[1] Preparation of Blood Hemoglobins of Vertebrates

By AUSTEN RIGGS

Hemoglobins of vertebrates are the most extensively studied of any proteins largely because of their ease of preparation. The red cells are easily separated from the plasma by centrifugation. Hemolysis leads immediately to a solution that is usually more than 90% pure. Nevertheless, precautions need to be taken to obtain preparations of maximum value. Hemoglobins from different organisms vary greatly in their stability and resistance to denaturation, tendency to oxidize to methemoglobin, solubility, and chromatographic and electrophoretic properties. What is appropriate for mammalian hemoglobins is frequently inappropriate for those of lower vertebrates. The latter are often less stable than those of mammals. Certain procedures found to be useful with hemoglobins from some animals will be described.

Erythrocyte Preparation

Blood from small animals is usually best obtained by cardiac puncture. Large animals can be bled from superficial veins, for example, those of the ears of rabbits and elephants. The caudal vein of fish is an excellent location for bleeding; it is usually easy, with a little practice, to insert a needle just ventral to the lateral line directly into the vein. Turtles can be bled by first drilling a circular hole in the plastron (.5 cm is adequate for medium-sized turtles).

Cold-blooded animals can be anesthetized with tricaine methane sulfonate (TMS-222, Crescent Research Chemicals, 5301 N. 37th Place, Paradise Valley, Arizona 85253) or by chilling on ice. Fish may be anesthetized by immersion in water or seawater with 50–100 ppm.[1] Sharks up to 180 kg can be anesthetized in 1 min by spraying TMS (1 g in 1 liter of seawater) into the mouth or spiracles.[2] Small amphibians can be anesthetized by immersion in concentrations of TMS of 1 : 1000 to 1 : 10,000.[3] The blood should be withdrawn into syringes containing an appropriate anti-

[1] G. W. Klontz, Proceedings of Symposium on Experimental Animal Anesthesiology, Brooks Air Force Base, (1964).
[2] P. W. Gilbert and F. G. Wood, *Science* **126,** 212 (1957).
[3] W. V. Lumb, "Anesthesia of Laboratory and Zoo Animals, Small Animal Anesthesia," pp. 269–310. Lea & Febiger, Philadelphia, Pennsylvania, 1963.

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coagulant, such as heparin (50 μl of sodium heparin, 5000 IU/ml, in 1.7% NaCl for a 1-ml syringe). The choice of anticoagulant is not important, provided that the red cells are adequately washed prior to lysis. Heparin is an anionic polysaccharide that has a high affinity for basic proteins such as hemoglobin[4] and so should be removed before lysis. Citrate and EDTA can act as allosteric anions modulating oxygen binding. Carboxypeptidases present in blood serum can produce spurious electrophoretic variants of hemoglobin[5] if not completely removed by adequate washing of the red cells. The blood should be refrigerated immediately, but not frozen.

Mammalian red cells can be washed with 0.85% NaCl, but it is frequently desirable to use a higher concentration for red cells from lower vertebrates. This is particularly true for small samples if the conditions for hemolysis are unknown. The red cells of the lamprey (*Petromyzon marinus*) are quite resistant to hemolysis and have been washed with NaCl concentrations as low as 0.4%. The following procedure has been found satisfactory for a wide variety of fish bloods.[6] Three to five volumes of ice cold saline (1.7% NaCl in 1 m*M* Tris, pH 8) are added to the blood, and the suspension is spun at 700 *g* for 10 min at 4°. The packed cells are washed three times in 10 volumes of this saline. The last packing of the red cells is done at 3000 *g*. The top layer of white cells can be removed at this time. A convenient way to do this is to use (sulfoethyl)cellulose, which binds white cells, but not red cells. The procedure[7] is to mix 1.0 ml of wet (sulfoethyl)cellulose with 1.0 ml of wet Sephadex G-25 (medium), pack on a 3 × 1 cm column, and equilibrate with 0.138 *M* NaCl, 0.005 *M* $MgCl_2$, 10 units of heparin per milliliter, and 0.01 *M* potassium phosphate, pH 7.5, or 1 m*M* Tris, pH 8; 1 ml of heparinized blood is applied to the column and eluted in 20 min with 5 ml of the equilibration buffer. More than 95% of the red cells are in this fraction, which is essentially free of both white cells and platelets. If the packed cells are not to be further processed they may be kept on ice for up to a few days. Long-term storage is best done by preservation in liquid nitrogen. The red cells of birds and mammals can be preserved intact for long periods as follows.[8] Two volumes of a glycol–citrate solution are mixed thoroughly with one volume of washed packed cells. The glycol–citrate solution (pH 8.4) is made by dissolving 60 g of trisodium citrate·2 H_2O and 400 ml of ethylene glycol

[4] L. B. Jaques, *Science* **206,** 528 (1979).
[5] H. R. Marti, D. Beale, and H. Lehmann, *Acta Haematol.* **37,** 174 (1967).
[6] U. E. H. Fyhn, H. J. Fyhn, B. J. Davis, D. A. Powers, W. L. Fink, and R. L. Garlick, *Comp. Biochem. Physiol.* **62A,** 39 (1979).
[7] M. Nakao, T. Nakayama, and T. Kankura *Nature (London), (New Biol.)* **246,** 94 (1973).
[8] J. L. Van de Berg and P. G. Johnston, *Biochem. Genet.* **15,** 213 (1977).

per liter. The procedure may require slight modification for use with cells from lower vertebrates. Storage temperatures of $-15°$ to $-25°$ may be adequate for many purposes. Much colder temperatures produce lysis. However, $-25°$ may not be cold enough to prevent a slow oxidation of the hemoglobin.

Extraction of hemoglobin from very small animals and embryos presents special problems. The following procedures for tadpole (*Rana catesbeiana*)[9] and chick embryo red cells[10] have been found to be useful. A healthy, anesthetized, tadpole of 3 cm body diameter can yield about 0.25 ml of blood by careful puncture of the ventricle with a No. 26 needle and a 1-ml tuberculin syringe. This requires prior dissection of the animal without significant bleeding. If 50 μl of an anticoagulant solution (see above) are placed in syringe, a small bubble can be used to monitor the pumping of the blood by the heart into the syringe: the bubble will be seen to contract discontinuously at each ventricular contraction. With practice the operator can keep the bubble volume constant by drawing slowly on the syringe barrel. However, this procedure is too slow when approximately 5000 tadpoles are required for amino acid sequence work.[11,12] Instead, tadpoles anesthetized with MS-222 are bled by inserting a microcapillary directly into the conus arteriosus[13] or, more crudely, by cutting the upper throat region with a razor blade and draining. The latter procedure requires subsequent filtration through cheesecloth. The microcapillary can be made by drawing out a Pasteur pipette and breaking the end to provide a sharp cutting edge. The capillary should be wet with anticoagulant. In this way a dozen or more tadpoles can easily be bled simultaneously.

Chick embryos have been bled as follows.[10] Five-day-old embryos were dissected out with the blood circulation from surrounding yolk, rinsed with Howard's Ringer solution (7.2 g of NaCl, 0.17 g of $CaCl_2$, 0.37 g of KCl to 1000 ml of H_2O) to remove residual yolk, transferred to another vessel with fresh solution, and finely minced. The liberated red cells were filtered through cheesecloth and washed at 4° six times with Howard's Ringer solution. The process of mincing tissue may well liberate proteases. The inclusion of 3.5 m*M* phenylmethylsulfonyl fluoride in the medium should help to protect against the hazards of proteolysis.[14]

[9] A. Riggs, *J. Gen. Physiol.* **35**, 23 (1951).
[10] J. L. Brown and V. M. Ingram, *J. Biol. Chem.* **249**, 3960 (1974).
[11] T. Maruyama, K. W. K. Watt, and A. Riggs, *J. Biol. Chem.* **255**, 3285 (1980).
[12] K. W. K. Watt, T. Maruyama, and A. Riggs, *J. Biol. Chem.* **255**, 3294 (1980).
[13] S. J. Aggarwal and A. Riggs, *J. Biol. Chem.* **244**, 2372 (1969).
[14] K. J. Fahrney and A. M. Gold, *J. Am. Chem. Soc.* **85**, 997 (1963).

