Hemoglobin Synthesis and RBC Testing Methods

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Today's Discussion

- Hemoglobin Structure
- Hemoglobin Assembly and Regulation
- Hemoglobin Dissociation Curve
- Carbon Dioxide Transport and Nitric Oxide Transport
- Dyshemoglobins
- Hemoglobin Testing

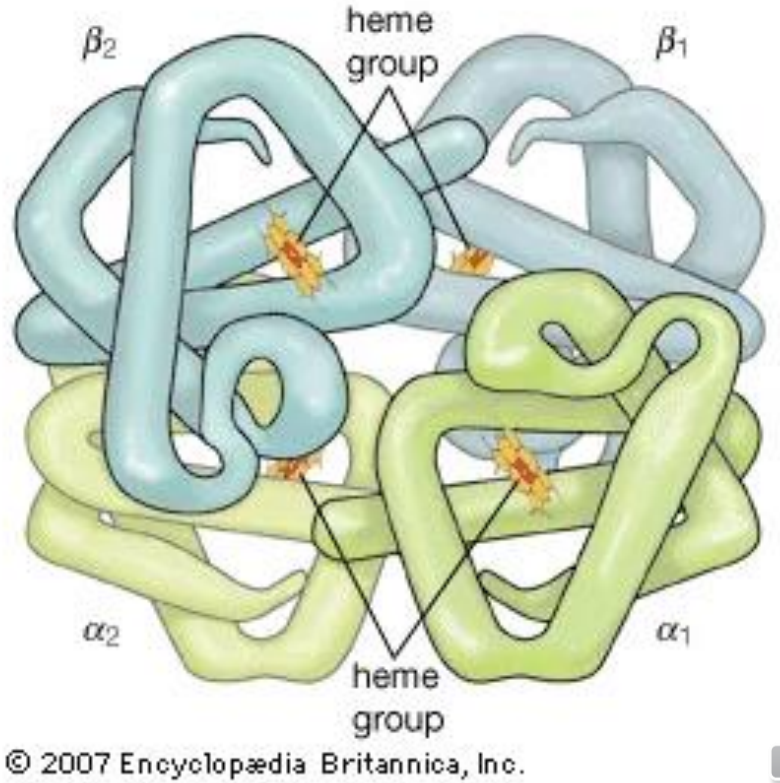
Hemoglobin Structure

Hemoglobin

- Protein that effectively transports oxygen from the lungs to the tissues, and transports carbon dioxide from the tissues to the lungs
- Not produced in mature RBCs
- Transported in RBCs for protection
  - In plasma, hemoglobin is denatured into iron and amino acids
  - In kidney, hemoglobin is excreted when salvage capacity is exceeded
- Concentration of hemoglobin in RBC= 32 g/dL
  - 95% of cytoplasmic content
  - Studies suggest close to 270 million hemoglobin molecules per RBC

Hemoglobin structure

- Globular protein consisting of:
  - 2 different pairs of polypeptide chains (4 polypeptide chains total
    - These are known as the “globin” chains
    - α-like and non-α like (β-like)
  - 4 heme groups
    - 1 heme group is embedded into each of the polypeptide chains
- 1 oxygen molecule carried by each heme molecule
  - 4 oxygen molecules carried per hemoglobin

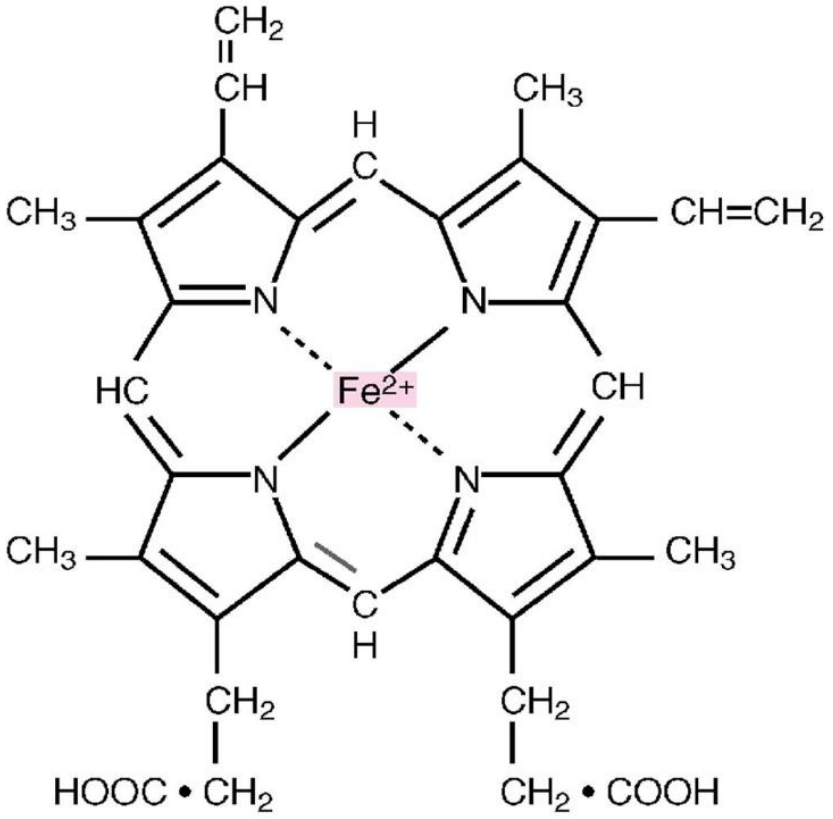


https://www.britannica.com/science/hemoglobin

Heme structure

- Consists of
  - Protoporphyrin IX
    - Ring of carbon, hydrogen, and nitrogen atoms
  - Central atom of divalent ferrous iron (Fe^{2+})
    - Reversibly binds with 1 oxygen molecule
    - When oxidized to ferric state (Fe^{3+}) can no longer bind oxygen

- Each heme group is in a pocket of the polypeptide chain near the surface of the hemoglobin molecule



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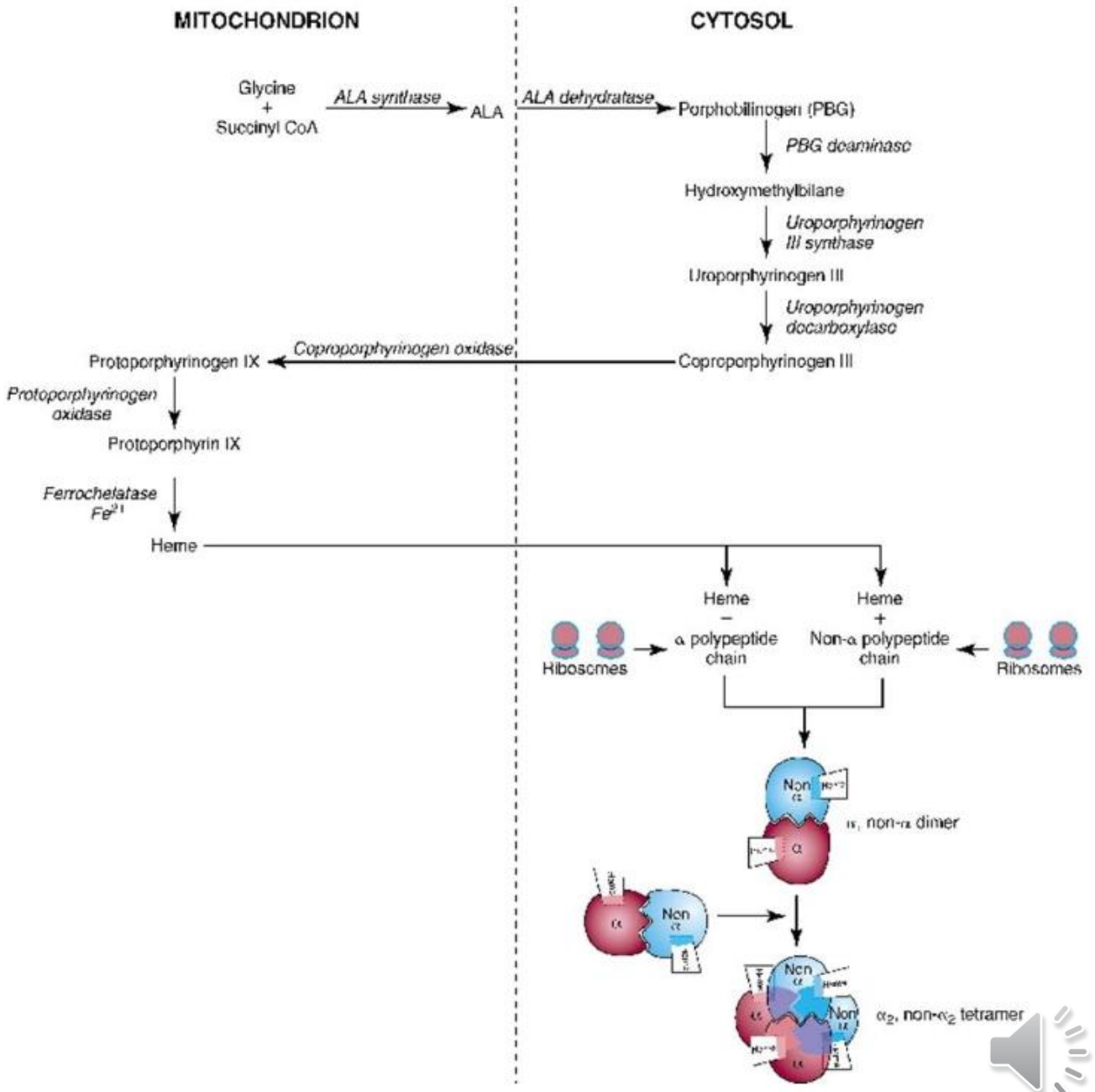
Heme Biosynthesis

