Hemoglobin electrophoresis

Hemoglobin electrophoresis

- Principle: proteins when applied to a membrane and exposed to a charge gradient, separate and can be visualized by protein or haem stain.

Hemoglobin electrophoresis

- Sample: Packed red cells; if whole blood used paraprotein or high concentration of polyclonal Ig may produce a band.
- Membrane: filter paper, cellulose acetate membrane, starch gel, citrate agar gel or agarose gel.
- Protein stain: see carbonic anhydrase band, behind HbA2.

Hemoglobin electrophoresis

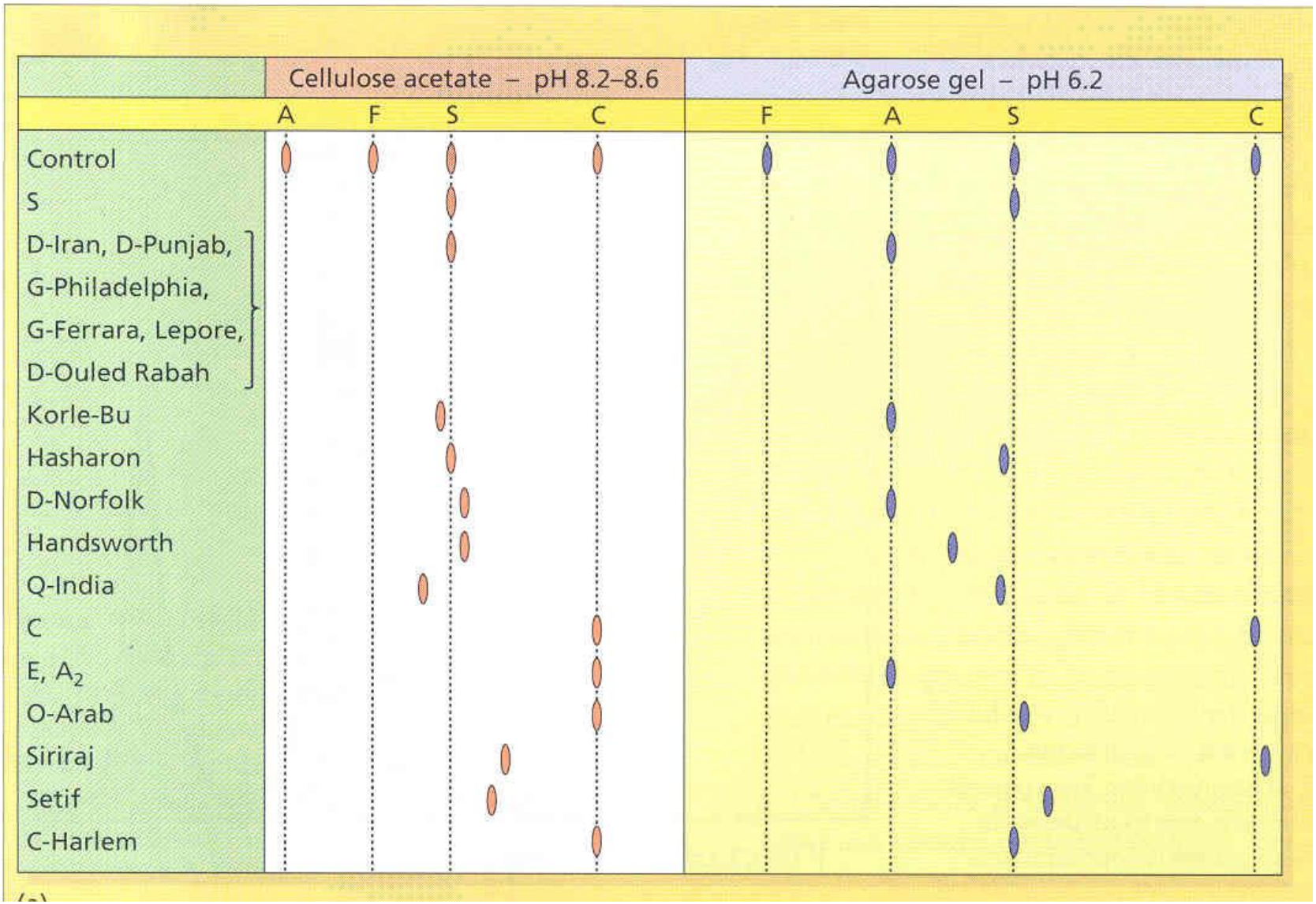
- Cellulose acetate at alkaline pH: initial procedure.
- Separation is largely determined by electrical charge.
- At this pH Hb is negatively charged and moves toward the positively charged anode.