Preparation of Hemolysate

Lysis

Although toluene and even the carcinogenic[15] carbon tetrachloride are still often used as hemolytic agents with human erythrocytes,[16] organic solvents should generally be avoided for several reasons. Some hemoglobins are readily denatured by these agents. For example, although this author found no deleterious effect of toluene lysis on the stability of human hemoglobin, exactly the same procedure caused oxidation and denaturation of the hemoglobin of the bullfrog, *Rana catesbeiana*. Cameron and George[17] noted spectrophotometric abnormalities after oxidation in the presence of traces of organic solvents. Even though the solubility of organic solvents in aqueous solutions is very low, it is not zero. Farnell and McMeekin[18] showed that hemolysis of human erythrocytes with toluene results in the binding of one molecule of toluene per molecule of hemoglobin. Since such a molecule will be bound tightly in a hydrophobic pocket of the hemoglobin, it may remain with the hemoglobin through further purification steps including chromatography. Farnell and McMeekin showed that the extent of crystallization of human carbonyl hemoglobin (HbCO) in 2.15 *M* phosphate, pH 7, is increased from 2–3% to over 90% in the presence of 0.07% toluene. Similar results were obtained with benzene, phenol, and *o*-xylene. Thus, these substances may usefully be added to some hemoglobins to enhance crystallization even though other hemoglobins may be denatured.

The preparation of hemolysates from mammalian red cells is particularly easy because nuclei are absent. Addition of 1.0–1.5 volume of distilled H_2O to 1 volume of packed cells with stirring gives virtually complete hemolysis within a few minutes. This procedure applied to fish red cells is likely to produce a gelatinous mass that will not separate upon centrifugation into a clear supernatant hemoglobin solution and a compact pellet. The jelly results from the liberation of nucleic acids by rupture of the nuclear envelope.

Three procedures have been used to minimize these difficulties. The first seeks to prevent rupture of the nuclear envelope.[19] For example, good results with tadpole erythrocytes from *Rana catesbeiana* have been obtained as follows.[20] The packed cells are lysed with 2 volumes of dis-

[15] J. R. Howe, *Lab. Pract.* **24,** 457 (1975).
[16] W. A. Schroeder and T. H. J. Huisman, "The Chromatography of Hemoglobin." Dekker, New York, 1980.
[17] B. F. Cameron and P. George, *Biochim. Biophys. Acta* **194,** 16 (1969).
[18] K. J. Farnell and T. L. McMeekin, *Arch. Biochem. Biophys.* **158,** 702 (1973).
[19] R. C. Krueger, I. Melnick, and J. R. Klein, *Arch. Biochem. Biophys.* **64,** 302 (1956).
[20] A. E. Herner and A. Riggs, *Nature (London)* **198,** 35 (1963).

tilled water at 0° for 5 min, and sufficient KCl is then added to give a final isotonic concentration of 0.17 *M*. The second procedure is to digest with a nuclease. For example, packed red cells from 5-day embryos of the Hyline strain of the White Leghorn chicken are lysed with 0.001 *M* phosphate,[10] pH 6.9, containing 0.02% saponin and 0.05% DNA nuclease II and incubated for 10 min at 37°, with vigorous mixing. The supernatant is removed after centrifugation, and the pellet is reextracted twice with 0.9% NaCl. The combined supernatants are then centrifuged at 15,000 *g* for 30 min. The saponin detergent may help to increase the yield but is nevertheless usually undesirable because it tends to denature some hemoglobins. It has long been known that saponin modifies the spectrum of methemoglobin.[21] As a general procedure, 1 m*M* Tris, pH 8, would be preferable to phosphate at pH 6.9 to avoid formation of methemoglobin. Furthermore, the elevated temperature is undesirable for many unstable animal hemoglobins. The following simpler procedure has been found to work well for a wide variety of fish red cells.[6]

The cells can be lysed in 3 volumes of ice-cold 1 m*M* Tris (pH 8) for 1 h. One-tenth volume of 1 *M* NaCl is then added before centrifuging at 28,000 *g* for 15 min at 4°. Sometimes it may be helpful to put the material through a glass–Teflon homogenizer chilled to 0° before centrifuging if maximum yields are desired. This helps break the nucleic acid matrix. Maintaining the pH at 8 helps prevent formation of methemoglobin. The addition of the 1 *M* NaCl changes the density of the medium so that the cellular debris can more readily be separated by centrifugation.

Stabilization with CO

The hemolysate, freed of cellular debris, is frequently rather unstable and the hemoglobins are often subject to slow oxidation. Maintaining the pH between 8 and 8.5 minimizes the oxidation but does not eliminate it. Untreated hemolysates should be kept cold and processed further as soon as possible unless it is desired to carry out functional studies on the intact hemolysate prior to further purification. If subsequent chromatography on DEAE-Sephadex or cellulose starting at a high pH is planned, the hemoglobin can often be kept in the oxy form, but attempts to chromatograph oxyhemoglobin on CM-cellulose or Sephadex starting at a pH < 7 are likely to produce disastrous quantities of methemoglobin. Saturation with carbon monoxide avoids this problem. The HbCO can readily be reconverted to HbO_2 later by exposure to sufficient light and oxygen. For this purpose the hemoglobin solution can be placed in a glass tonometer (300 ml volume) capable of being evacuated. The vessel is placed in an ice bath, and the gas phase is made 100% in oxygen. With gentle agitation the

[21] H. S. Baar and E. M. Hickmans, *J. Physiol. (London)* **100**, 3P (1941).

solution is illuminated with a convenient light source, such as the Sylvania Model SG-50 "Sun Gun" with a DWY lamp (120 v, 650 W). Since much heat is produced by this lamp, it is extremely important to keep the hemoglobin at 0°. The lamp should not be held closer than about 20 cm, and even then the exposure should be for only a few seconds. The gas phase should be replaced immediately by a fresh supply of oxygen and the process repeated. All excess oxygen should be removed both from the gas phase and the solution prior to warming.

Stripping and Ion Removal

After the discovery that organic phosphates bind to hemoglobin[22] and modulate oxygen binding,[23,24] much attention was devoted to procedures for removal of these agents. Dialysis against distilled water is completely ineffective in removing organic phosphates.[25] Indeed, a unique electrophoretic band of human oxyhemoglobin led to the original discovery of 2,3-diphosphoglycerate (2,3-DPG) binding.[22] Passage of the hemolysate through a column of Sephadex G-25 (1.5 × 45 cm) equilibrated with 0.1 *M* NaCl removes about 98% of the 2,3-DPG from a mouse hemolysate.[25] Since binding of 2,3-DPG to oxyhemoglobin is pH dependent and decreases greatly above pH 7, Berman *et al.*[26] found that phosphates could be effectively removed with an even shorter column of Sephadex G-25 (1.5 × 22 cm) equilibrated with 0.1 *M* NaCl if the pH of the sample was raised to 7.5. Although this procedure is effective for human hemoglobin A, the pH may not be high enough for the removal of phosphate from some hemoglobins. Hemoglobin Deer Lodge, for example, binds phosphates even at pH 9.[27] It is recommended, therefore, that the pH of stripping be maintained at or above pH 8, and pH 9 may be necessary for some hemoglobins. Hemoglobin so purified generally oxidizes faster than in the original hemolysate. The presence of trace quantities of copper enormously increases the rate of autoxidation of hemoglobin.[28] Such amounts are often found in hemolysates and in reagent grade buffers unless special precautions are taken. The rate of oxidation depends on the kind of hemoglobin and on the copper concentration. At pH 7.2 in 0.05 *M* Tris, one copper (CuII) is bound to every two hemes in horse hemoglobin with an apparent binding constant of $3 \times 10^5\ M^{-1}$. All of this is used to

[22] Y. Sugita and A. Chanutin, *Proc. Soc. Exp. Biol. Med.* **112,** 72 (1963).
[23] R. Benesch and R. E. Benesch, *Biochem. Biophys. Res. Commun.* **26,** 162 (1967).
[24] A. Chanutin and R. Curnish *Arch. Biochem. Biophys.* **121,** 96 (1967).
[25] S.Tomita and A. Riggs, *J. Biol. Chem.* **246,** 547 (1971).
[26] M. Berman, R. Benesch, and R. E. Benesch, *Arch. Biochem. Biophys.* **145,** 236 (1971).
[27] J. Bonaventura, C. Bonaventura, B. Sullivan, and G. Godette, *J. Biol. Chem.* **250,** 9250 (1975).
[28] J. M. Rifkind, *Biochemistry* **13,** 2475 (1974).

oxidize the heme rapidly.[29] Human hemoglobin oxidation requires a higher copper:heme ratio. Addition of 10^{-5} *M* EDTA or triethylenetetramine to a solution of purified horse oxyhemoglobin in (6.3×10^{-5} *M* heme) at pH 7.0, 4°, was completely effective in preventing oxidation for a period of 10 days.[29] Rifkind[29] has devised the following procedure for the rigorous removal of copper. Since contamination from reagents and glassware might contribute to the copper content of hemoglobin in preparations, Suprapur NaCl (E. Merck, Darmstadt, Germany) and filtered deionized water of 15 megohm resistance were used. The glassware was acid-washed and exhaustively rinsed with distilled water. The Sephadex columns were washed with EDTA prior to sample application.