- Occurs in the mitochondria and cytoplasm of BM erythroid precursors
  - Pronormoblast → polychromatic erythrocyte
- Steps:
  - 1. Begins in the mitochondria
    - Glycine + succinyl CoA →aminolevulinic acid (ALA)
      - Catalyzed by aminolevulinate synthase
  - 2. ALA moves into the cytoplasm
    - ALA catalyzed by aminolevulinic acid dehydratase → porphobilinogen (PBG)
      - Then converted to hydroxymethlbilane → → coproporphyrinogen III
  - 3. Coproperphyrinogen III into Mitochondria
    - Several steps occur
    - Fe^{2+} + protoporphyrin IX → heme
      - Catalyzed by ferrochelatase (heme synthase)



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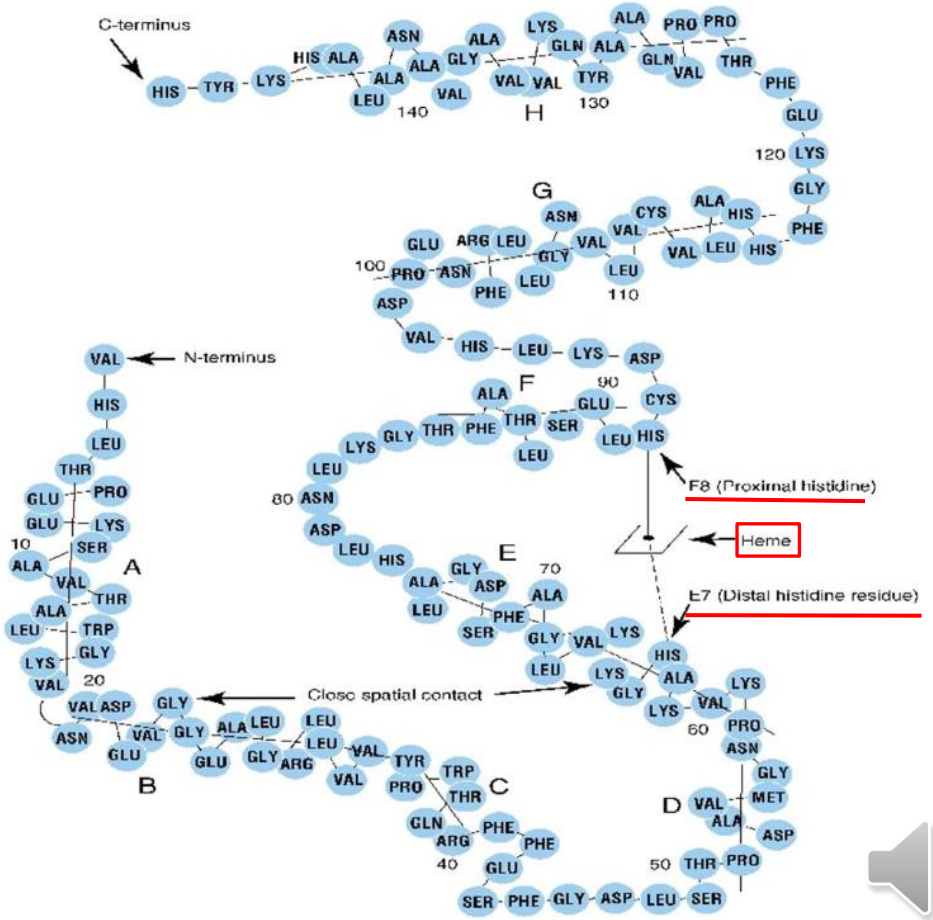
Globin Structure

- Four globin chains comprising each hemoglobin molecule consist of two identical pairs of unlike polypeptide chains
  - 141-146 amino acids each
  - Different chains give rise from amino acid variations
- Chains are designated by a Greek letter
  - α alpha
  - β beta
  - γ gamma
  - δ delta
  - ε epsilon
  - ζ zeta
  - ϑ theta
- Globin chains loop to form a cleft pocket for heme
  - Suspended between the E and F helices of each chain



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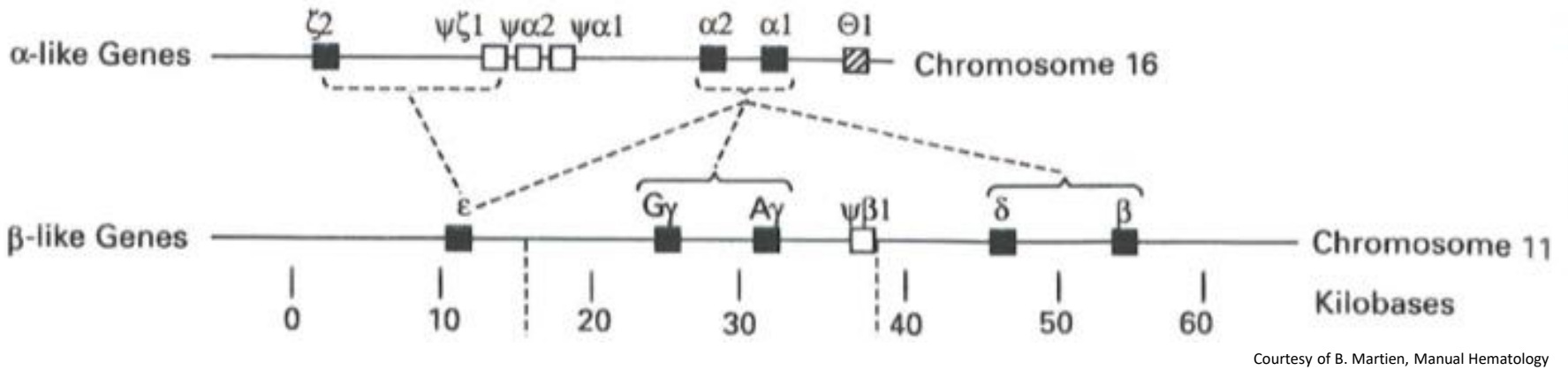
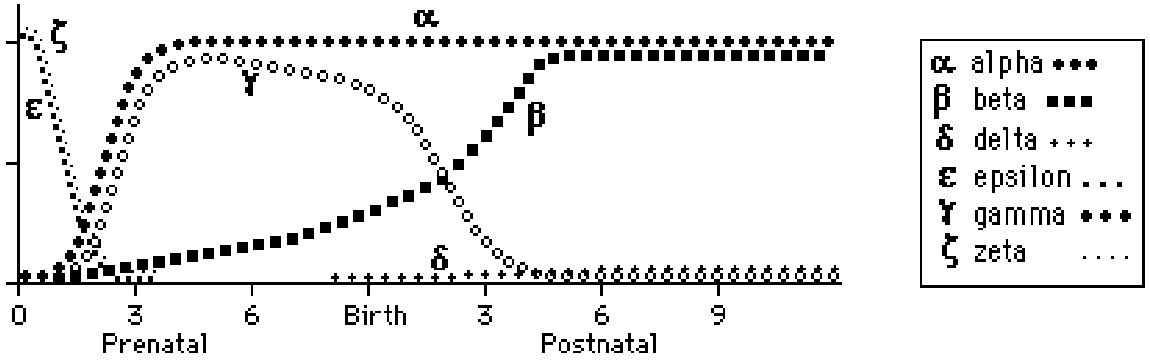
Globin Synthesis



Courtesy of B. Martien, Manual Hematology

Chromosome 16 (α-like genes)	Chromosome 11 (β-like genes)
• Alpha and zeta	• Beta, gamma, delta, epsilon

