique is used in two distinct approaches. The first is quantitative analysis of the various Hb fractions by ion-exchange HPLC, which is now done in routine hospital laboratories mostly by using fully automated systems. The second is reverse-phase (RP)-HPLC, which is of interest for more specialized studies (*see* **Note 1**).

2. Materials and Methods

2.1. Ion-Exchange HPLC Separation of Hbs

Cation-exchange HPLC is the method of choice to quantify normal and abnormal Hb fractions ***(1–4)***. This is the method of reference for measuring glycated Hb for monitoring diabetes mellitus. It is also generally used for measuring of the levels of HbA_2, HbF, and several abnormal Hbs.

According to some researchers, this method could even replace electrophoretic techniques for primary screening of Hbs of clinical significance ***(3,5–7)*** or, at least, should be an additional tool for the identification of Hb variants ***(8)***. Automated apparatuses have been developed for large series measurement. I describe the Bio-Rad Variant Hemoglobin Testing System (Bio-Rad, Hercules, CA), using the β Thalassemia Short program as an example of this type of equipment.

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2.1.1. Bio-Rad Variant Hb Testing System

The Bio-Rad apparatus is a fully automated HPLC system, using double wavelength detection (415 and 690 nm). The β Thalassemia Short program is the most widely used system for HbA_2 and HbF measurements, but other elution methods, including specific columns, buffers, and software, are available from the manufacturer according to the test to perform. This program has been designed to separate and determine, in 5 to 6 min, area percentage for HbA_2 and HbF and to provide qualitative determinations of a few abnormal Hbs. Windows of retention time have been established for presumptive identification of the most commonly occurring Hb variants. The β Thalassemia Short program uses a 3.0 × 0.46 cm nonporous cation-exchange column that is eluted at 32 ± 1°C, with a flow rate of 2 mL/min, by a gradient of pH and an ionic strength made of two phosphate buffers provided by the manufacturer. This material and procedure have been used worldwide in many laboratories over the last several years. Since recommendations for experimental procedure are fully detailed by the manufacturer, I describe only a few additional notes of practical import.

1. Blood is collected on adenine citrate dextrose (ACD).
2. Samples for analysis (about 0.2% Hb) are obtained by hemolysis of 20 μL of blood in 1 mL of a buffer containing 5 g/L of potassium hydrogenophthalate, 0.5 g/L of potassium cyanide, 2 mL of a 1% solution of saponine, and distilled water. This procedure for sample preparation, which is currently used for HPLC determination of HbA_{1c}, avoids some of the Hb components present in low amounts (about 1%) eluted together with HbF in the HbF retention time window ***(8)***.
3. Twenty microliters of hemolysate is applied onto the column for analysis.

Under these experimental conditions an excellent agreement is found between chromatographic measurement of HbF, down to 0.2%, and resistance to alkali denaturation, up to 15% ***(9)***. Presumptive identification of the most commonly occurring variants (Hb S, HbC, HbE, and HbD Punjab) is made using the retention time windows named S-Window, D-Window, A_2-Window, and C-Window, which have been specified by the manufacturer. Aged Hb specimens display some degraded products that are eluted in the P2 and P3 windows (e.g., glutathione-Hb) (**Table 1**).

Slight differences in the elution time of the various Hb components are observed from column to column and from one reagent batch to another, which should be taken into account by a program supplied by the manufacturer. The elution time of an Hb component varies also slightly according to its concentration in the sample. For a given column, a more accurate calibration than that proposed by the manufacturer could be obtained using HbA_2 as reference. The concentration of this Hb, which varies between narrow limits, prevents significant modification of its elution time.

Table 1
Analyte Identification Window[a]

Analyte name	Retention time (min)	Band (min)	Window (min)
F	1.15	0.15	1.00 -1.30
P2	1.45	0.15	1.30-1.60
P3	1.75	0.15	1.50-1.90
A0	2.60	0.40	2.20-3.30
A2	3.83	0.15	3.68-3.98
D-window	4.05	0.07	3.98-4.12
S-window	4.27	0.15	4.12-4.42
C-window	5.03	0.15	4.88-5.18

[a] Example provided by manufacturer.

Two methods are available for comparing data when the elution time of HbA_2 differs between two runs done with a different column or reagent batch. The first consists of slightly modifying the experimental procedure (temperature or pH) to reproduce exactly the elution times of the previous runs. The second method consists of establishing a normalized retention scale taking as references two Hbs eluted within a linear part of the gradient.

The elution patterns of more than 100 variants have been published, but, in my opinion, these data should be used as a confirmatory test for characterization of a variant after a careful multiparameter electrophoretic study ***(8)*** rather than as a primary identification method.