The following procedure,[30] derived from the Dintzis method,[31] was found useful for a wide variety of Amazonian fish hemoglobins. The red cell lysate (≤ 5 ml) in 1 m*M* Tris pH 8.5 is passed through a Sephadex G-25 medium column (2×50 cm) equilibrated with the same buffer. The hemoglobin fraction without additional concentration is then deionized by passage through a 1.5×30 cm column consisting of the following resins, top to bottom: 2 cm of Dowex 1 (acetate form), 2 cm of Dowex 50-W (ammonium ion form), and 20 cm of Bio-Rad AG501-X8(D) mixed-bed ion-exchange resin. The resulting deionized oxyhemoglobin from most species of fish was quite stable and could be kept for a week or more at 4° without significant formation of methemoglobin.

Substantial quantities of methemoglobin are sometimes present *in vivo*. Indeed, turtles are sometimes found with 50–90% of their hemoglobin in this form.[32] This can be removed either with dithionite[33] or enzymically.[34,35] The decomposition and reaction products of dithionite cause undesirable changes in the hemoglobin. This problem has been overcome by Bauer and Pacyna,[33] whose procedure is convenient for routine use.

Dithionite Procedure.[33] All steps are done at 4°. A concentrated, deionized hemoglobin preparation (6–9 g/dl) in 0.5–1.0 ml is added to 1 ml of deaerated, deionized water containing 10 mg of purified sodium dithionite. *Within 60 sec* this solution is applied to a 1.5×50 cm column of a mixed-bed ion-exchange resin (Bio-Rad AG501-X8(D), Bio-Rad, Richmond, California). The solution is chased down the column with deionized water at 150 ml/hr. The emerging sample is oxyhemoglobin in de-

[29] J. M. Rifkind, L. D. Lauer, S. C. Chiang, and N. C. Li, *Biochemistry* **15,** 5337 (1976).
[30] R. L. Garlick, B. J. Davis, M. Farmer, H. J. Fyhn, U. E. H. Fyhn, R. W. Noble, D. A. Powers, A. Riggs, and R. E. Weber, *Comp. Biochem. Physiol.* **62A,** 239 (1979).
[31] Y. Nozaki and C. Tanford, this series, Vol. 11, p. 715.
[32] B. Sullivan and A. Riggs *Nature (London)* **204,** 1098 (1964).
[33] C. Bauer and B. Pacyna, *Anal.Biochem.* **65,** 445 (1975).
[34] A. Hayashi, T. Suzuki, and M. Shin, *Biochim. Biophys. Acta* **310,** 309 (1973).
[35] T. Suzuki, R. E. Benesch, S. Yung, and R. Benesch, *Anal. Biochem.* **55,** 249 (1973).

ionized water. It is important for this procedure that any salts be removed from the sample first. If this is not done, much of the hemoglobin may precipitate on the column. This procedure is much simpler than enzymic procedures, but the latter are still needed where experiments are to be done with oxyhemoglobin under conditions where methemoglobin forms rapidly.[34] The Bauer–Pacyna procedure has been found useful not only for mammalian hemoglobins, but also for those of amphibians and fish.[36]

Enzymic Procedures. The following procedure[35] is suitable for reducing methemoglobin, but the reagents must be removed by Sephadex chromatography prior to functional studies. The system has 2 μmol of NADH per mole of heme iron and 100 μg of pig heart diaphorase (Boehringer–Mannheim, New York) and 5×10^{-3} μmol of methylene blue per milliliter of 0.05 *M* Tris, pH 7.45, at room temperature. The reaction must be carried out anaerobically. This can be done conveniently in a Thunberg vessel with a 1 cm quartz cuvette, such as the Model 190 cell of Hellma Cells, Inc. (Hall Station, Jamaica, New York 11424.) The hemoglobin (in 0.05 *M* Tris, pH 7.45) is placed in the cuvette, with the reagents in the side arm; the vessel is evacuated or flushed with nitrogen, and the sidearm contents are then mixed with the hemoglobin solution. The reaction is complete within 15 min and can be followed at 555 nm. The solution is then cooled to 4° and chromatographed on Sephadex G-25. The second procedure[34] uses an NADPH generating system (NADP, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase) and has been widely used in association with the automated measurement of oxygen equilibria. It is described in this volume [25].

Detection and Isolation of Hemoglobin Components

General Considerations: Subunit Dissociation

All vertebrate blood hemoglobins except those of the primitive Agnatha[37] can be described as tetramers of two types of subunit, $\alpha_2\beta_2$. Tetramers can dissociate to dimers and those of some hemoglobins can also dissociate readily to monomers: $\alpha_2\beta_2 \rightleftarrows 2\alpha\beta \rightleftarrows 2\alpha + 2\beta$. These dissociation equilibria have important consequences for the isolation of hemoglobin components and for the determination of the number of components. A mixture of two hemoglobins, for example ligated human hemoglobins A($\alpha_2\beta_2^A$) and S($\alpha_2\beta_2^S$) show only two chromatographic or electrophoretic

[36] A. Riggs, unpublished observations.

[37] K. O. Pedersen, *in* "The Ultracentrifuge" (T. Svedberg and K. O. Pedersen, eds.), p. 355. Oxford Univ. Press, London and New York.

fractions. No hybrid species, $\alpha_2\beta^A\beta^S$, is detected. The reason for this is not that such hybrid species do not exist, but rather that the separatory procedures act on the tetramer–dimer dissociation equilibrium in such a way that the $\alpha\beta^A$ and $\alpha\beta^S$ dimers are separated from one another.[38,39] This sorting out depends on the magnitude of the tetramer–dimer dissociation constant, $K_{4,2}$, typically about 1 μM.[40] The value is about 10^{-12} M for deoxy or unligated human hemoglobins and is probably similar for the hemoglobins of other mammals.[41,42] For this reason deoxygenation effectively "freezes" the distribution of species, and so the hybrid deoxyhemoglobins, such as $\alpha_2\beta^A\beta^S$, appear as unique electrophoretic species.[43,44] Such hybrid hemoglobins have also been detected as the CO-derivative by isoelectric focusing and electrophoresis at $-20°$ to $-30°$.[45]

Thus. either very large or small values of $K_{4,2}$ can greatly alter the electrophoretic or chromatographic properties. Human hemoglobins Kansas and Hirose are examples of variants with greatly enhanced dissociation. The values of $K_{4,2}$ are about 10^{-4} M and probably about 1 M for HbCO Kansas and Hirose, respectively.[46,47] This means that these hemoglobin variants can be isolated readily by Sephadex G-100 chromatography alone.[47,48] Dissociation constants very much lower than 1 μM, however, can lead to the isolation of multiple hybrid hemoglobins. This occurs with many fish hemoglobins for which the $K_{4,2}$ values for HbO_2 are estimated to be at least 100 times smaller than for human hemoglobin.[49,50] Since many fish hemolysates contain multiple kinds of subunits in substantial proportions, hybrid hemoglobins are quite common.[51,52] Electrophoresis and chromatography can give rise to as many as 25 distinguish-

[38] G. Guidotti, W. Konigsberg, and L. C. Craig, *Proc. Natl. Acad. Sci. U.S.A.* **50,** 774 (1963).
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[43] H. F. Bunn and M. McDonough, *Biochemistry* **13,** 988 (1974).
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[48] H. F. Bunn, *J. Clin. Invest.* **48,** 126 (1969).
[49] M. Brunori, B. Giardina, E. Chiancone, C. Spagnuolo, I. Binotti, and E. Antonini, *Eur. J. Biochem.* **39,** 563 (1973).
[50] S. J. Edelstein, B. McEwen, and Q. H. Gibson, *J. Biol. Chem.* **251,** 7632 (1976).
[51] A Riggs, *Comp. Biochem. Physiol.* **62A,** 257 (1979).
[52] P. A. Mied and D. A. Powers, *J. Biol. Chem.* **253,** 3521 (1978).

able fractions.[53,54] Some hemoglobins, such as those of the bullfrog tadpole, dissociate significantly not only to dimers, but also to monomers.[55] Since electrophoretic and chromatographic properties of the α and β subunits are very different, unique fractions corresponding to these subunits can be isolated chromatographically.[13,56]

These general considerations raise questions about the meaning and significance of isolated components. It is a mistake to assume that the proportions of chromatographically or electrophoretically distinct components necessarily reflect the proportions of unique molecular species that exist as such within the red cell. The total concentration of hemoglobin is 100–1000 times higher in the cell than in these separatory procedures, so that the proportions of dissociated species will usually be smaller. Moreover, the procedure itself, acting on a dissociation equilibrium, will not necessarily produce amounts of fractions in the equilibrium proportions. Further problems also exist. For example, tadpole and adult bullfrog hemoglobins arise in different cell populations.[57,58] During metamorphosis, both populations exist and a hemolysate will contain both classes of hemoglobins. An electrophoretic component not detected in the hemolysates of either tadpole or adult[59] appears to be a hybrid hemoglobin. Since the parent hemoglobins occur in different cells, such a hybrid hemoglobin is an artifact of no physiological significance.