Globin Synthesis



Normal Hemoglobins

A	$\alpha_2\beta_2$	– ≈95 % adult	Gower 1	$\zeta_2\varepsilon_2$	embryonic Hgb; ↑ O_2 transport to embryo
A_{1c}	$\alpha_2\beta$-(glycosylation)$_2$	– ≈3 % adult (↑ in diabetes m)	Gower 2	$\alpha_2\varepsilon_2$	
A_2	$\alpha_2\delta_2$	– ≈2% adult	Portland	$\zeta_2\gamma_2$	
F	$\alpha_2\gamma_2$	– major fetal Hgb 3–9 th month; ↑ O_2 transport from placenta; <1 % adult	H	β_4	– non-functional
			Barts	γ_4	– trace at birth; non-functional

Courtesy of B. Martien, Manual Hematology

Globin synthesis

- Production of globin chains occurs in erythroid precursors
  - Pronormoblast → circulating polychromatic erythrocyte
- Transcription of globin genes → mRNA occurs in the nucleus
- Translation of mRNA → globin polypeptide chain occurs on the ribosomes in the cytoplasm
- α-like globin transcription produces more mRNA than β-like globin gene
  - Less efficient translation of α-like globin mRNA
- α-like globin and β-like globin are produced in equal amount

Hemoglobin Assembly and Regulation

Hemoglobin Assembly

- Globin is released from ribosomes, combines with heme to form a heterodimer
- 2 heterodimers then form to make a tetramer of two α-like chains (α or ζ) and two non-α chains (β,γ,δ,ε)
  - Produce a complete hemoglobin molecule
  - Combinations of these produce 6 normal hemoglobins
- The non-α chains (-) have a charge difference that determines their affinity to bind to α chains (+)
  - α (+) chain has the highest affinity for β (-) chain
    - Followed by gamma (γ) then delta (δ)

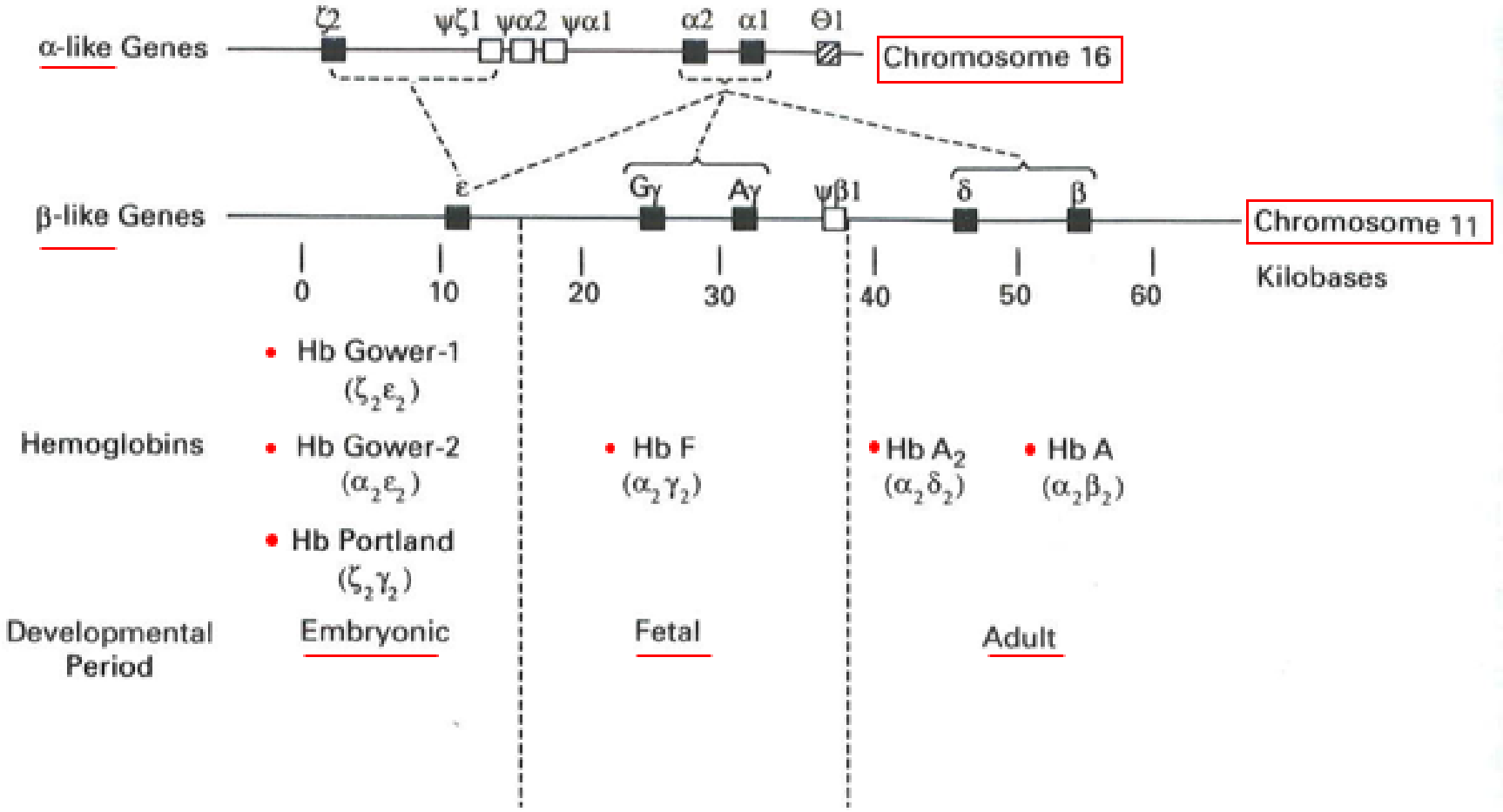
TABLE 7.2 Normal Hemoglobins

Stage	Globin Chain	Hemoglobin
Intrauterine		
Early embryogenesis (product of yolk sac erythroblasts)	$\zeta_2 + \epsilon_2$	Gower-1
	$\alpha_2 + \epsilon_2$	Gower-2
	$\zeta_2 + \gamma_2$	Portland
Begins in early embryogenesis; peaks during third trimester and begins to decline just before birth	$\alpha_2 + \gamma_2$	F
Birth		
	$\alpha_2 + \gamma_2$	F, 60%–90%
	$\alpha_2 + \beta_2$	A, 10%–40%
Two Years through Adulthood		
	$\alpha_2 + \gamma_2$	F, 1%–2%
	$\alpha_2 + \delta_2$	A_2, <3.5%
	$\alpha_2 + \beta_2$	A, >95%

Hemoglobin Regulation

- Heme regulation
  - Key rate limiting step is the initial reaction of glycine and succinyl CoA to form ALA, catalyzed by ALA synthase
  - Heme inhibits transcription of ALA synthase gene
    - Leads to a decrease in heme production (negative feedback mechanism)
- Globin regulation
  - Highly regulated to balance between globin and heme
    - Excess of globin chain, protoporphyrin IX, or iron can damage the cell and decrease the life span
  - Mainly controlled at the transcription level by a complex interaction of DNA sequences and soluble transcription factors
- Systemic regulation
  - Hypoxia detected by the peritubular cells of the kidney
  - Result in increased EPO secretion