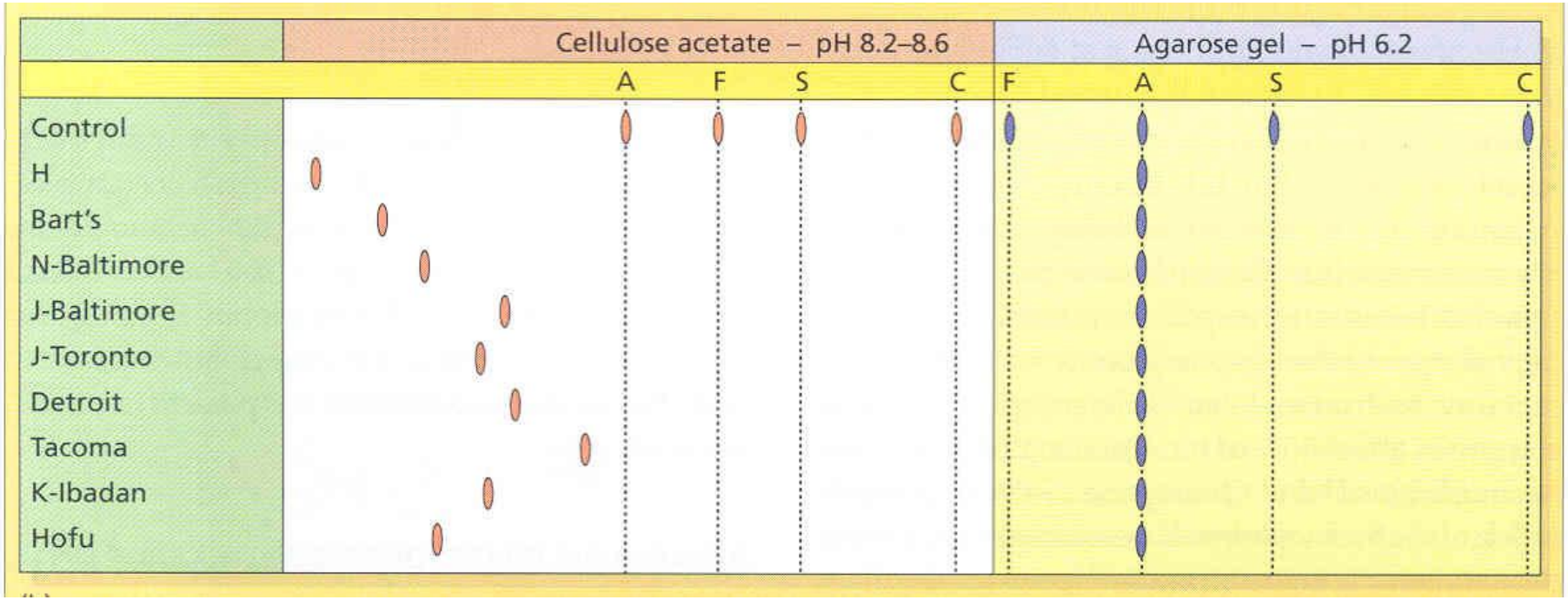
Hemoglobin electrophoresis

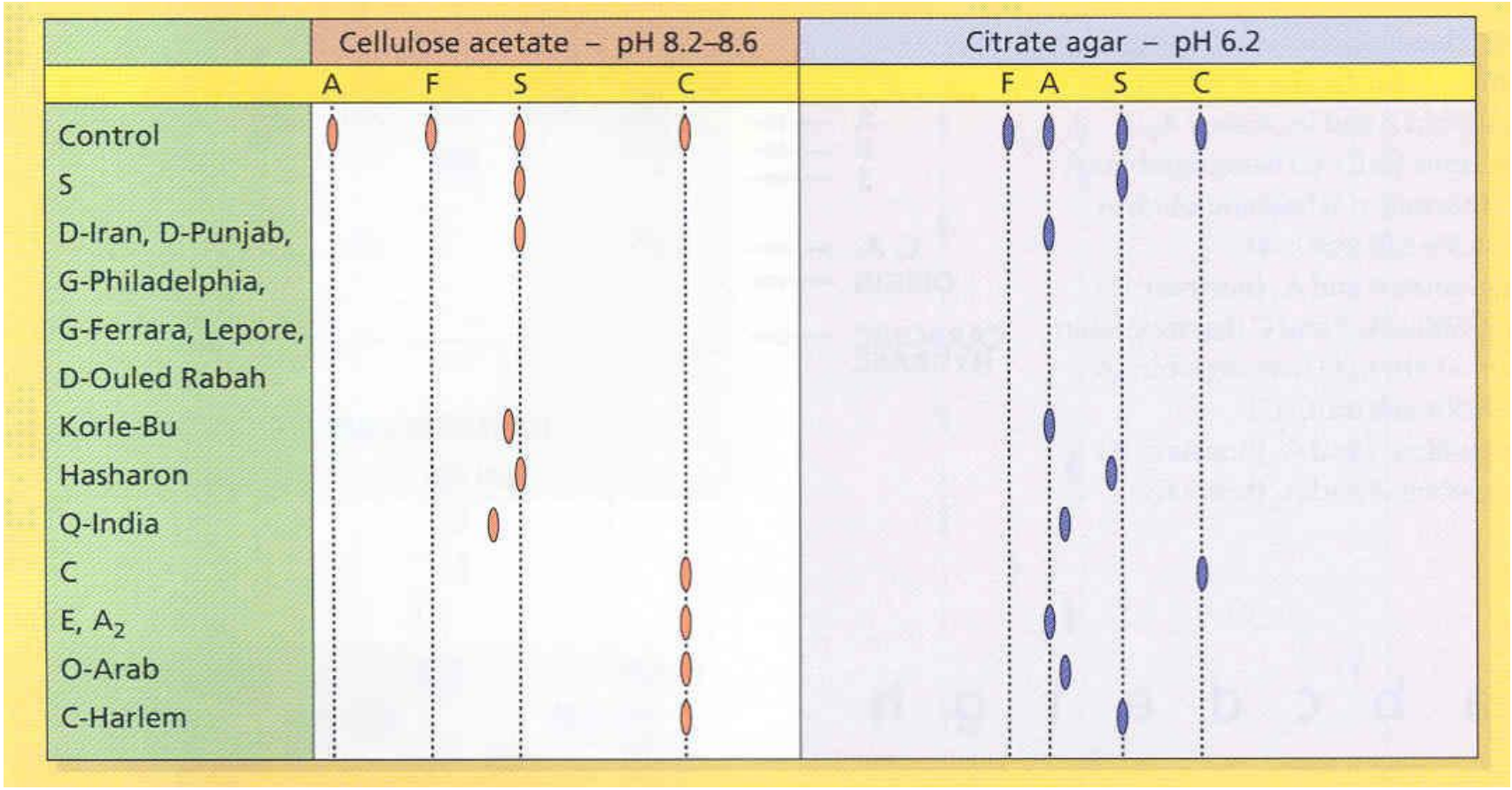
- With good technique: Hb F levels >2% can be recognized; split A2 can be seen (seen with alpha chain variant)