Analytical Gel Electrophoresis

Procedures. The most widely used technique for studying heterogeneity of hemoglobin in hemolysates is that of polyacrylamide gel electrophoresis. Either slab or disc gels may be used. However, if disc gels are used to compare hemoglobins that are very similar electrophoretically, an internal standard, such as bovine serum albumin, should be provided in each tube. The many techniques of analytical gel electrophoresis are described in detail by Gabriel[60] and need not be repeated here. However, specific formulations and procedures found useful for hemoglobins will be summarized for tube gels. They differ little from those originally de-

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[60] O. Gabriel, this series, Vol. 22, p. 565.

TABLE I
STOCK SOLUTIONS[a] FOR 7.5% ACRYLAMIDE GELS

	Solution	Volume ratio	Components per 100 ml of solution
Resolving gel, pH 8.9	A	1	Acrylamide, 30.0 g Bisacrylamide, 0.8 g
	B	1	1 *N* HCl, 24.0 ml Tris, 18.15 g TEMED[b] 0.4 ml
	C	1	Riboflavin, 1.0 mg or
	Water	1	Ammonium persulfate, 0.1 g
Stacking gel, pH 7.2	D	2	Acrylamide, 5.0 g Bisacrylamide, 1.25 g
	E	1	1 *M* H_3PO_4 12.8 ml Tris 2.85 g TEMED[b] 0.1 ml
	C	2	Ammonium persulfate 0.1 g
			Components per 1000 ml of solution
Upper buffer, pH 8.9			Tris, 6.32 g Glycine, 3.94 g
Lower buffer, pH 8.1			Tris, 12.1 g 1 *N* HCl, 50.0 ml

[a] All solutions should be stored at 4°. Solution C should be prepared fresh daily. Riboflavin is usually best because of effects of residual traces of persulfate on the protein.
[b] TEMED, *N,N',N',N'*-tetramethylethylenediamine.

scribed by Davis[61] and Ornstein.[62] A 7.5% acrylamide gel, pH 8.9, generally works well with hemoglobins with the cathode on top and the anode below so that the hemoglobins migrate downward. The formulations are provided in Table I. The procedure is as follows:

1. Coat the inside of the tubes with a 3% solution of Kodak Photoflo 600 and allow to dry in air.
2. Seal the bottoms of the tubes with Parafilm and insert into a rack capable of holding them exactly vertical.
3. Mix the components of the resolving gel and degas quickly (20 sec).
4. Add 0.9–1.0 ml of this mixture with a syringe or calibrated Pas-

[61] B. J. Davis, *Ann. N. Y. Acad. Sci.* **121,** 404 (1964).
[62] L. Ornstein, *Ann. N. Y. Acad. Sci.* **121,** 321 (1964).

teur pipette, and carefully put a 0.5-cm layer of water above the mixture *without disturbing* the surface.

5. Set the rack close to a set of fluorescent lamps if riboflavin is used and allow to polymerize (approximately 1 hr). If ammonium persulfate is used it is important that no excess remain at the time of sample application.
6. Remove the top water layer by inverting the rack on a paper towel. Be sure that all water is removed.
7. Mix stacking gel components (5 ml is enough) and add approximately 0.2 ml to each tube, then overlay with water as in step 4.
8. The gels can be stored overnight if covered with plastic wrap.
9. Remove top layer of water.
10. Insert gel tubes into apparatus from the bottom after wetting the outside of the tubes with water for lubrication. Remove the Parafilm.
11. Fill the bottom reservoir (anode) with lower buffer to the appropriate level, and place a drop of this buffer on the bottom of each tube to avoid air bubbles.
12. With tubes in position fill upper reservoir (cathode).
13. Sample application: Mix hemoglobin (absorbance = 1.0 at 580 nm), upper buffer, and glycerol in the volume proportions of 1:2:1. It is often desirable to make the upper buffer component for the mixture 0.1 *M* in 2-mercaptoethanol and to add a small quantity of dithionite. The mixture should be saturated with CO. No more than about 50 μl should be applied to each gel. Then add 5 μl of bromophenol blue solution (0.05% in upper buffer) as tracking dye. An alternative procedure for small samples is to combine 1 drop of Hb solution (absorbance approximately 1.0 at 580 nm), 1 drop of glycerol, 2 drops of upper buffer, and 1 drop of upper buffer containing 0.1 *M* 2-mercaptoethanol with a crystal of sodium dithionite.
14. Electrophoresis is done at 4° for 1–2 hr or until tracking dye is near bottom of tube. The voltage gradient should be about 16 V/cm with 1.5 mA per tube (with Bio-Rad apparatus Model 150A). If 12 tubes (12.5 × 5 mm i.d.) are used, the voltage would be about 200 V with about 18 mA. Although the driving force is the voltage difference, it is frequently convenient to carry out the electrophoresis at constant current because this will limit local heating. However, the voltage should be recorded.
15. Disconnect power supply and remove tubes. Gels may be removed from tubes by gently inserting a long needle between glass tubes and gel with water flowing from needle. Do this at each end.

Stains. The most specific and sensitive stain is undoubtedly benzidine. Unfortunately, it is an extremely potent carcinogen, known to produce bladder cancer in man.[15] Alternatives that have been proposed include 3,3′-dimethoxybenzidine (=*o*-dianisidine)[63] and 3,3′,5,5′-tetramethylbenzidine; these, too, appear to be carcinogenic, although less so.[15] If the investigator must use one of these compounds to detect minute quantities of hemoglobin (approximately 1 μg), the procedures and precautions given by Broyles *et al.*[64] and the OSHA standards[65] should be followed, at least for benzidine itself. Broyles *et al.*[64] found that benzidine at 0.07% in 1 *M* acetic acid provided an adequately sensitive stain if staining is lengthened to 20 min. They obtained quantitative results on amounts between 0.9 and 50 μg by the use of a gel scanner. The sensitivity increases 10–20% if the hemoglobin is converted to the CNMetHb form prior to electrophoresis. Although the OSHA regulations do not apply to concentrations of less than 0.1%, Broyles *et al.*[64] suggest that the precautions should not be relaxed. Their work was done while wearing full length protective clothing with gloves overlapping the sleeves and a respiratory mask that filters both particulates and organic vapors. All operations including weighing were done in a fume hood. Used staining solutions were accumulated in a marked closed vessel and finally mixed with diesel oil and incinerated at a high temperature. Quantitative results have also been obtained for 3,3′,5,-5′-tetramethylbenzidine, althought at a lower sensitivity.[66]

For most purposes benzidine or its derivatives are not necessary, and other dyes can be used. Staining with Coomassie Brilliant Blue R-250 (1.25 g in 250 ml of methanol, 250 ml of H_2O, 46 ml of acetic acid) works well and has good sensitivity. Gels can be destained with 14% methanol, 7% acetic acid. This wash solution can be decolorized with activated charcoal and filtered for reuse. An alternative staining technique with Coomassie Brilliant Blue of greater sensitivity is the following[67]: To one volume of 0.2% (w/v) aqueous solution of Coomassie Brilliant Blue is added an equal volume of 2 *N* H_2SO_4; the preparation is mixed well, set aside for 3 hr, and then filtered through Whatman No. 1 paper. One-ninth volume of 10 *N* KOH is added to the filtrate, which turns from brown to dark purple. Trichloroacetic acid (100% w/v) is added to make the final

[63] J. A. Owen, N. J. Silberman, C. Got, *Nature (London)* **182,** 1373 (1958).

[64] R. H. Broyles, B. M. Pack, S. Berger, and A. R. Dorn, *Anal. Biochem.* **94,** 211 (1979).

[65] General Industry OSHA Safety and Health Standards (29 CFR1910), OSHA 2206 (revised January, 1976), section 1910.1010, Benzidine, pp. 550–555, U.S. Dept. of Labor Occupational Safety and Health Administration.