Hemoglobin Ontogeny



Courtesy of B. Martien, Manual Hematology

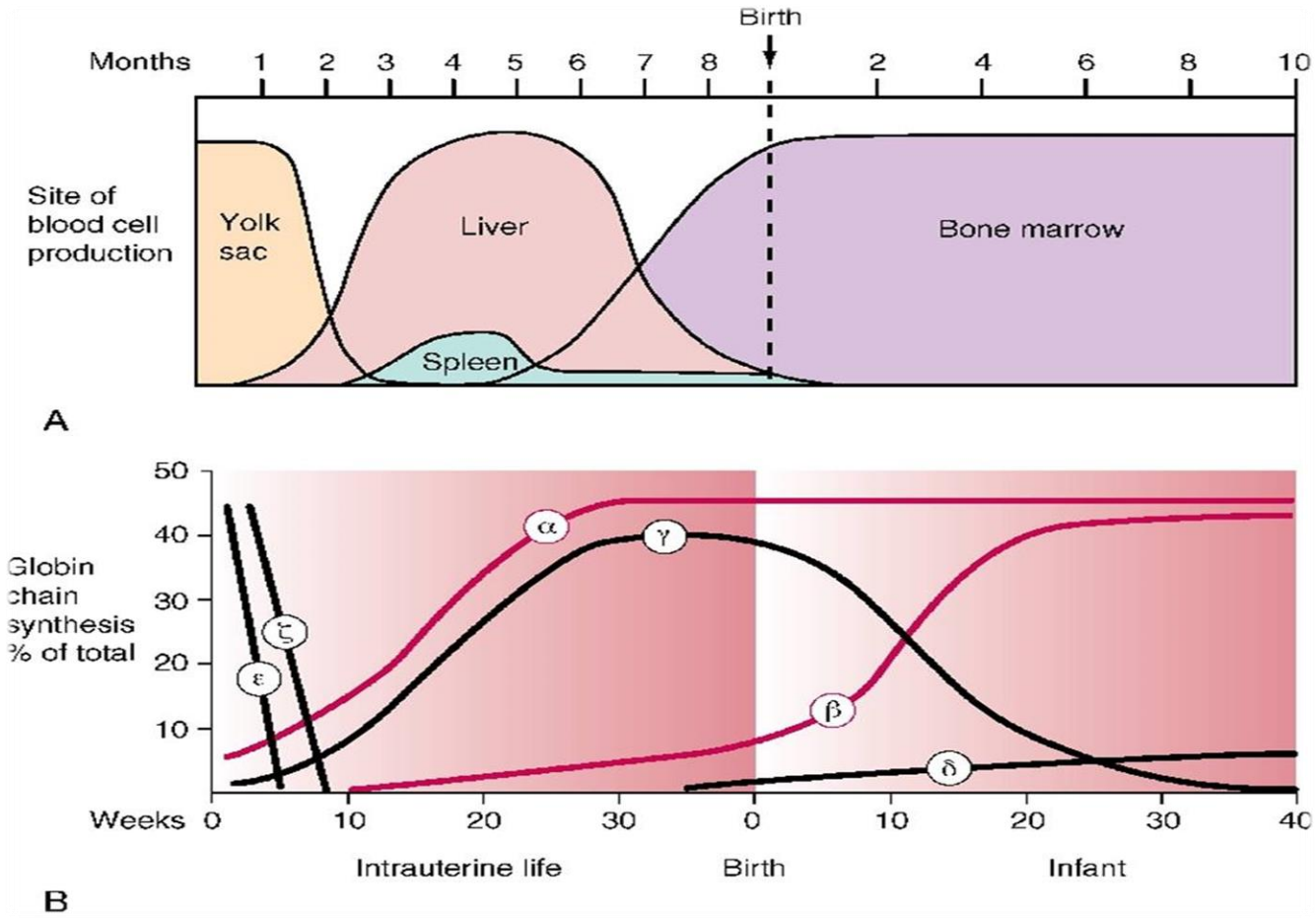
Hemoglobin Development

Birth through Adulthood

- 6 months after birth, gamma chain synthesis gradually decreases
  - Gamma chain gene silenced by transcriptional repressors
  - Replaced by beta chain synthesis
    - "γ-β switching"
  - HbA ($\alpha_2\beta_2$) is produced
- Delta globin gene is activated at birth and pairs with alpha globin
  - HbA_2 ($\alpha_2\delta_2$)

	Adult	Newborn
Hb A ($\alpha_2\beta_2$)	95%	10-40%
Hb A_2 ($\alpha_2\delta_2$)	<3.5%	0.2%
Hb F ($\alpha_2\gamma_2$)	<1-2%	60-90%

Timeline of Globin Chain Production



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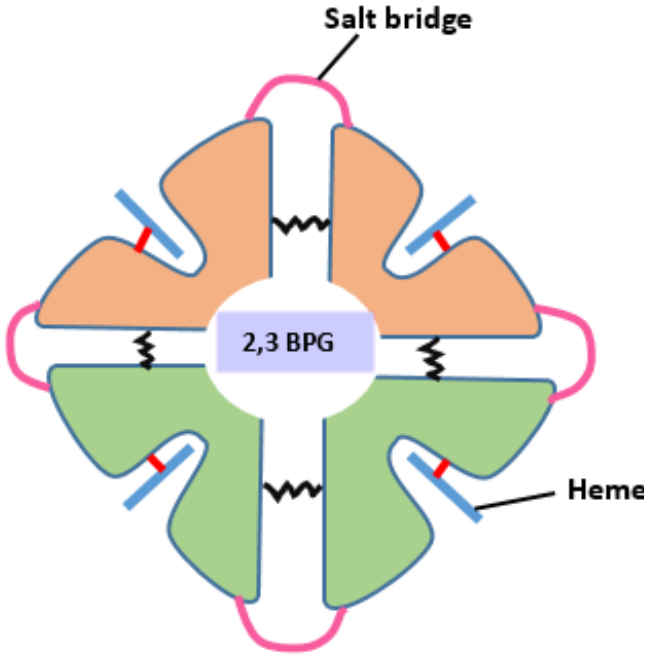
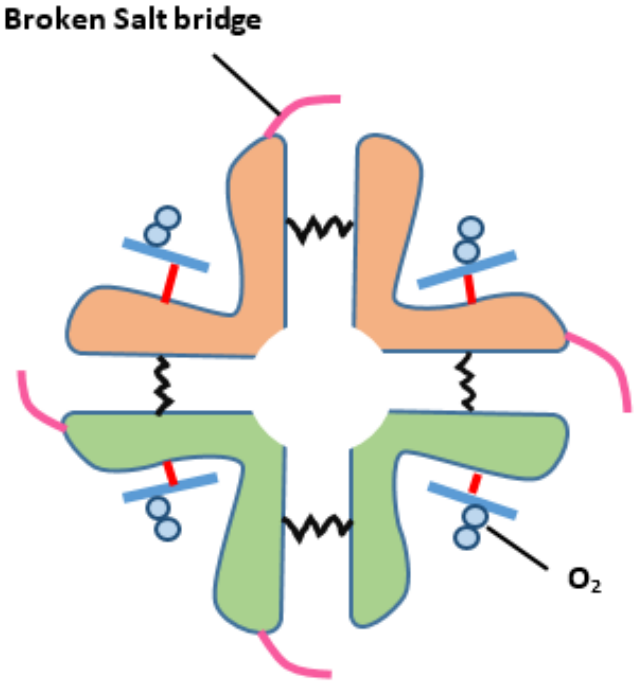
Hemoglobin Function

- During oxygenation, 4 heme molecules reversibly bind 1 oxygen molecule
- Affinity of hemoglobin for oxygen relates to partial pressure of oxygen (PO2)
  - P_{50} = the amount of oxygen needed to saturate 50% of hemoglobin
- Hb with no O_2 has little affinity for O_2
- Each subsequent O_2 bound, affinity increases
- Lungs
  - High O_2 tension → affinity of Hb for O_2 is high
    - Hb rapidly oxygenated
- Tissues
  - Low O_2 tension→ affinity of Hb for O_2 is low
    - Hb rapidly releases O_2





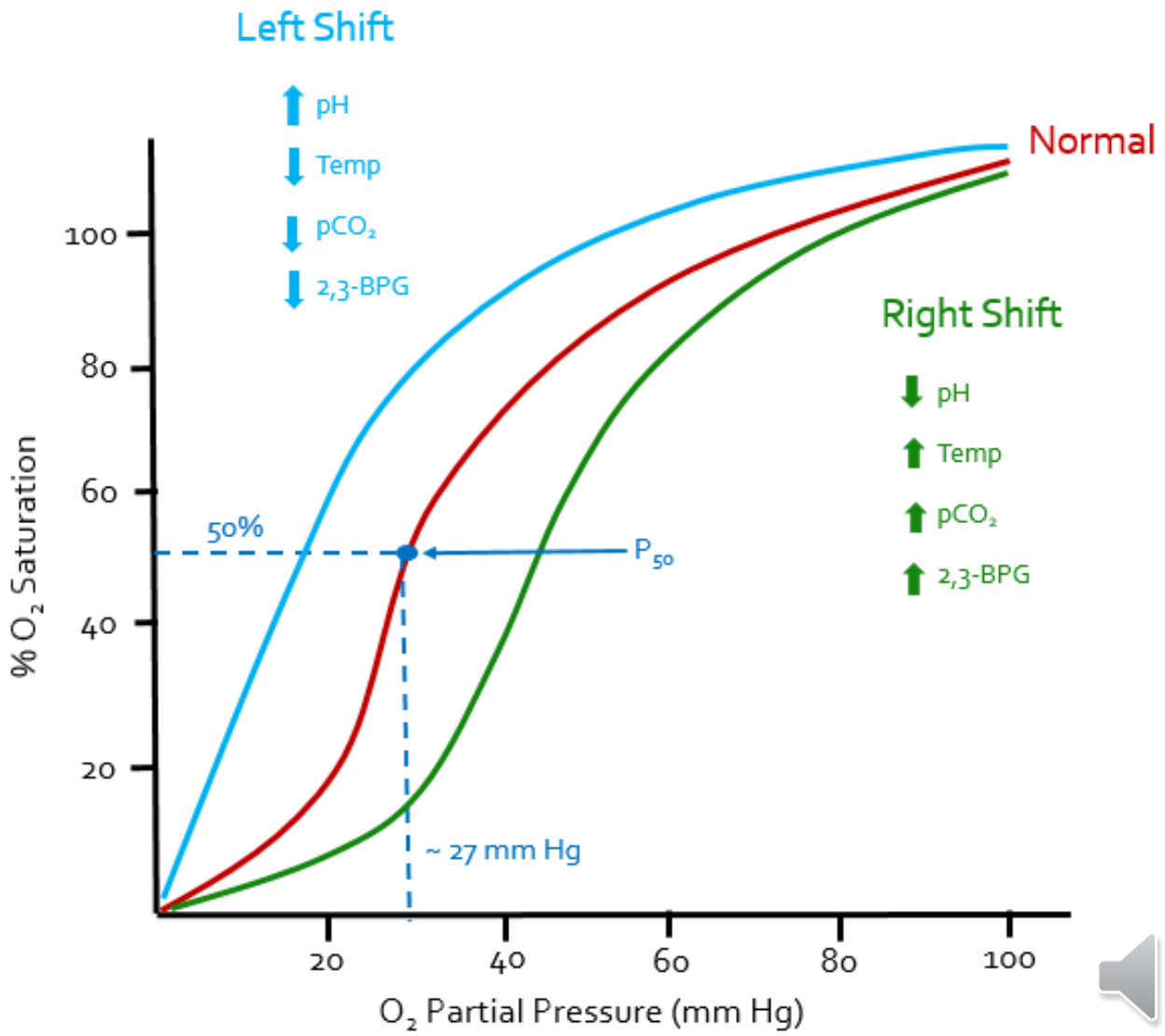
Deoxygenated State
-hemoglobin tetramer tense or T structure
-stabilized by 2,3-BPG between β globin chains and salt bridges