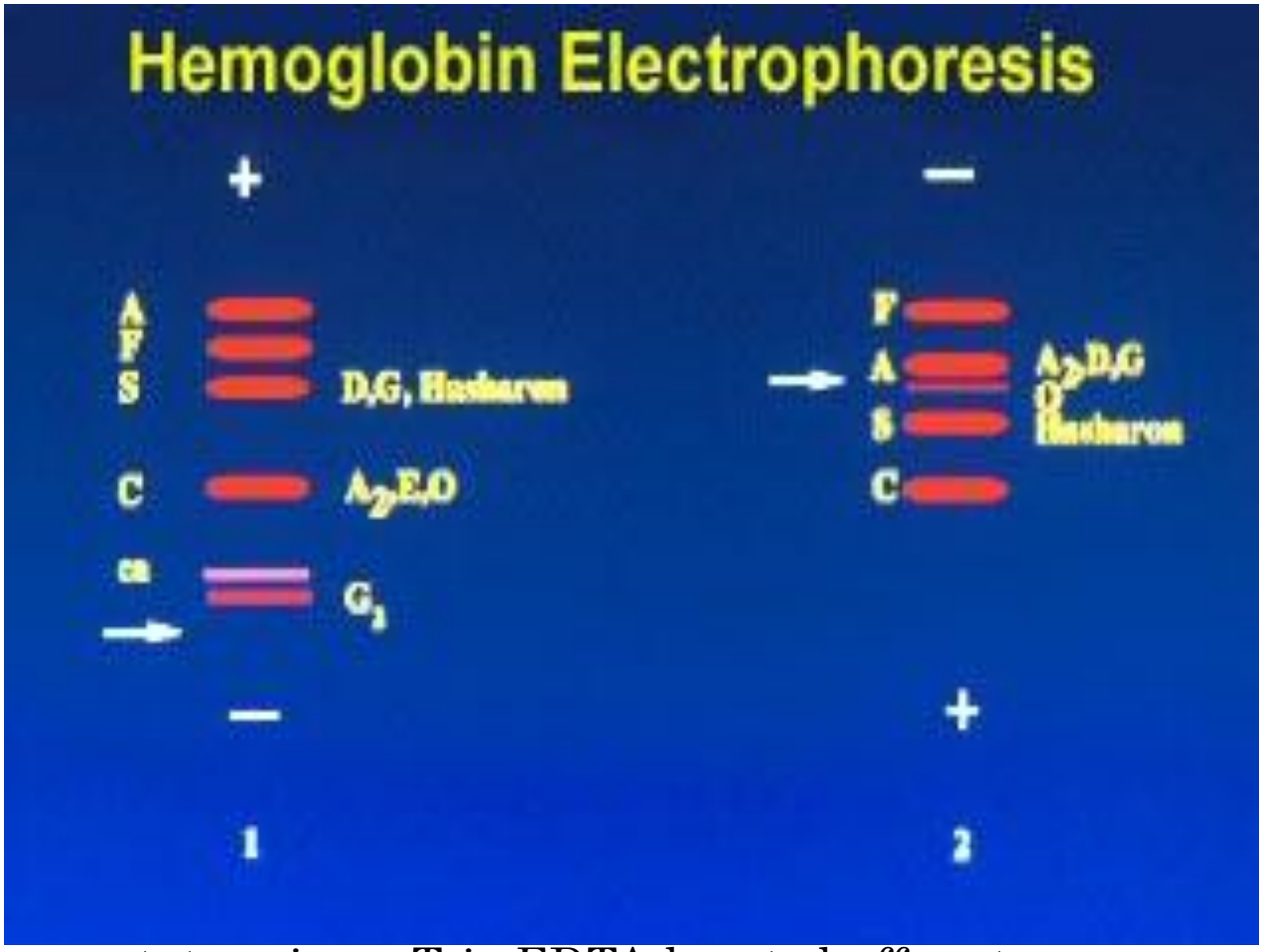
Hemoglobin electrophoresis

- Next step: Citrate agar or agarose gel at acid pH


Cellulose acetate – pH 8.2–8.6
Agarose gel – pH 6.2
A
F
S
C
F
A
S
C
Control
S
D-Iran, D-Punjab,
G-Philadelphia,
G-Ferrara, Lepore,
D-Ouled Rabah
Korle-Bu
Hasharon
D-Norfolk
Handsworth
Q-India
C
E, A2
O-Arab
Siriraj
Setif
C-Harlem


Cellulose acetate – pH 8.2–8.6
Agarose gel – pH 6.2
A
F
S
C
F
A
S
C
Control
H
Bart's
N-Baltimore
J-Baltimore
J-Toronto