[66] H. H. Liem, F. Cardenas, M. Tavassoli, M. B. Poh-Fitzpatrick, and U. Müller-Eberhard, *Anal. Biochem.* **98,** 388 (1979).

[67] R. W. Blakesley and J. A. Boezi, *Anal. Biochem.* **82,** 580 (1977).

light blue solution 12% (w/v). Gels placed in this solution achieve maximal development in 5–8 hr. Bands with as little as 5–10 μg of protein can be seen within 30 min.

Amido Black can also be used: 1% (w/v) in 7.5% (w/v) acetic acid. Filter before use. The excess stain can be removed by washing the gels in 7.5% acetic acid, but this is usually time consuming (approximately 36–48 hr).

Isoelectric Focusing

Gel Preparation. Isoelectric focusing on disc gels depends on the formation of a stable pH gradient. The limits of resolution depend on the distribution and nature of the ampholytes. Resolution of hemoglobins that differ in isoelectric point by only 0.02 pH unit can be achieved under certain circumstances, and almost always if they differ by 0.05 pH unit. In addition, the technique can be used to investigate subunit interactions and the changes in isoelectric point that result from deoxygenation—a reflection of the Bohr effect.[68] Thus, the technique is capable of providing a considerably greater amount of information than merely that required to identify the components of a heterogeneous system. For isoelectric focusing of deoxyhemoglobins, Model M 137A unit of the MRA Corporation (1058 Cephas Rd., Clearwater, Florida 33515) has proved to be convenient because samples can be applied anaerobically. The following procedure refers to this apparatus and is derived from the technique described by Righetti and Drysdale[69] and Drysdale *et al.*[70] The standard tubes for the MRA apparatus are 3 mm i.d. × 10 cm. These tubes are suitable for focusing 10–100 μg of protein per gel and can be obtained in quartz for direct ultraviolet scanning. A medium preparative unit (M137P) is suitable for 2–5 mg per gel, and a large-scale unit with three tubes, 15 × 2 cm, can fractionate up to 50 mg per gel. Tubes as small as 1.5 mm × 10 cm can be used to detect 1–10 μg of protein per gel. Five stock solutions (solutions A–E) are prepared as follows: (A) 5.0 g of acrylamide and 0.2 g of *N,N'*-methylenebisacrylamide dissolved in distilled H_2O to 25 ml; refrigerate; keeps about a week; (B) dissolve 0.5 g of ammonium persulfate per 2.5 ml of distilled H_2O; refrigerate; keeps no more than 3 days; (C) 0.01 *M* phosphoric acid (anolyte); (D) 0.01 *M* sodium hydroxide (catholyte); (E) 0.01 *M* KCN. Store in brown bottle. For 12 gel tubes mix the following solutions *in the order given:* (1) 14.1 ml of H_2O; (2) 4.8 ml of solution A; (3) 30 μl of *N,N',N',N'*-tetramethylethylenediamine (TEMED); (4) 0.9 ml of the appropriate ampholyte (LKB) for the pH range desired.

[68] H. F. Bunn and A. Riggs, *Comp. Biochem. Physiol.* **62A**, 95 (1979).
[69] P. Righetti and J. W. Drysdale, *Biochim. Biophys. Acta* **236**, 17 (1971).
[70] J. W. Drysdale, P. Righetti, and H. F. Bunn, *Biochim. Biophys. Acta* **229**, 42 (1971).

Usually this will be the pH 6–8 ampholyte (LKB). The mixture should be degassed by evacuation for approximately 20 sec. Then, (5) add 0.3 ml of solution B. Mix and *immediately* apply to tubes arranged vertically in rack with a double piece of Parafilm on the bottom of each. A long-tipped Pasteur pipette should be used for the application. The tubes should be filled to about 0.5 cm from the top. If there are air bubbles in the tubes, they can sometimes be dislodged with a sharp tap. *Carefully* float a layer of water above the gel filling of the tube. Let the gels polymerize at room temperature for 30– 40 min. If gelling is not achieved in this time the most probable cause is low catalytic activity of the ammonium persulfate, which slowly decomposes even in the dry state with production of ozone; in aqueous solution it decomposes with production of H_2O_2 and O_2.[71]

Prefocusing of the Gels. The tubes wetted with H_2O should be inserted into the apparatus so that equal lengths (about 0.5 cm) extend beyond the holding grommets. Coolant at 1° should be circulated through the water jacket for 15 min *before* applying current. The upper and lower chamber should now be filed to the mark with 0.01 *M* NaOH and 0.01 *M* phosphoric acid, respectively. The filling takes about 120 ml in each chamber. Be sure that all gel tube ends make contact with the buffers and that no air bubbles block the end. Make sure that all elements associated with the safety switch are dry to prevent an electrical short. Seat the top electrode; make sure that the safety switch is engaged (faint click). Now apply a current of about 0.5 mA per gel for 20 min.

Sample Application and Electrofocusing. After switching off the current and unplugging the power supply, the samples can be applied. Sample preparation is as follows: Prepare 200 μl of salt-free hemoglobin at a concentration of 10 mg/ml. Add 1 drop of 0.01 *M* KCN neutralized to pH 7.0. Add 1 drop of the appropriate ampholyte (2% solution in 10% sucrose or glycerol). Flush with CO. Apply 10–20 μl per gel tube. Apply current of 1 mA until voltage reaches 300–350 V. Set power supply at constant voltage and focus for about 3 hr or until bands appear sharp. After turning off power supply and removing gels from tubes (see gel electrophoresis above), place gels in 13 × 100 mm tubes and stain with 0.2% bromophenol blue in 50 ml of ethanol, 4 ml of acetic acid, and 45 ml of H_2O. Let stand for 20 min, then destain over 3–4 days with twice daily changes of the wash solution: 30 ml of ethanol, 5 ml of acetic acid, 65 ml of H_2O.

Quantification

Many methods have been used to determine the quantity of hemoglobin. Some, such as Fe and pyridine hemochromogen techniques do not

[71] N. V. Sidgwick, "The Chemical Elements and Their Compounds," p. 938. Oxford Univ. Press, London and New York, 1950.

depend on the initial ligand state of the hemoglobin. Others, such as oxygen binding capacity, measure only one form, i.e., oxyhemoglobin. Spectrophotometric techniques are clearly the most convenient to use and are capable of providing the proportions of different forms, HbO_2, Hb, MetHb. However, they must ultimately be calibrated by means of some primary technique such as Fe determination or pyridine hemochromogen. In principle, the latter is more specific because it excludes extraneous iron, but this should not be a problem for hemoglobin that has been purified as described above.

Although extinction coefficients have been accurately determined for human hemoglobin in various forms, corresponding measurements for the hemoglobins from other animals are almost nonexistent. Most spectral measurements of the quantity of animal hemoglobins simply assume that the extinction coefficients determined for human hemoglobin are appropriate: anthropomorphic spectrophotometry. Although the absorption spectra of animal hemoglobins are quite similar to those of human hemoglobin, they are probably seldom identical. The extinction coefficients of the hemoglobin of one fish ("spot") are reported[72] to be identical to those of human hemoglobin, but details are not provided. Spectral differences have been reported among the deoxyhemoglobins of a number of fish: the absorption maxima vary between 553 and 560 nm.[73] The early literature on hemoglobin has many references to spectroscopic differences,[74] but these differences have not been investigated by modern spectrophotometry. In view of the sparse data it seems necessary tentatively to utilize the extinction coefficients established for human hemoglobins.

The Pyridine Hemochromogen Method. This method, developed by de Duve,[75] requires some care in its execution. A weighed sample of authentic hemin should always be used as a standard. A suitable hemoglobin or hemin sample should be in the concentration range 20–60 μM (heme). The sample is prepared by adding 3.0 ml of an alkaline pyridine solution to 1.0 ml of the hemoglobin or hemin solution in a 1 cm cuvette and mixing thoroughly. The pyridine solution is made by mixing 100 ml of pyridine (redistilled over ninhydrin, bp 115°) with 30 ml of 1 N NaOH and making up to 300 ml with distilled H_2O. An excess of solid sodium dithionite is dissolved with stirring in a 2.0-ml aliquot to yield the reduced pyridine hemochromogen, which is stable for only a few minutes. The absorbance at 557 nm should be recorded immediately. The other 2.0-ml ali-

[72] C. Bonaventura, B. Sullivan, and J. Bonaventura, *J. Biol. Chem.* **251,** 1871 (1976).

[73] M. Farmer, H. J. Fyhn, U. E. H. Fyhn, and R. W. Noble, *Comp. Biochem. Physiol.* **62A,** 115 (1979).