Oxygenated State
-hemoglobin tetramer relaxed or R structure
-oxygen binds, change in hydrophobic interactions at contact point disrupts salt bridges and release 2,3 BPG

Hemoglobin Dissociation Curve

Hemoglobin-Oxygen Dissociation Curve

- Normal curve
  - 27 mm Hg in 50% oxygen saturation of hemoglobin molecule
- Left shift
  - High affinity of Hb for O_2
  - Seen in the lungs
  - 50% saturation occurs at <27 mm Hg
- Right shift
  - Low affinity of Hb for O_2
  - Seen in tissues
    - Muscles and placenta
  - 50% saturation occurs at >27 mm Hg
- Bohr effect: shift in concentration due to pH (or H^+ concentration)
  - Facilitates ability of hemoglobin to exchange oxygen and carbon dioxide (CO_2)

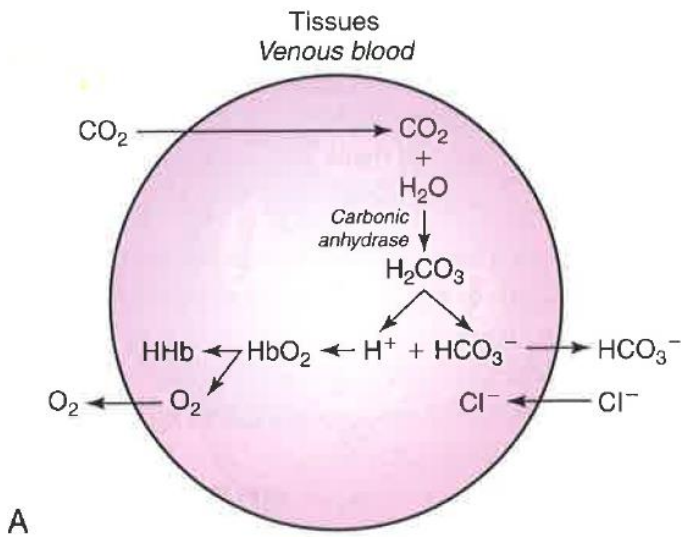


Courtesy of A. Zelasco Manual Hematology

Carbon Dioxide Transport and Nitric Oxide Transport

Carbon Dioxide Transport

- 2nd crucial function of hemoglobin
- In venous blood, CO_2 diffuses into RBC and combine with H_2O → carbonic acid (H_2CO_3)
  - Catalyzed by carbonic anhydrous
- H_2CO_3 dissociates to release H^+ and bicarbonate (HCO_3^-)
- H^+ binds oxygenated Hb due to Bohr Effect
- As bicarbonate concentration increases in blood, it then diffuses across RBC membrane into plasma
- Chloride shift- chloride (Cl^-) diffuses into cell to maintain electroneutrality across membrane
- In the lungs, oxygen diffuses and binds deoxygenated hemoglobin (HHb)
- H^+ released from Hb and binds with bicarbonate → carbonic acid
  - Converted to H H_2O and CO_2 which is diffused out of the cell and expelled from the lungs
- Bicarbonate into cell, chloride out of cell



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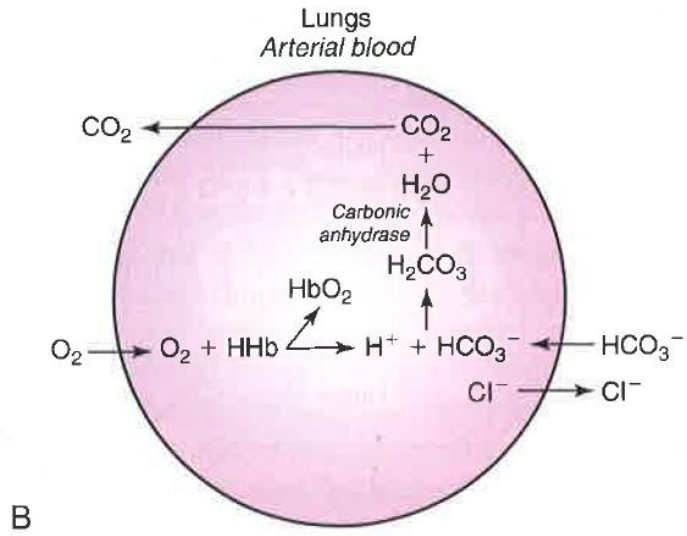

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Nitric Oxide Transport

- 3rd function of hemoglobin
- Binding, inactivation and transport of nitric oxide
- Nitric oxide is released by endothelial cells and causes relaxation of vascular wall smooth muscle and vasodilation
  - Very short half-life when in this free nitric oxide form
- Some enter RBC and bind cysteine in beta chain of Hb
  - S-nitrohemoglobin
    - Preserves and transports nitric oxide to hypoxic microvascular areas
      - Causes vasodilation and increase in blood flow
  - Areas of increased oxygen
    - Hb bind and inactivate nitric oxide
    - Vasoconstriction and decrease in blood flow
  - Areas of decreased oxygen
    - Release nitric oxide
    - Increase in blood flow and vasodilation

Dyshemoglobins

Dyshemoglobins

- Dyshemoglobin- dysfunctional hemoglobin that are unable to transport oxygen
- Types
  - Methoglobin
  - Sulfhemoglobin
  - Carboxyhemoglobin
- Accumulate to toxic levels, after exposure to certain drugs or environmental chemicals or gasses
- Most acquired, small fraction of methoglobinemias are inherited

Methemoglobin (MetHb)

- Formed by reversible oxidation of heme iron to ferric state (Fe^{3+})
- Small amount normally formed during oxygenation/deoxygenation of Hb
  - Limited to 1% by NADH-methemoglobin reductase (NADH-cytochrome b5 reductase 3 pathway)
- Methemoglobinemia
  - Increase in methemoglobin
  - Acquired or hereditary (rare)
- Brownish-red color

Sulfhemoglobin

- Irreversible oxidation of hemoglobin by drugs/ exposure to sulfur chemicals
  - Drugs: sulfanilamids, phenacetin, nitrites, and phenylhydrazine
  - Forms sulfur atom to pyrrole ring to heme ring and creates a green pigment
- Ineffective for oxygen transport
- Cannot be converted to HbA and is persistent for the life of the cell

Carboxylhemoglobin (COHb)

- Carbon monoxide (CO) + heme iron
  - CO has a 240x affinity for heme iron than O_2
  - Shifts curve to the left
- "Silent killer"
- Produced endogenously- car exhaust, tobacco smoke, industrial pollutants, coal and charcoal burning
  - Normally <2 % CO Hb
  - Smokers up to 15% CoHb
- Symptoms include headache, dizziness, disorientation and severe symptoms include coma, seizure, hypotension, death
- Gives blood a cherry red color (can show on victims skin)