Detroit
Tacoma
K-Ibadan
Hofu

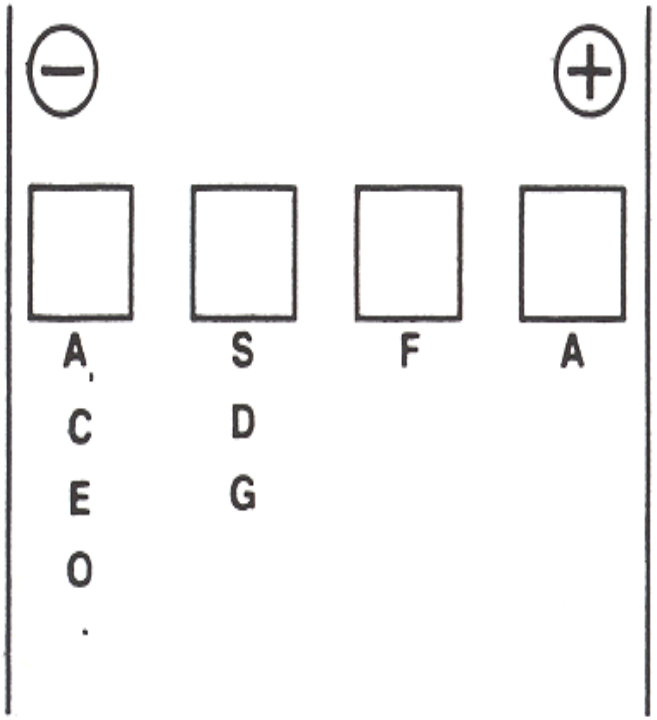

Cellulose acetate – pH 8.2–8.6
Citrate agar – pH 6.2
A
F
S
C
F
A
S
C
Control
S
D-Iran, D-Punjab,
G-Philadelphia,
G-Ferrara, Lepore,
D-Ouled Rabah
Korle-Bu
Hasharon
Q-India
C
E, A_2
O-Arab
C-Harlem



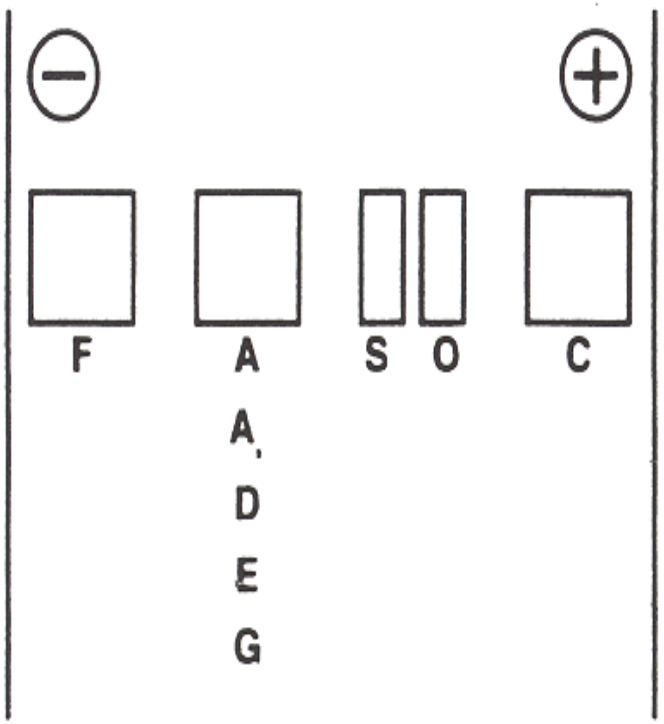
On cellulose acetate using a Tris-EDTA-borate buffer at an alkaline pH 7.4. In this system hemoglobins migrate according to their charge as shown in the diagram.