2.1.2. Alternative Methods

When a dedicated machine is not available for Hb analysis, or when the chromatographic separation is done for "preparative" purposes, alternative techniques have to be used. These procedures are suitable for conventional HPLC equipment. Several anion-exchange and cation-exchange HPLC columns may be used for Hb separation; some are silica based and others are synthetic polymers. These methods have been well standardized for several years ***(10,11)***.

PolyCat A (Poly LC, Columbia, MD) is one of the more popular phases for Hb separations ***(6)***. It consists of 5-µm porous (100-nm) spherical particles of silica coated with polyaspartic acid. For analytical purposes, a 5.0 × 0.40 cm column is used; elution is obtained at 25°C with a flow rate of 1 mL/min, by developing in 20 min at pH 6.58 a linear gradient of ionic strength from 0.03 to 0.06 *M* NaCl in a 50 m*M* Bis-Tris, 5 m*M* KCN buffer. The presence of KCN is necessary to convert methemoglobin into cyanmethemoglobin, which displays ion-exchange chromatographic properties similar to those of oxyhemoglobin (*see* **Note 2**).

2.2. HPLC Analysis of Globin Chains

2.2.1. Analysis of HbF Composition (*see* **Note 3**)

The solvent system, acetonitrile–trifluororoacetic acid (TFA), which is used for RP-HPLC, dissociates the Hb molecule into its subunits and removes the heme group. This method is therefore used to analyze or separate the globin chains. This kind of study may be useful in the investigation of many human Hb disorders. For instance, the determination of HbF composition ($^{G}\gamma$:$^{A}\gamma$ ratio) is of interest in several genetic and acquired disorders.

A good separation is obtained between the $^{G}\gamma$ and $^{A}\gamma^{I}$, with most of the RP columns by using a very flat acetonitrile gradient. By contrast, it is often much more difficult to separate $^{G}\gamma$ from $^{A}\gamma^{T}$, a frequent allele of $^{A}\gamma^{I}$. Among the procedures that have been successfully proposed for this analysis, one of the most popular is the RP-HPLC method described by Shelton et al. ***(12)***. They used a Vydac C4 column (The Separation Group, Hesperia, CA) eluted at a flow rate of 1 mL/min by developing in 1 h a linear gradient from 38 to 42% acetonitrile in 0.1% TFA with detection at 214 nm. Under these conditions, the chains were eluted in the following order: β, α, $^{A}\gamma^{T}$, $^{G}\gamma$, and $^{A}\gamma^{I}$. In recent years, a modification introduced in the manufacturing process of this type of column ***(13)*** made necessary the use the higher acetonitrile concentrations to elute the γ-chains. Unfortunately, it also resulted in the low resolution of $^{A}\gamma^{T}$.

2.2.1.1. RP Perfusion Chromatography

Perfusion chromatography involves a high-velocity flow of the mobile phase through a porous chromatographic particle ***(14–16)***. The Poros R1® media (Applied Biosystems, Foster City, CA) used in this technique consists of 10-µm-diameter particles. These particles are made by interadhering under a fractal geometry poly(styrene-divinylbenzene) leading to throughpores of 6000- to 8000-Å-diameter microspheres with short, diffusive 500- to 1000-Å-diameter pores connected to them. As a result, relatively low pressures are obtained under high flow rates. The Poros R1® beads may be considered a fimbriated stationary phase having retention properties somewhat similar to those of a classic C4 support ***(15)***. The column (10 × 0.46 cm) is packed on a conventional HPLC machine at a flow rate of 8 mL/min using the Poros self-pack technology® according to the manufacturer’s protocol. More than a thousand runs may be performed without alteration of the resolution.

2.2.1.1.1. Sample Preparation

1. Samples containing about 0.1 mg of Hb/mL are obtained by lysis, in 1 mL water (or 5 m*M* KCN), of 2–5 µL of washed red blood cells (RBCs).
2. Membranes are removed by centrifuging at 6000*g* for 10 min.

3. According to the HbF level, 20–100 µL of these hemolysates are applied onto the column. To avoid additional chromatographic peaks owing to glutathione adducts, 10 µL of a 50 m*M* solution of dithiothreitol in water is added per 100 µL of sample. An in-line stainless steel filter (0.5-µm porosity) needs to be used to protect the column.

2.2.1.1.2. Equipment. Any conventional HPLC machine can be used. In the method described here, the analyses were performed on a Shimadzu LC-6 HPLC machine equipped with an SCL-6B system controller, an SIL-6B autoinjector, and a C-R5A integrator (Shimadzu, Kyoto, Japan). A flow rate of 3.0–4.5 mL/min was convenient for synchronization of injection, integration, and column equilibration.