Hemoglobinopathy

- Hemoglobinopathy- disease state involving hemoglobin molecule
  - Result from a mutation in one or more genes that affect hemoglobin synthesis
  - Genes that are mutated either:
    - Code for proteins that make up hemoglobin molecule (globin or polypeptide chain)*
    - Are involved in synthesizing or regulating synthesis of the globin chains *
- Qualitative= **Hemoglobinopathies**
  - Synthesis is normal/near normal
  - Altered amino acid sequence within globin chain
    - Alter the structure and function
- Quantitative= **Thalassemia**
  - Reduction in hemoglobin synthesis
  - Reduction of specific hemoglobin can cause anemia
    - Stimulates production of other hemoglobins not affected to compensate for the anemia

Hemoglobin Testing Methods

Hemoglobin Solubility test

- AKA Sickle Solubility
- Screening and confirmatory test
- Used to identify Hb S
  - Capitalizes on ↓ solubility of deoxygenated Hb S in a solution
- Method: *
  - Blood is added to buffered salt solution containing:
    - Detergent based lysing agent (saponin)
      - Dissolves membrane lipids – release Hb from RBC
    - Reducing agent (sodium hydrosulfite (dithionite))
      - Reduces Fe from ferrous to oxidative ferric state – unable to bind oxygen
        - Lowers the oxygen tension which causes a change in the Hb if it is HbS
  - Deoxygenated Hb S polymerizes
    - Solution will appear turbid due to precipitate of tactoid crystals

Hemoglobin Solubility Test

- Turbidity is qualitatively determined from the inability to visualize black type lines on a white background
  - Turbid- positive for Hb S
  - Clear- negative for Hb S
- False positives
  - Hyperlipemia, rare hemoglobinopthaties, too much blood is added
- False negatives
  - Patient < 6 months old*, patient has a low HCT

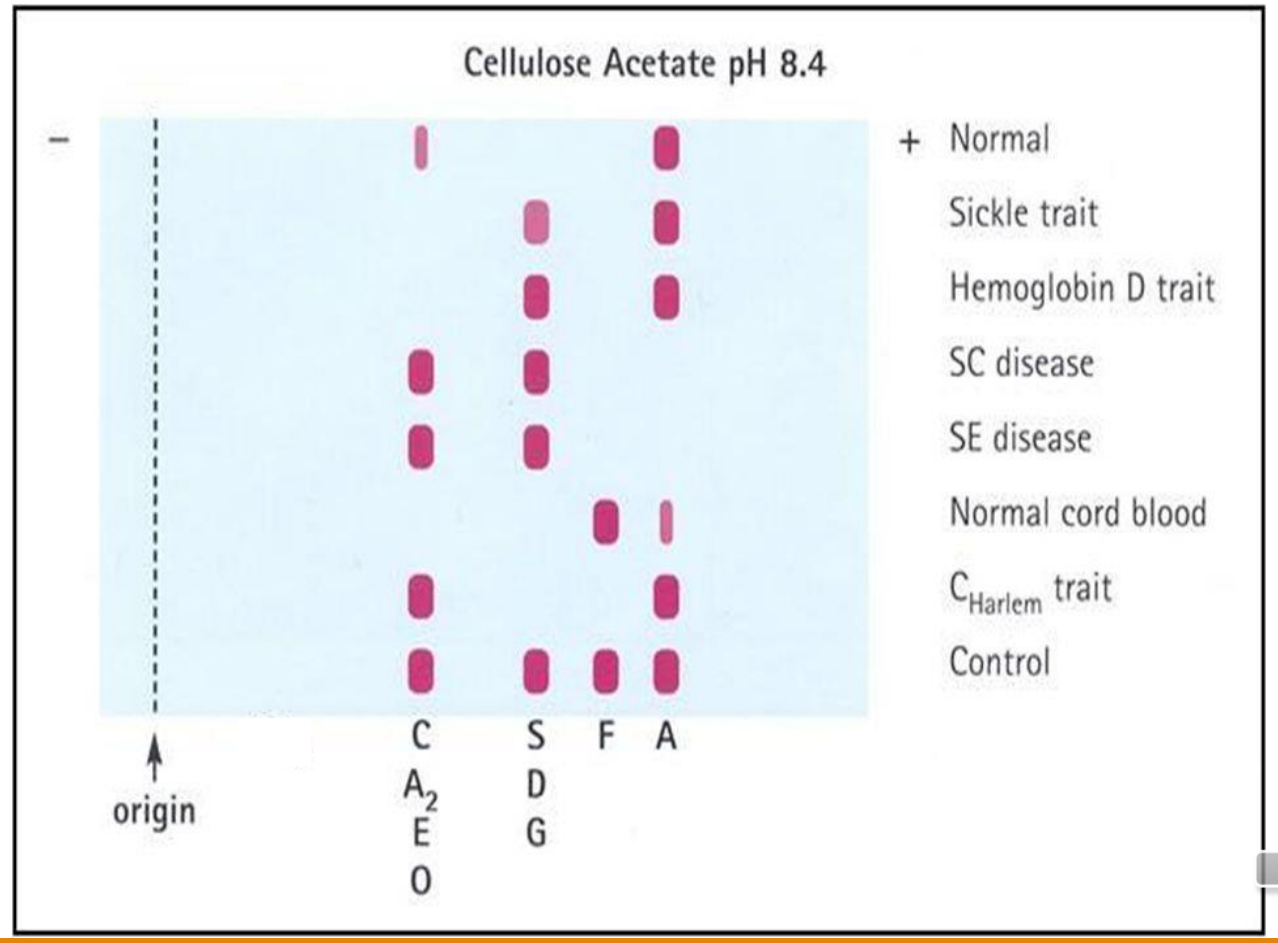
https://www.researchgate.net/figure/showing-the-reactivity-pattern-of-the-rapid-sickle-cell-hemoglobin-s-dithionate_fig2_329079444

Gel Electrophoresis

- Hemoglobin electrophoresis- The separation of hemoglobin molecules in an electric field based on differences in molecular charge
- Two types:
  - Alkaline electrophoresis
  - Acid electrophoresis

Alkaline Electrophoresis

- Performed on agarose medium (pH 8.4)
- Hemoglobin molecules (negative charge) migrate towards anode (positive pole)
- Drawback: Some hemoglobin have the same charge → same electrophoretic mobility patterns
- Undergo acid electrophoresis for definitive separation



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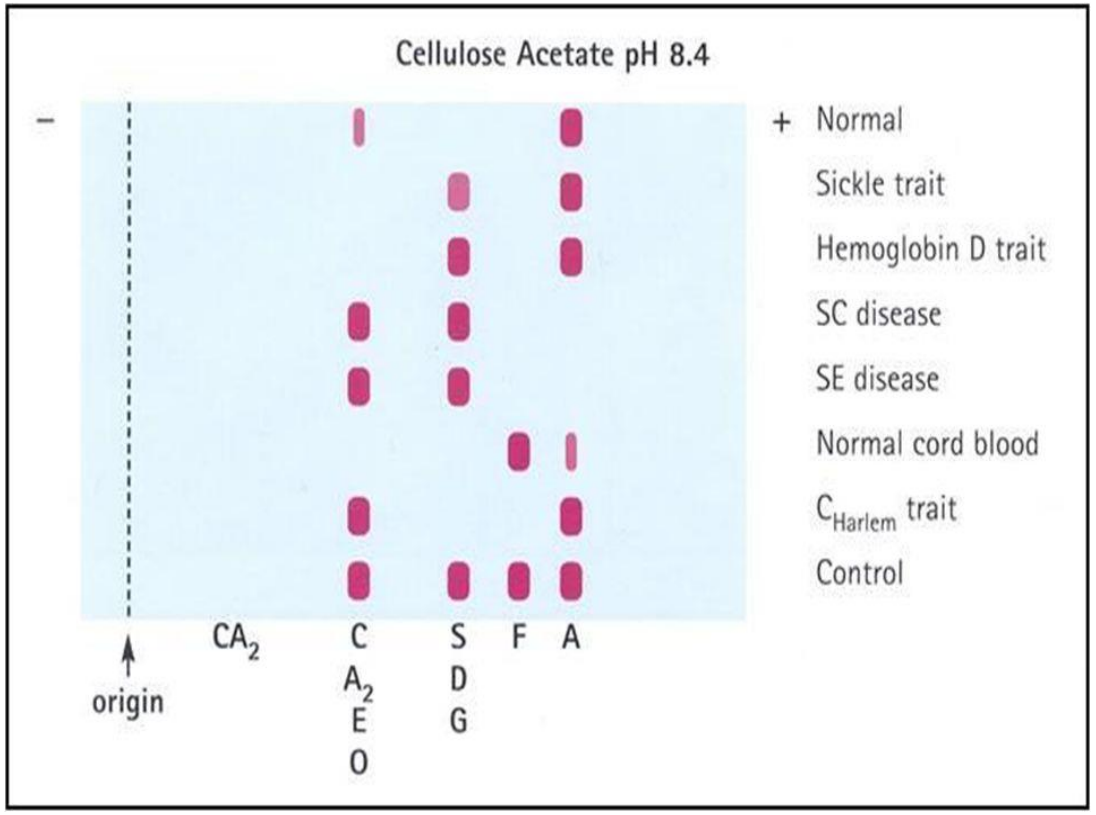
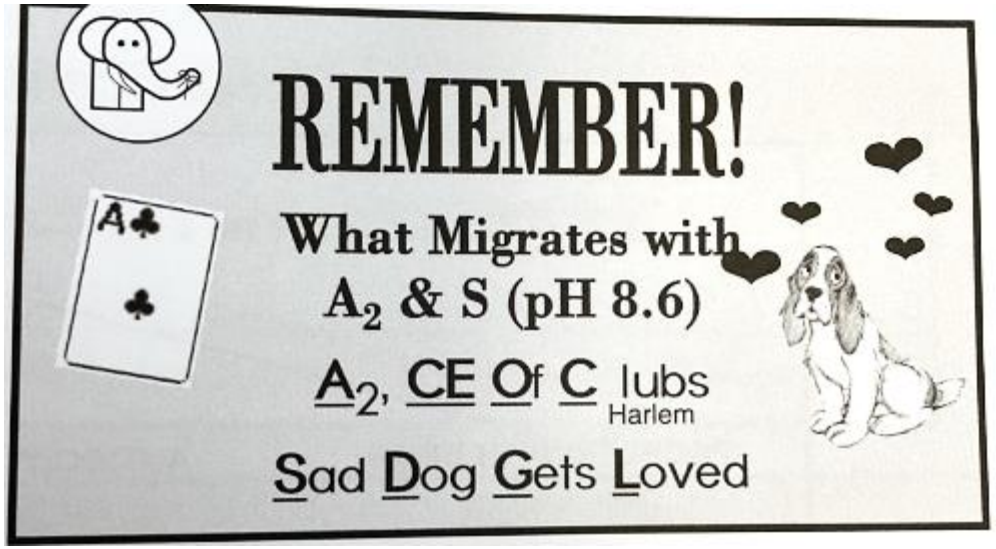
Alkaline Electrophoresis