In agar gel using an acetic acid-acetate buffer at an acid pH 6.0. In this system hemoglobins migrate only partly due to their charge but also due to a complicated interaction with the agar called electroendosmosis.

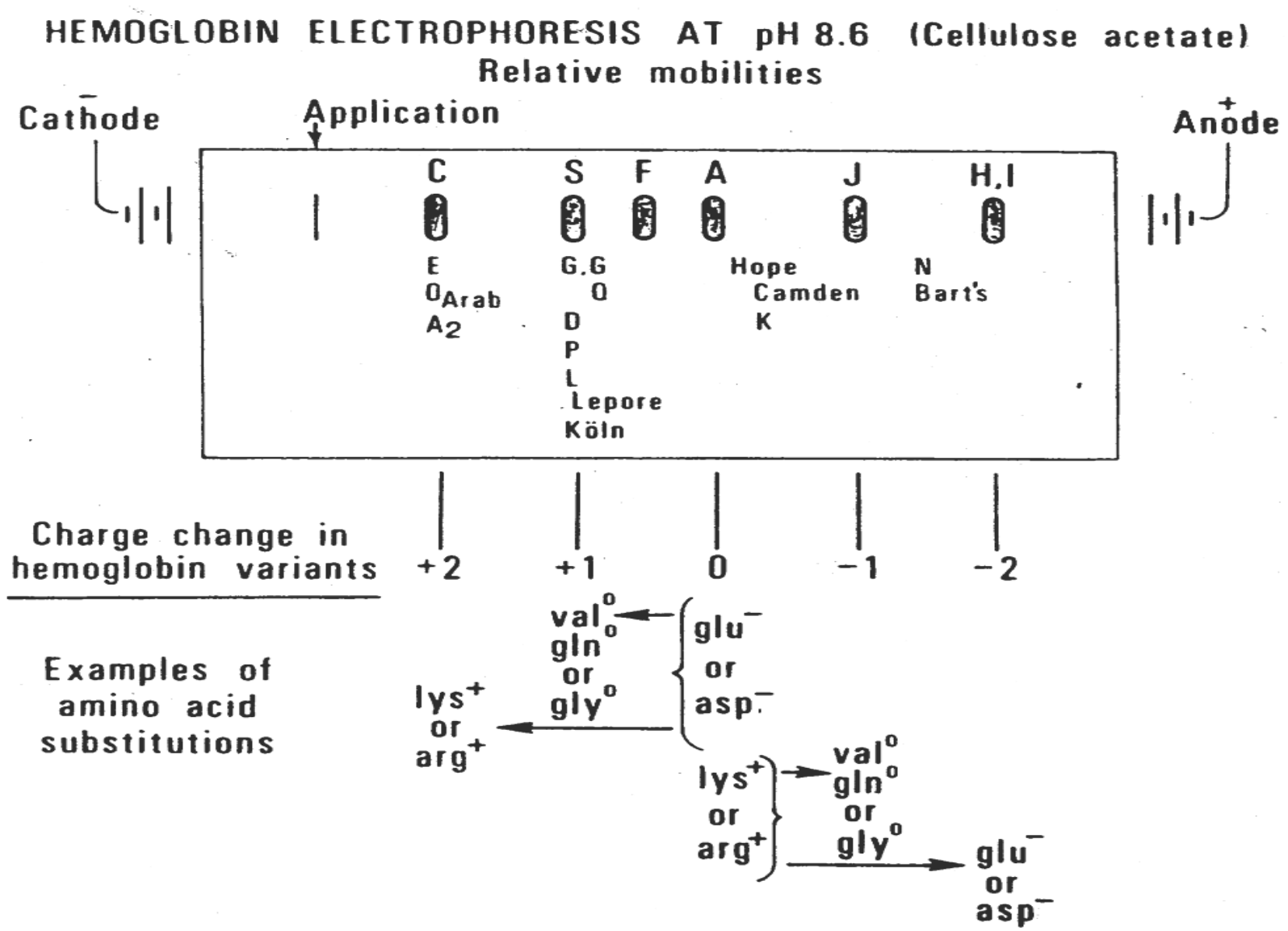
HEMOGLOBIN ELECTROPHORESIS



Paragon (Alkaline) Hb



Paragon Acid Hb


HEMOGLOBIN ELECTROPHORESIS AT pH 8.6 (Cellulose acetate)
Relative mobilities
Cathode
Application
Anode
C
S
F
A
J
H,I
E
O Arab
A2
G,G
O
D
P
L
Lepore
Köln
Hope
Camden
K
N
Bart's
Charge change in hemoglobin variants
+2
+1
0
−1
−2
Examples of amino acid substitutions
val o
gln o
or
gly o
glu −
or
asp −
lys +
or
arg +
lys +
or
arg +
val o
gln o
or
gly o
glu −
or
asp −