[74] J. Barcroft, "The Respiratory Function of the Blood," Part II, "Haemoglobin," pp. 48–49. Cambridge Univ. Press, London and New York, 1928.

[75] C. de Duve, *Acta. Chem. Scand.* **2,** 264 (1948).

quot is kept for 12–24 hr, and the same procedure is followed to ensure that all the heme has been extracted from the protein. The extraction effectiveness can be tested by determining the absorbance ratio, $A_{557}:A_{540}$, which should be 3.55 for the hemochromogen.[75] The millimolar extinction coefficient is 32.0 at 557 nm for reduced pyridine hemochromogen.

Iron Determination. A convenient method for the determination of iron is that of Cameron,[76] in which the protein is digested by perchloric acid–hydrogen peroxide and the iron liberated is determined as the ferrous-*o*-phenanthroline complex stabilized in 10% pyridine. The reagents (all analytical grade) are: 70% perchloric acid, redistilled (G. Frederick Smith Chemical Co.); 30% hydrogen peroxide, 10% aqueous hydroxylamine; 1,10-phenanthroline, 0.5% in 50% ethanol; and pyridine. The hydroxylamine-HCl should be recrystallized from 92% acetic acid to remove traces of iron. It may be necessary to purify the pyridine by passing it through a column of silica gel and subsequent distillatin from potassium hydroxide. Standard iron solutions can be made from $Fe(NH_4)_2(SO_4)_2 \cdot 6\ H_2O$ (Mohr's salt) or by dissolving pure iron wire in concentrated HCl.

A hemoglobin sample in not more than 0.1 ml containing 10–50 μg of iron is introduced by micropipette into a 10-ml volumetric flask, and 0.1 ml of perchloric acid and 0.1 ml of hydrogen peroxide solution are added. The flask is heated at 100° for 30 min, then cooled and 0.1 ml of 10% aqueous hydroxylamine is added. After 5 min, add 1.0 ml of the *o*-phenanthroline reagent, and then *at once* add 1.0 ml of pyridine. The solution is made up to 10 ml with iron-free water. The absorbance is measured at 509 nm, and the reagent blank is subtracted. The extinction coefficient, ϵ_{mM}^{509nm}, is 11.0.

Quantification of Hemoglobin Mixtures by Spectrophotometry. The most widely used spectrophotometric reference for hemoglobin is the CNMetHb form, which has become the clinical standard.[77] The following procedure is suitable for hemoglobin solutions. Dissolve 200 mg of $K_3Fe(CN)_6$, 50 mg of KCN, and 140 mg of KH_2PO_4 in distilled water and make up to 1 liter with H_2O. The pH should be 7–7.4, and the solution should be stored in a brown borosilicate bottle. van Assendelft[77] utilizes a detergent in this preparation [0.5 ml of Sterox-SE (Hartman-Leddon, Philadelphia) or Nonidet P-40 (Shell International Chemical Co., The Hague, Netherlands)] because his technique is intended for use with whole blood. However, if the hemoglobin is purified as described above, this is unnecessary. To 5.0 ml of above solution add 20 μl of the hemoglobin solution, let stand 15 min, then measure absorbance at 540 nm in a

[76] B. F. Cameron, *Anal. Biochem.* **11**, 164 (1965).

[77] O. W. van Assendelft, "Spectrophotometry of Haemoglobin Derivatives," p. 152. Royal Vangorcum, Ltd., Assen, The Netherlands, 1970.

TABLE II
MOLAR EXTINCTION COEFFICIENTS ($\times 10^{-4}$)[a]

λ (nm)	DeoxyHb		OxyHb		HbCO	
	1	2	1	2	1	2
540	1.03	1.03	1.46	1.43	1.38	1.43
560	1.28	1.27	0.867	0.85	1.17	1.21
570	1.11	1.10	1.18	1.19	1.44	1.42
576	0.97	0.98	1.58	1.53	1.08	1.12
630	0.11	0.10	0.014	0.02	0.022	0.02

[a] Values are from van Assendelft and Zijlstra.[79] Columns 1 give values from Benesch *et al.*[78] corrected for the millimolar extinction coefficient value of 11.0 at 540 nm for cyanomethemoglobin. Columns 2 give values determined by van Assendelft and associates.[77,79] The small differences indicate the uncertainty with which the values are known for oxy- and carbonylhemoglobins.

1-cm cuvette. If hemoglobin is very concentrated use 10 μl of Hb solution, and if very dilute use only 2.5 ml of the diluent solution. If the minimal molecular weight on a heme basis is 16125, the amount of hemoglobin may be calculated by multiplying the absorbance by 1.47, by the dilution factor, and by the total volume to give the amount in milligrams. Multiplication of the absorbance by 9.09×10^{-5} will give the molar concentration.

Benesch *et al.*[78] have determined the extinction coefficients of oxyhemoglobin, carbonylhemoglobin, and deoxyhemoglobin and of methemoglobin at various pH values. Their values, based on a millimolar extinction coefficient of 11.5 for cyanomethemoglobin at 540 nm, have been corrected by van Assendelft and Zijlstra[79] to conform to the currently accepted value, 11.0 for cyanomethemoglobin; the results are given in Tables II and III, together with values given by van Assendelft and Zijlstra.[77,79] The values for methemoglobin in Table III were determined at room temperature. The pH for the ionization of the water molecule in aquomethemoglobin is temperature dependent, therefore these values will vary with temperature. Since the apparent enthalphy for this ionization varies substantially with different animal hemoglobins,[80] some caution is needed in the use of Table III at temperatures that differ from 20–25°. The ΔH values for the ionization vary between 3400 cal/mol for human hemoglobin and 9160 cal/mol for pigeon hemoglobin.[80]

Some additional cautions need to be observed in the use of these extinction coefficients. Small quantities of both HbCO and methemoglobin

[78] R. E. Benesch, R. Benesch, and S. Yung, *Anal. Biochem.* **55**, 245 (1973).
[79] O. W. van Assendelft and W. G. Zijlstra, *Anal. Biochem.* **69**, 43 (1975).
[80] J. G. Beetlestone and D. H. Irvine, *J. Chem. Soc.* **1964**, 5090 (1964).

TABLE III
MOLAR EXTINCTION COEFFICIENTS ($\times 10^{-4}$) OF METHEMOGLOBIN AS A FUNCTION OF pH[a]

pH	540 nm	560 nm	570 nm	576 nm	630 nm
6.2	0.583	0.357	0.340	0.342	0.394
6.4	0.583	0.358	0.341	0.344	0.392
6.6	0.586	0.363	0.349	0.354	0.391
6.8	0.596	0.372	0.363	0.368	0.388
7.0	0.610	0.387	0.383	0.388	0.384
7.2	0.629	0.406	0.407	0.414	0.376
7.4	0.652	0.430	0.435	0.445	0.363
7.6	0.679	0.460	0.471	0.485	0.344
7.8	0.714	0.497	0.514	0.534	0.310
8.0	0.754	0.542	0.564	0.593	0.293
8.2	0.799	0.589	0.619	0.652	0.268
8.4	0.844	0.636	0.672	0.713	0.243
8.6	0.886	0.679	0.722	0.765	0.220
8.8	0.922	0.716	0.766	0.813	0.199

[a] Values were determined by Benesch *et al.*[78] as corrected by van Assendelft and Zijlstra.[79]

are likely to be present in all hemoglobin samples. Human blood from nonsmokers in CO-free air contains about 0.45% HbCO[81] from the methylene bridges of porphyrins during heme degradation. The methemoglobin for Tables II and III was prepared by oxidation with $K_3Fe(CN)_6$ followed by chromatography on Sephadex G-25 on 0.1 *M* NaCl and 5°.[78] Under these circumstances, the ferrocyanide produced accompanies the hemoglobin[78] and about 1% of the heme is reduced on the column, and so oxyhemoglobin is formed.[82] After 3–5 days the HbO_2 content rises by as much as 4%. The ferrocyanide anion probably binds to the same site to which diphosphoglycerate binds.[83] Keilin[84] has cautioned that some cyanomethemoglobin forms if methemoglobin is oxidized with ferricyanide in the light because ferricyanide under goes slow photodecomposition.[85]

Tomita *et al.*[86] have devised a useful method for estimating the methe-

[81] R. D. Stewart, E. D. Baretta, L. R. Platte, E. B. Stewart, J. H. Kalbfeisch, B. van Yserloo, and A. A. Rimm, *Science* **182,** 1362 (1973).