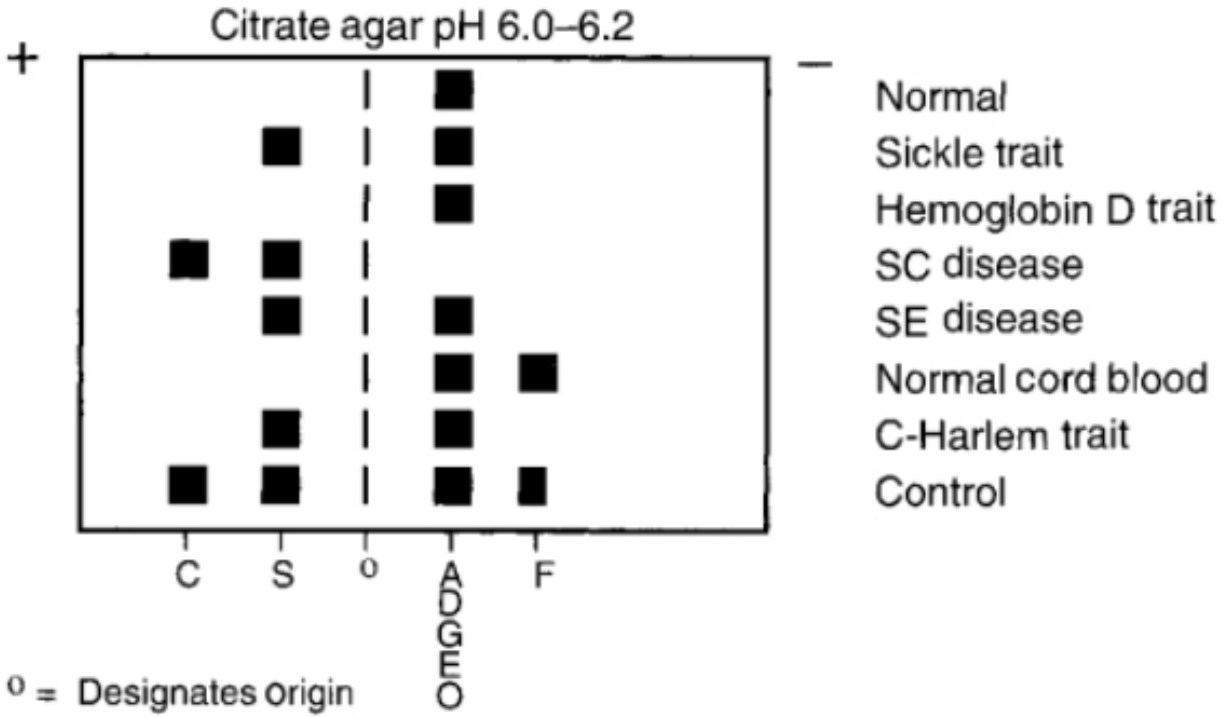
Hemoglobin Migration (pH 8.6) (- to +)

- **C** (crawl), **S** (slow), **F** (fast), and **A** (accelerate)



Acid Electrophoresis

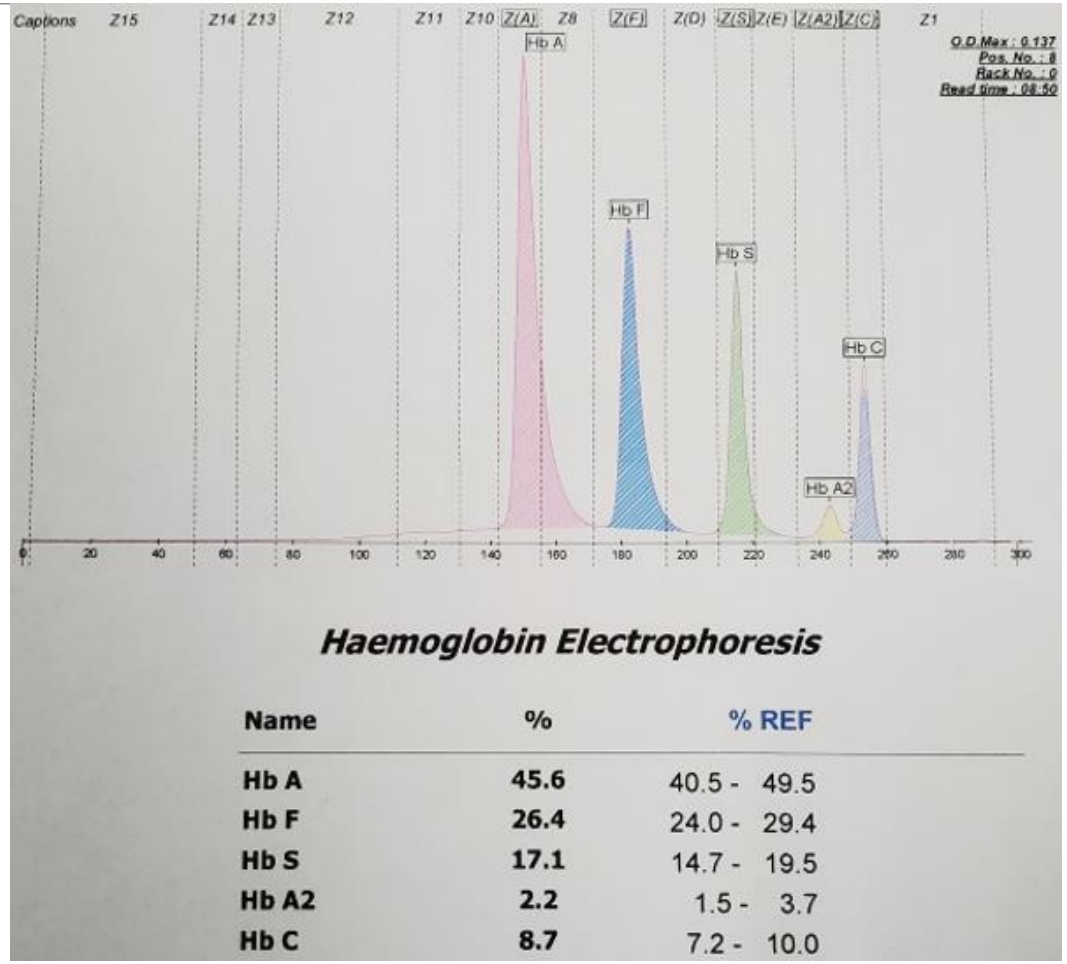
- Performed on a citrate agar at an acid pH
- Definitive hemoglobin separation