Group	Principal hemoglobins
A	A, M, some unstable Hbs
F	F
S	S, D, G, Lepore
C	C, E, A2, O Arab

Isoelectric focusing

- Principle: net charge of a protein depends on the pH of the surrounding solution. At low pHcarboxylic gp is uncharged and amino gp is charged with a net + charge and vice versa. In IEF, various Hb are separated according to their isoelectric point (pI), the point at which they have no charge

Isoelectric focusing

- Bands are sharper
- Hbs that can not be distinguished from each other by electrophoresis can be separated by IEF eg D and G variants

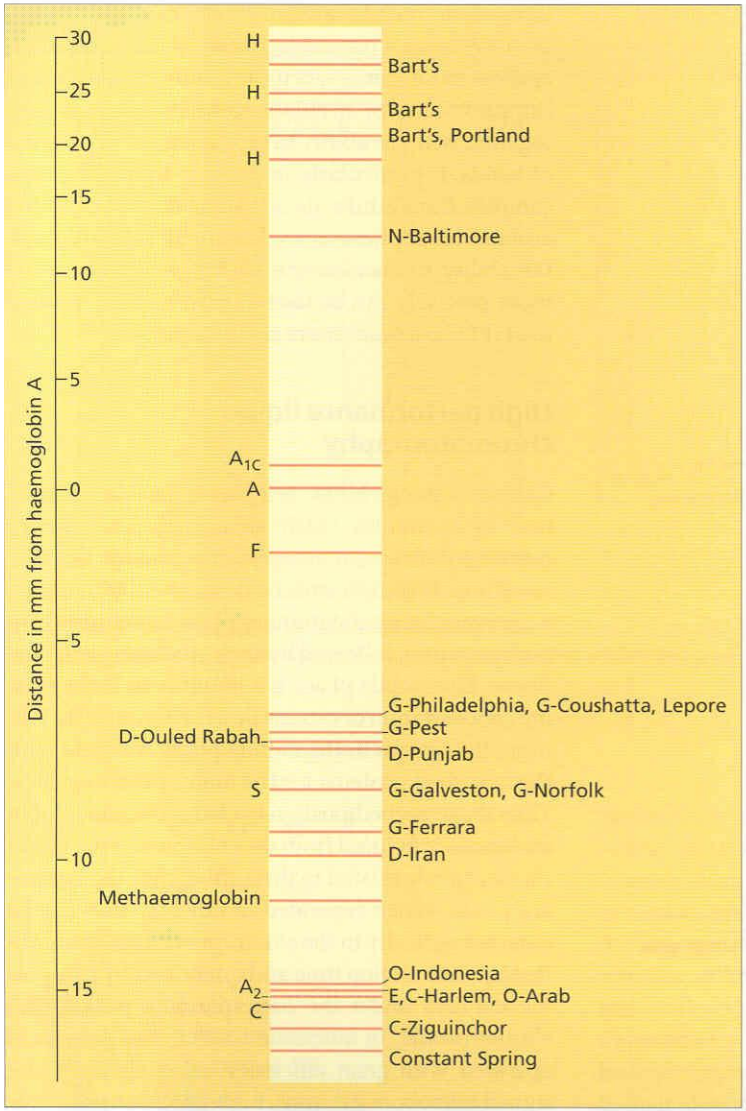
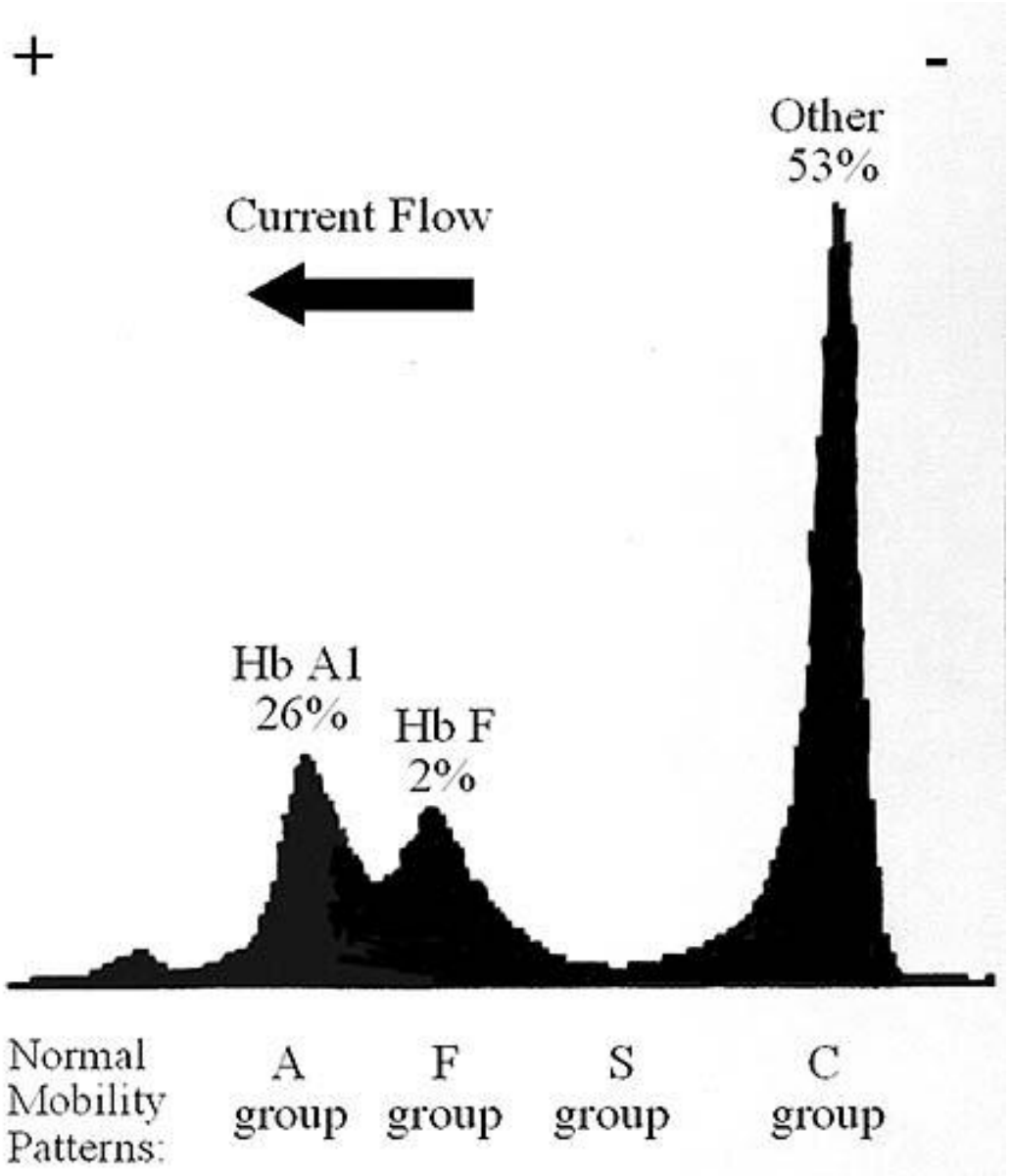
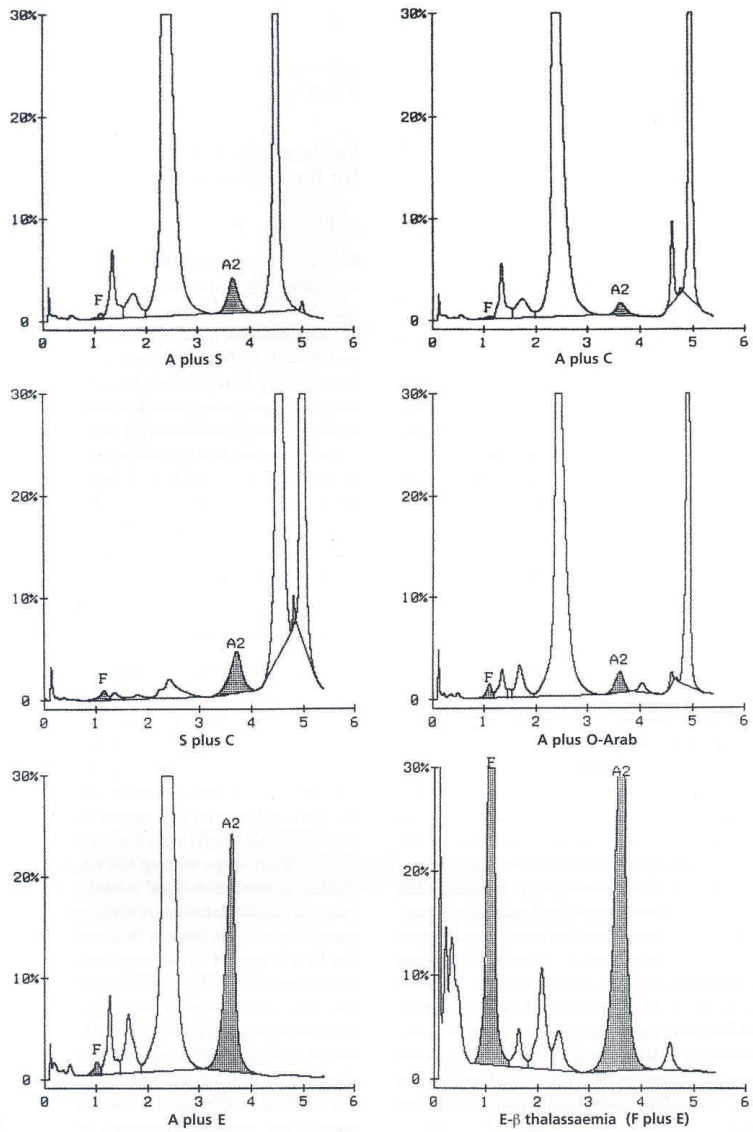