2.2.1.1.3. Experimental Procedure (*see* **Note 4**). Using a flow rate of 3 mL/min, the various γ-chains are isolated by developing in 9 min a linear gradient from 37 to 42% acetonitrile in a 0.1% solution of TFA in water. In practice, this is done by using two solvents (A: 35% acetonitrile, 0.1% TFA in water; B: 50% acetonitrile, 0.1% TFA in water) and a linear gradient from 15 to 45% B. Before injection, the column is equilibrated by a 10 column volume wash with the starting solvent, thus allowing completion of a cycle of analysis every 14 min. Elution is followed at 214 nm (wavelength at which double bonds absorb), and the recorder is set to 0.08 AUFS. Higher flow rates may be used, but the slope of the gradient will need to be increased in proportion. Keeping the same initial and final acetonitrile concentrations as above, elution is achieved in 6 min at a flow rate of 4.5 mL/min and in 4 min at a flow rate of 6.0 mL/min.

2.2.2. RP-HPLC Analysis of Globin Chains (*see* **Note 5**)

Globin chain analysis is also important as an additional test that allows discrimination between Hb variants for the identification of structural abnormalities. Several RP-HPLC procedures have been proposed ***(10,14,17,18)***.

On a conventional HPLC apparatus, a 20 × 0.46 cm column packed with Lichrospher 100 RP8 (Merck, Darmstadt, Germany) is used. Samples are prepared as described in **Subheading 2.2.1.1.1.** Elution is obtained at 45°C with a flow rate of 0.7 mL/min using a 90-minute linear gradient of acetonitrile, methanol, and NaCl made by a mixture of two solvents ***(18)***. Solvent A contains acetonitrile, methanol, and 0.143 *M* NaCl, pH 2.7 (adjusted by a few drops of 1 *N* HCl), in the proportion of 24, 38, and 36 L/L, respectively. Solvent B is made from the same reagents but in the proportion of 55, 6, and 39 L/L, respectively. The gradient starts with 10% B and ends with 70% B. The design of the gradient may be modified according to the machine, the geometry of the column, and the separation to be achieved. Elution can be followed at 214 or 280 nm. Globin chains are eluted in the same order as on the Vydac C4 column.

A kit for globin chain analysis with similar performance is also commercially available from Bio-Rad (ref. 270.0301).

2.2.3. Scaled Up Methods for Chain Separation

For biosynthetic or structural studies, milligram amounts of globin chains need to be separated. This can be achieved either by scaling up the RP-HPLC procedure using semipreparative size columns or by cation-exchange -HPLC done in the presence of dissociating concentrations of urea.

2.2.3.1. Semipreparative Size RP-HPLC Columns

2.2.3.1.1. Samples. Globin solution rather than Hb solution is used. Globin is prepared from a 1% Hb solution obtained by hemolysing washed RBCs in distilled water. Stromas are removed by centrifuging at 6000*g* for 30 min, and the globin is precipitated by the acid acetone method. Usually, the sample is made from 1 to 2 mg of globin dissolved in 250 µL of 0.1% TFA, which requires the use of a 500-µL injection loop.

2.2.3.1.2. Chromotographic Procedure. A 240 × 10 mm Vydac C4 column (ref. 214TP510) is used. Elution is obtained by a gradient of acetonitrile in 0.1% TFA made by two solvents (solvent A contains 35% acetonitrile and solvent B 45%). A typical elution program, using a flow rate of 1.2 mL/min, consists of a 10-min equilibration at 35% B, 70 min of a linear gradient from 35 to 55% B, 30 min of a linear gradient from 55 to 90% B, and 5 min of an isocratic step at 90% B for cleaning the column. Elution of the column is followed at 280 nm with a full scale of 0.16 absorbance units (AU).

2.2.3.2. Cation-Exchange HPLC in Presence of 6 *M* Urea Using a PolyCat Column

Procedures that are modified from the classic CM cellulose chromatography described by Clegg et al. ***(19)*** may be transposed to the HPLC technology ***(20)***. The retention capacity of this type of column is higher than that of RP supports, allowing the handling of larger samples. I describe here a method using a PolyCat 300-Å, 10-µm particle column (150 × 4 mm).

2.2.3.2.1. Reagents and Buffers. Two buffers are used. Buffer A consists of 6 *M* urea, 0.1 *M* sodium acetate, and 0.4% β-mercaptoethanol, with the pH adjusted to 5.8 by acetic acid. Buffer B consists of 6 *M* urea, 0.25 *M* sodium acetate, and 0.35% β-mercaptoethanol, with the pH adjusted to 5.8 by acetic acid. Both buffers need to be filtered through a membrane with 0.45-µm porosity before being used. In addition, an in-line stainless steel filter (0.5-µm porosity) is needed to protect the column.