[82] R. E. Linder, R. Records, G. Barth, E. Bunnenberg, C. Djerassi, B. E. Hedlund, A., Rosenberg, E. S. Benson, L. Seamans, and A. Moscowitz, *Anal. Biochem.* **90,** 474 (1978).

[83] J. V. Kilmartin, *Biochem. J.* **133,** 725 (1973).

[84] D. Keilin, *Nature (London)* **190,** 717 (1961).

[85] J. S. Haldane, *J. Physiol. (London)* **25,** 230 (1900).

[86] S. Tomita, Y. Enoki, M. Santa, H. Yoshida, and Y. Yasumitsu, *J. Nara Med. Assoc.* **19,** 1 (1968).

moglobin content of a dilute oxy- or carbonylhemoglobin solution. Their method depends on conversion to the CNMetHb form and is independent of pH. A few crystals of KCN are added to 5 ml of the hemoglobin solution. Let stand 20 min at 20–25° (not at 38° as originally described[86]), then measure the absorbance, A_1, at one of the following wavelengths: if HbO_2, at 542 nm or 576 nm; if HbCO, at 540 or 570 nm. Then add a few crystals of $K_3Fe(CN)_6$ to the same solution warmed to 38°. After 20 min (for HbO_2) or 40 min (for HbCO) measure the absorbance, A_2, at the same wavelength. The percentage of methemoglobin, α, is given by the expression

$$\alpha = \left(\frac{k}{k-1} - \frac{1}{k-1} \times \frac{A_1}{A_2}\right) \times 100$$

where k is 2.285 (λ = 576 nm, HbO_2), 1.319 (λ = 542 nm, HbO_2), 1.776 (λ = 570 nm, HbCO), 1.298 (λ = 540 nm, HbCO). k is the ratio of the extinction coefficients $\epsilon_{HbO_2}/\epsilon_{CNMetHb}$.

Gel Chromatography

Some human hemoglobin variants can be readily isolated by chromatography on Sephadex G-100 or a similar matrix. These are hemoglobins that are largely or entirely dissociated to dimers in the ligated state. Thus, both hemoglobins Kansas and Hirose can be effectively isolated by this procedure. For example, chromatography of a mixture of carbonylhemoglobins A and Hirose on a 1.6 × 86.5 cm column of Sephadex G-100 equilibrated with 0.05 *M* Tris, 0.1 *M* NaCl, 1 m*M* EDTA, pH 7.6, at 26° yielded two distinct peaks, the second of which was the dimeric variant.[47] The major hemoglobin components, B and C, of the adult bullfrog may be similarly separated, but for a different reason.[87] Component C polymerizes by disulfide bond formation, but component B remains tetrameric. Components B and C were separated as follows. The mixture was incubated at 25° in 0.1 *M* phosphate buffer, pH 7.5, for 60 hr as HbO_2. During this time all of component C polymerizes to octamers and larger aggregates. A small amount of the polymer precipitates and can be removed by centrifugation. Approximately 0.7–1.0 g of hemoglobin in 15–20 ml of 0.1 *M* phosphate, pH 7.5, were applied to a column of Sephadex G-200 (5 × 100 cm) arranged for upward flow at 65 ml/hr. Two fractions were isolated; the first contained polymers of various sizes ($s_{20,w} \geq 7$), and the second was tetrameric ($s_{20,w} \simeq 4$). The latter could not be made to polymerize by further oxidaton and consisted entirely of component B.

Since disulfide polymers are of wide occurrence in amphibian and reptilian hemoglobins, similar procedures may be useful for them. Further-

[87] T. O. Baldwin and A. Riggs, *J. Biol. Chem.* **249**, 6110 (1974).

more, since some hemoglobins from amphibians and birds aggregate beyond the tetramer upon deoxygenation[88–90] anaerobic chromatograpahy may be useful for isolating certain of these components.

Ion-Exchange Chromatography

The most useful media for the isolation of pure components of hemoglobin by ion exchange are those that contain either the anionic diethylaminoethyl (DEAE) or cationic carboxymethyl (CM) groups, with a matrix of either cellulose or Sephadex. In general, hemoglobins are eluted from these materials either with a pH gradient or at constant pH with a salt gradient. The chromatography of human hemoglobin variants has been described in great detail by Schroeder and Huisman.[16] Many of their procedures can be used directly or adapted for other hemoglobin systems.

Sephadex and cellulose have very different physical properties. Columns of cellulose derivatives are denser than those of Sephadex, and with some systems the hemoglobin components are eluted in more compact zones. Sephadex columns are spongy and very sensitive to pressure and flow rate. A common procedure has been to use CM-Sephadex or CM-cellulose with a positive pH gradient. Since the starting pH is usually <7, chromatography with HbO_2 results, as discussed earlier, in much methemoglobin and the formation of spurious chromatographic fractions. Although it is possible to camouflage this effect by addition of KCN, it is often better to avoid methemoglobin as much as possible, especially if the desired product is HbO_2 for functional studies. This can be done by saturation of the hemoglobin and all buffers with CO and its subsequent removal by light (see above). Alternatively, the use of DEAE-Sephadex or cellulose with HbO_2, at a high starting pH (>8) usually results in virtually no methemoglobin formation. For these reasons DEAE-Sephadex and DEAE-cellulose are widely used.

CM-Cellulose. The microgranular preswollen CM-52 (Whatman) provides very reproducible results. Most procedures described are similar to that of Huisman and Wrightstone,[91] a modification of which is described here. If oxidation to methemoglobin does not matter, 100 mg of KCN per liter can be added to all buffers. Otherwise, the hemoglobin and buffers should be saturated with CO. Equilibrate 100 g of CM-52 with 0.01 *M*

[88] R. Elli, A. Guiliani, L. Tentori, E. Chianconi, and E. Antonini, *Comp. Biochem. Physiol.* **36,** 163 (1970).

[89] T. Araki, T. Okazaki, A. Kajita, and R. Shukuya, *Biochim. Biophys. Acta* **351,** 427 (1974).

[90] J. S. Morrow, R. J. Wittebort, and F. R. N. Gurd, *Biochem. Biophys. Res. Commun.* **60,** 1058 (1974).

[91] T. H. J. Huisman and R. N. Wrightstone, *J. Chromatogr.* **92,** 391 (1974).

phosphate, pH 6.7, overnight; discard the supernatant and repeat twice at 2-hr intervals. A slurry of the cellulose and buffer in the proportion 2:1 is poured to make a column of 1.8 × 35 cm and equilibrated with this buffer at a flow rate of about 30 ml/hr. The hemoglobin (approximately 50 mg) should be dialyzed overnight against the equilibration buffer prior to application. The column can readily be scaled up for larger quantities. Thus, a 2.8 × 50 cm column can readily handle 1.2 g of hemoglobin in a hemolysate containing approximately equal quantities of hemoglobins A and Kansas.[92] Although pH 6.7–6.8 is sufficiently low for most mammalian hemoglobins, pH 6.5 has been found necessary for tadpole hemoglobins.[13] The sample can be applied in one of two ways. The sample can be stirred into the first 5 mm of the column; the glass above the column should be washed with 0.5 ml of buffer. Alternatively, the sample can be applied to the top of the column without disturbing it. The 1.8 × 35 cm column can be eluted by using a 250-ml constant-volume mixing chamber initially containing 0.01 *M* phosphate, pH 6.9, into which flows 0.01 *M* phosphate of a higher pH (usually 7.4). After 24 hr, the pH of the buffer entering the mixing chamber is increased by 0.2 pH unit. This procedure has been used for the analysis of a variety of normal and abnormal hemoglobins.[16] The elution position depends to a substantial extent on the surface topology of charge distribution and the accessibility of groups to the ion-exchange groups. It is striking that four human hemoglobin variants, Hb C,Hb E, Hb Agenogi, and Hb O-Arab, with the same substitution of Glu → Lys at $\beta 6$, $\beta 26$, $\beta 90$, and $\beta 121$, are eluted at quite different positions.[16] Generally, however, the elution pH is related to the electrophoretic mobility at pH 8.9: the higher the mobility, the lower is the pH of elution.

The chromatography of hemoglobin Kansas is instructive.[92] A linear gradient is made between 1 liter of 0.01 *M* phosphate at pH 7 and 1 liter of the same buffer at pH 7.5. The chromatogram illustrates some of the problems that can occur. Since Hb Kansas is a variant with a neutral substitution ($\beta 102$ Asn → Thr) the separation might appear not to depend on a net charge difference. However, HbCO Kansas is largely dimeric.[46,92] The tetramer to dimer reaction is accompanied by an uptake of 0.6 H^+ per mole of tetramer,[46] so the dimers are more positively charged than the tetramers and have a different surface topology. This small difference may be enough to provide the excellent resolution. Two chromatographic peaks are resolved, the second of which is the Kansas variant. The first fraction, however, initially assumed to be hemoglobin A,[92] now turns out to be a mixture containing both β^A and β^{Kansas} subunits. Close observation of the column reveals that this fraction initially comprises two distinct

[92] J. Bonaventura and A. Riggs, *J. Biol. Chem.* **243**, 980 (1968).

bands, which soon fuse as fraction 1.[93] One of the bands turns out to be Hb A; the other, a hybrid.