Rodak's Hematology 6th Edition Figure 24.7

Capillary Electrophoresis

- Separation of hemoglobin type by charge in an alkaline buffer
- Hemoglobin is charge in a capillary electrophoresis tube
  - Will flow towards positive electrode
  - Eluding off at certain points in the tube
    - Will create these nice peaks where they elude off

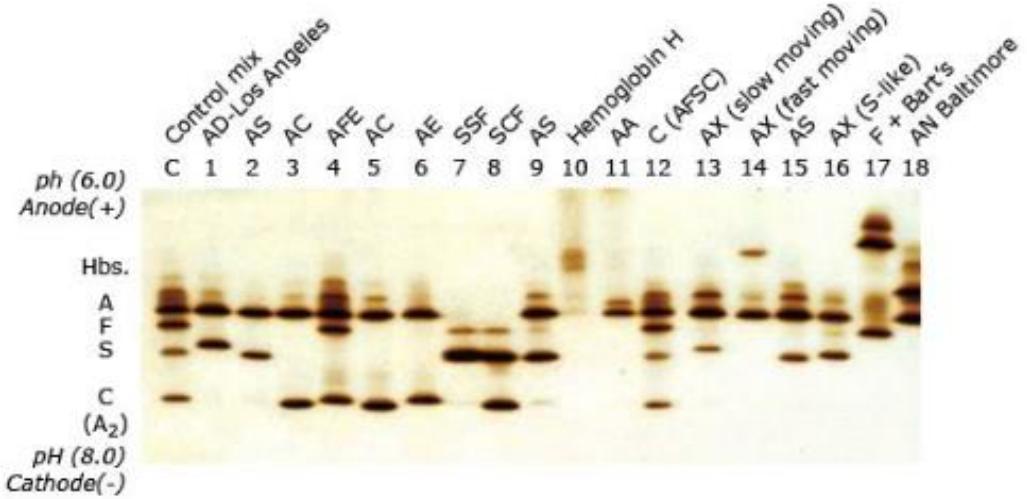


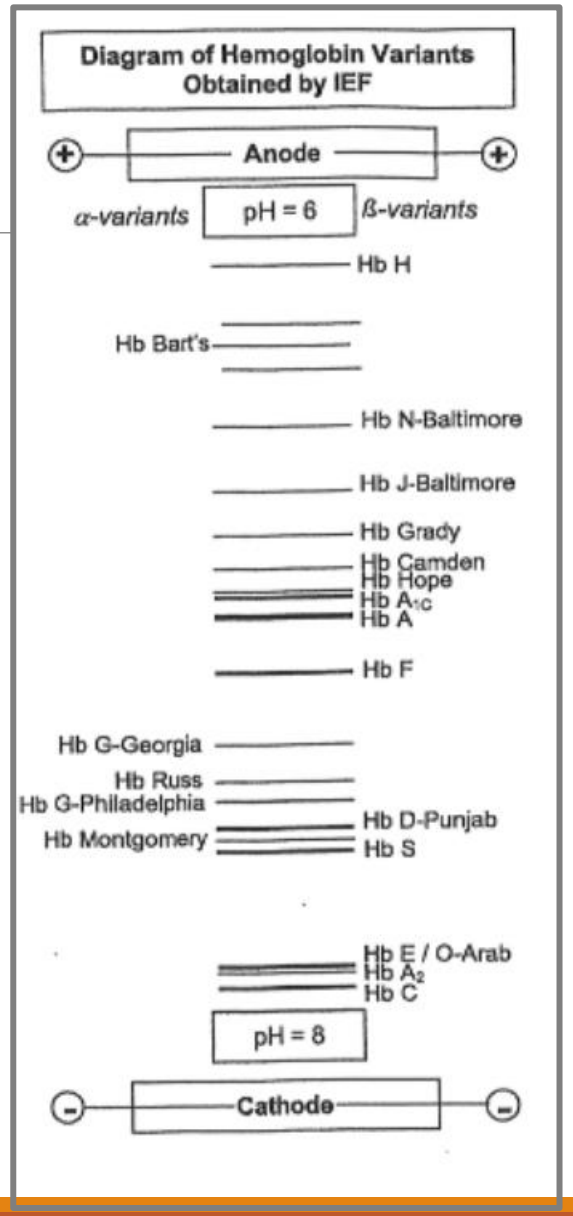
HPLC

- High performance liquid chromatography
- Separates normal and abnormal Hb types in a cation exchange column under high pressure
  - Individual molecules elute at different and characteristic rates
    - Allows for separation and identification of hemoglobin variants
- ID and quantify low levels of Hb A2 and Hb F
  - Comigration of A2 and E can occur
- Used in the diagnosis of thalassemias

IEF

- Confirmatory test
- Expensive and complex
- Electrical current push Hb molecules across pH gradient
  - Charge changes as it goes through pH gradient
  - Hb stops when it reaches isoelectric point (net charge zero)
    - Isoelectric position
- Can separate Hb pH differences as little as pH 0.02



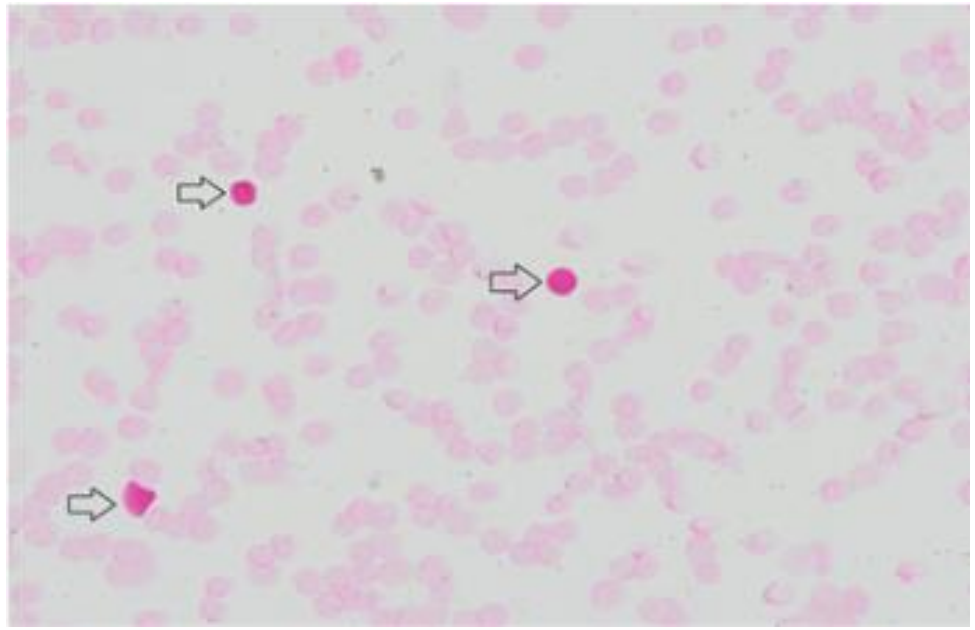


Kleihauer-Betke Special Stain

- Test Principle:
  - Blood smear is stained to examine for cells containing Hb F
    - Hb A and its variants are eluted from the RBCs when immersed in acidic solution while Hb F remains intracellular due to its acid resistance
- Peripheral blood films are ethanol fixed and immersed in a citrate-acid buffer
  - Adult hemoglobin- eluted in a citrate-acid buffer
  - HB F- resist acid elution and remain in the cell
- Peripheral blood films are then stained
  - Adult Hb will appear as “ghosts” cells
  - Hb F cells will take up the stain

Kleihauer-Betke Special Stain

- Test is used to
  - Determines if the Hb F distribution in RBCs is pancellular or heterocellular
  - Estimate the volume of fetal-maternal hemorrhage
    - Determine how much fetal blood has been lost as well as how much fetal blood the mother has been exposed to
    - Important when a Rh-negative mother is to deliver a Rh-positive baby
    - Quantification of fetal blood is used to determine the dose of Rhogam is needed for the mother



Courtesy of Dr. Genevieve Crane MD, PhD

Cyanmethemoglobin Method

- Also called the hemiglobincyanide method
- Quantitative method used to determine the hemoglobin concentration of a sample
- Procedure
  - Whole blood is added to reagent (Drabkin solution)*
    - Potassium ferricyanide converts hemoglobin from ferrous → ferric state
      - Forms methemoglobin
    - Methemoglobin combines with potassium cyanide to form the stable pigment cyanmethemoglobin
    - Absorbance of cyanmethemoglobin is read at 540 nm
      - Directly proportional to hemoglobin concentration

References

Rodak's Hematology, Clinical Principles and Applications 6th Edition

Harmening Clinical Hematology and Fundamentals of Hemostasis 4th edition

Additional material Courtesy of Andrew Zelasco, MLS and Barbara Martien, MLS