2.2.3.2.2. Samples. Up to 5–10 mg of globin, prepared by the acid acetone method, is dissolved in 200–600 mL of buffer A.

2.2.3.2.3. Chromatographic Procedure. Elution is obtained by a gradient of ionic strength developed with the two buffers. A typical elution program, using a flow rate of 1.0 mL/min, consists of a 10-min equilibration at 0% B, 5 min of a linear gradient from 0 to 25% B, 50 min of a linear gradient from 25 to 100% B, and 5 min of an isocratic step at 100% B for cleaning the column. Elution of the column is followed at 280 nm with a full scale of 0.32 UA.

3. Notes

1. Why should one method be preferred over another? The choice of a separation method between RP or ion-exchange chromatography depends on the purpose of the separation. Ion-exchange is the only chromatographic method that allows preparation of native Hb fractions. The presence of cyanide ions in the buffers (or during sample preparation) will nevertheless hinder any further oxygen-binding study. If the aim of the separation is to obtain Hbs suitable for functional studies, the technique will have to be modified accordingly by removing cyanide from all the steps. It may be of interest in some cases to work with carbonmonoxy-hemoglobin, since Hb is very stable under this form and procedures are available to return to the oxyform. For several applications, salts in excess also need to be removed. RP separation methods always lead to denatured proteins that cannot be used for functional studies. Techniques involving an ionic strength gradient can only be used for analytical purposes. By contrast, using fully volatile buffers, such as the acetonitrile-TFA system, the isolated globin fractions can be vacuum dried and readily used for further structural studies such as mass spectrometry measurements.
2. To isolate amounts of Hb in the milligram range, larger columns (15.0×0.46 cm) may be used. According to the separation to be achieved, the dimensions of the column, and the apparatus used, slightly different experimental conditions may have to be designed. Elution is followed at 415 nm for analytical purposes or at 540 nm in preparative runs. This buffer system is not suitable for ultraviolet (UV) detection. The use of an in-line stainless steel filter (0.5-µm porosity) is recommended to increase the column life expectancy. Reproducibility requires careful preparation of the buffers and temperature control. Since in these chromatographic methods the elution is recorded at one of the wavelengths of absorption of the heme, any factor modifying the absorption spectrum of the Hb molecule will hinder accurate quantitative measurement. For instance, unstable Hb variants, which lose their heme groups or lead to hemichrome formation, will be underestimated. HbMs, which are hardly converted into cyanmethemoglobin, display a much higher extinction coefficient than oxyhemoglobin at 415 nm and a lower one at 540 nm. As a consequence, HbMs will be overestimated when measured at the first wavelength, and underestimated at the second one. A modified experimental procedure allowing for a simultaneous measurement of HbF, glycated Hb, and several other Hb adducts has been proposed by using a combination of pH and ionic gradients ***(11)***.

3. In my laboratory, for routine determination of the γ-chain composition, we replaced this procedure with an RP perfusion chromatography using a Poros R1® column (Applied Biosystems) ***(14)***.
4. To obtain good reproducibility, we recommend using the same glassware for preparing the solvents. Solvents may be kept refrigerated at 4°C for a few days. Accurate balance of the TFA between both solvents is important to avoid baseline drift. Acetonitrile must be of HPLC grade with low UV absorbancy in the 210-nm region. With this Poros R1 column, the α-chain is eluted before the β-chain. Resolution may be improved by modifying the geometry of the column or the design of the gradient. A 10 × 0.2 cm column may be used to improve separation between the various γ- or adult chains. In this case, with a flow rate of 1 mL/min, after 5 min of equilibration at 5% B, the column is eluted using a 15-min linear gradient between 5 and 25% B of the described solvents. This is followed by a 2-min isocratic elution at 25% B.
5. Several columns may be used, but I have found that a method adapted from that described in **ref. *17*** leads to a good resolution. Other columns or techniques may nevertheless be more appropriate for some specific separations. When chromatographic methods are used for globin chain quantification, it is important to consider the absorption coefficient of the various chains at the wavelength of detection. In some cases, it may be identical, such as when comparing the various γ-chains. In other cases, the absorption may differ considerably; for example, at 280 nm, γ-chains, because of their 3 Trp residues, have a higher ε coefficient than β-chains (2Trp) and α-chains (1 Trp). Abnormal Hbs containing a number of aromatic residues different from the normal may also display modified absorption coefficient.

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