Further problems occur in the chromatography if dissociation to monomers occurs. Thus, the chromatograms of tadpole hemoglobins on CM-cellulose[13,56] have a fraction that elutes at pH 6.5 consisting of β subunits and another fraction eluting at pH > 7.6 consisting of α subunits. Simple equimolar mixing of these gives a component corresponding to one that elutes at about pH 7.2.[13]

DEAE-Sephadex. This material has been extensively used for hemoglobin since the work of Dozy *et al.*[94] DEAE-Sephadex A-50, beads, 40–120 μm, can be used to form a 0.9 × 50 cm column (suitable for 50 mg of HbO_2) equilibrated with 0.05 *M* Tris, pH 8.5. The hemoglobin can be eluted with a gradient between 0.05 *M* Tris, pH 8.5, and 0.05 *M* Tris, pH 6.5. The gradient can be established with a closed mixing chamber of 500 ml volume initially with pH 8.3, 0.05 *M* Tris-HCl and into which a lower pH buffer is delivered. In this system, the elution of the hemoglobin depends largely on the buffer pH.

The procedure has been scaled up and modified for tadpole hemoglobin as follows[95]: A hemolysate (6–8 ml) from bullfrog tadpoles is dialyzed overnight against 0.05 *M* Tris, pH 7.7. For 1.3 or 5.6 g of Hb one can use 3.8 × 42 cm (flow, 30 ml/hr) or 8.8 × 40 cm (flow, 170 ml/hr) columns, respectively. For each column 300 ml of the initial buffer is passed through the column, and then a linear gradient is started. For the smaller column the gradient uses 1 liter each of pH 7.7 and 6.7 buffers; for the larger column, 5.4 liters of each buffer are required. When the gradient is almost complete, four bands are visible on the column. The first three bands are eluted with 0.05 *M* Tris, pH 6.6, and the last band is eluted with the same buffer containing 0.07 *M* NaCl.

The problems of tetramer–dimer–monomer dissociation do not seem to be present with this procedure, in contrast to that described for the chromatography of tadpole hemoglobin on CM-cellulose. It is significant that all attempts to isolate Hb Kansas on DEAE-Sephadex have failed completely in contrast to the excellent separation on CM-cellulose. It would appear that subunit dissociation properties reflected in the chromatography of both tadpole Hb and Hb Kansas on CM-cellulose are not expressed on DEAE-Sephadex in spite of the fact that the two hemoglobins are significantly dissociated at the high pH of the DEAE-Sephadex chromatography.

[93] Q. H. Gibson, A. Riggs, and T. Imamura, *J. Biol. Chem.* **248,** 5976 (1973).
[94] A. M. Dozy, E. F. Kleihauer, and T. H. J. Huisman, *J. Chromatogr.* **32,** 723 (1968).
[95] K. W. K. Watt and A. Riggs, *J. Biol. Chem.* **250,** 5934 (1975).

DEAE-Cellulose. The use of this material for human hemoglobin variants has been described by Abraham *et al.*[96] and Schroeder and Huisman[16] and will be summarized here. Several advantages over DEAE-Sephadex are apparent: the procedure is often faster, the columns are more easily poured, and the separated chromatographic bands are more compact. Whatman DE-52 is granular, preswollen, and ready to use; 100 g should be mixed with 300 ml of 0.2 *M* glycine, the pH adjusted to 7.8 with 2 *M* HCl, and a 1 × 25 cm column prepared (suitable for 30 mg of Hb). Elution of the hemoglobin is accomplished with a salt gradient as follows: A closed 500-ml vessel leading to the column is prepared with 0.005 *M* NaCl, 0.2 *M* glycine. Entering this vessel is 0.2 *M* glycine containing 0.03 *M* NaCl. At the end of the gradient, the column can be washed with 0.2 *M* NaCl, 0.2 *M* glycine.

The following procedure, derived from earlier techniques,[97,98] has routinely been used for gram quantities of carp hemoglobin.[99] A 2.5 × 25 cm column of DE-52 (Whatman), is prepared at 4° with Tris-HCl buffer (10 m*M* Cl^-), pH 8.2 (measured at 20°). The hemoglobin is stripped and deionized as described in the section on preparation of hemolysate. The first component (A) elutes with this buffer in 2–3 hr, followed by the second component (B). Elution of fraction C is accomplished by elution with Tris-HCl buffer, pH 8.2, 12.5 m*M* in Cl^-. The hemoglobin can normally be run as HbO_2. This procedure illustrates the use of DEAE-cellulose for chromatography of hemoglobin with high isoelectric points—often with relatively little change in initial conditions.

Preparation of Human Hemoglobin A_0 on DEAE-Sephadex. For many studies it is useful to be able to prepare large quantities of the major component, A_0, of human hemoglobin, in which the formation of methemoglobin is minimized. Since methemoglobin forms most rapidly in dilute solution, Williams and Tsay[100] have devised an effective procedure for preparing the major component and simultaneously concentrating it in less than 36 hr. Their procedure produces oxyhemoglobin A_0 at a concentration of 6 g/dl with a methemoglobin content of about 0.5%. The procedure is as follows.

Blood (40 ml) is drawn into Vacutainers (Becton-Dickenson, Inc.) containing the ACD anticoagulant. The red cells are washed four times with 0.9% NaCl, and the top layer of white cells is removed. The red cells are combined with 2–3 volumes of 0.05 *M* Tris, pH 8.0, and dialyzed for

[96] E. C. Abraham, A. Reese, M. Stallings, and T. H. J. Huisman, *Hemoglobin* **1**, 27 (1976–1977).
[97] A. L. Tan, A. DeYoung, and R. W. Noble, *J. Biol. Chem.* **247**, 2493 (1972).
[98] R. G. Gillen and A. Riggs, *J. Biol. Chem.* **247**, 6039 (1972).
[99] A. DeYoung, and R. W. Noble, private communication, 1980.
[100] R. C. Williams and K.-Y. Tsay, *Anal. Biochem.* **54**, 137 (1973).

1 hr against 1 liter of the same buffer. The Tris is the "ultra-pure" grade (Mann Laboratories, Inc.). The dialysis tubing is boiled with 10^{-4} *M* EDTA and is then washed exhaustively with deionized water before use. All steps of the preparation are done at 4°. The lysate is removed from the dialysis bag and centrifuged at 10,000 *g* for 30 min. The solution is removed from the center two-thirds of the tube and diluted with an equal volume of 0.05 *M* Tris, pH 8.0; the centrifugation process is repeated. About 40 ml of solution (approximately 2.0 g of hemoglobin) are applied to a 4 × 60 cm column of DEAE-Sephadex A-50 thoroughly equilibrated with 0.05 *M* Tris, pH 7.6 ± 0.03. Elution is done with this buffer at no more than 160 ml/hr. The eluent is directed to a fraction collector by a three-way valve until the desired fraction emerges. This fraction is then directed through one channel of a two-channel peristaltic pump and is mixed with an equal volume of 0.05 *M* Tris, pH 8.4, coming through the other channel. The mixture than enters a short collection column of DEAE-Sephadex A-50. After collection of the chosen fraction (A_0), the column is removed and eluted with 0.2 *M* NaCl in 0.1 *M* Tris, pH 7.4. The use of such a collection column should be generally useful for many hemoglobins, especially those of lower vertebrates, which may be particularly unstable.

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[2] Preparation of Myoglobins

By JONATHAN B. WITTENBERG and BEATRICE A. WITTENBERG

This chapter presents procedures for the isolation of intracellular oxygen-binding proteins of tissues, called tissue hemoglobins in the widest sense. All of these, except *Ascaris* and yeast hemoglobin, are monomers or dimers having a minimum molecular weight of 18,000 with similar optical spectra and chemical reactivity. Strictly, only muscle hemoglobin should be called myoglobin; by extension the term is often applied to other tissue hemoglobins as well. Isolation of leghemoglobin, the tissue hemoglobin of plants, has been treated in this series.[1] Monomeric blood

[1] M. J. Dilworth, this series, Vol. 69 [74].

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