Fig. 2.12 Mobilities of various haemoglobins on an isoelectric focusing plate. Haemoglobins with similar mobility on haemoglobin electrophoresis can be distinguished from each other: haemoglobins A_2, C and E can be distinguished (but E cannot be distinguished from C-Harlem and O-Arab); haemoglobins S, D-Punjab and G-Philadelphia can be distinguished from each other (and also from D-Iran and G-Galveston but G-Philadelphia has the same pI as G-Coushatta and Lepore). (Modified from reference 7.)

HPLC

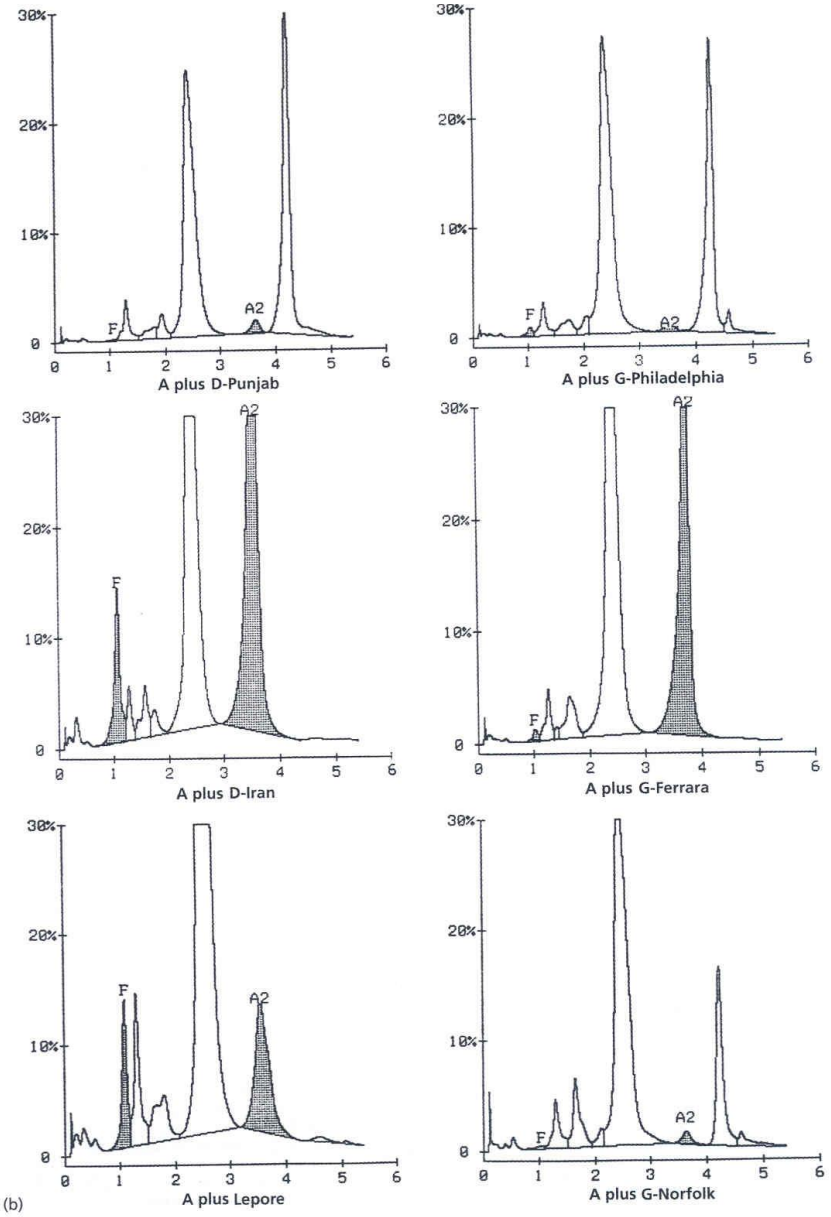
- Retention time of different Hb varies
- Retention time of A2 and E are the same


+
-
Other
53%
Current Flow
Hb A1
26%
Hb F
2%
Normal
Mobility
Patterns:
A
group
F
group
S
group
C
group



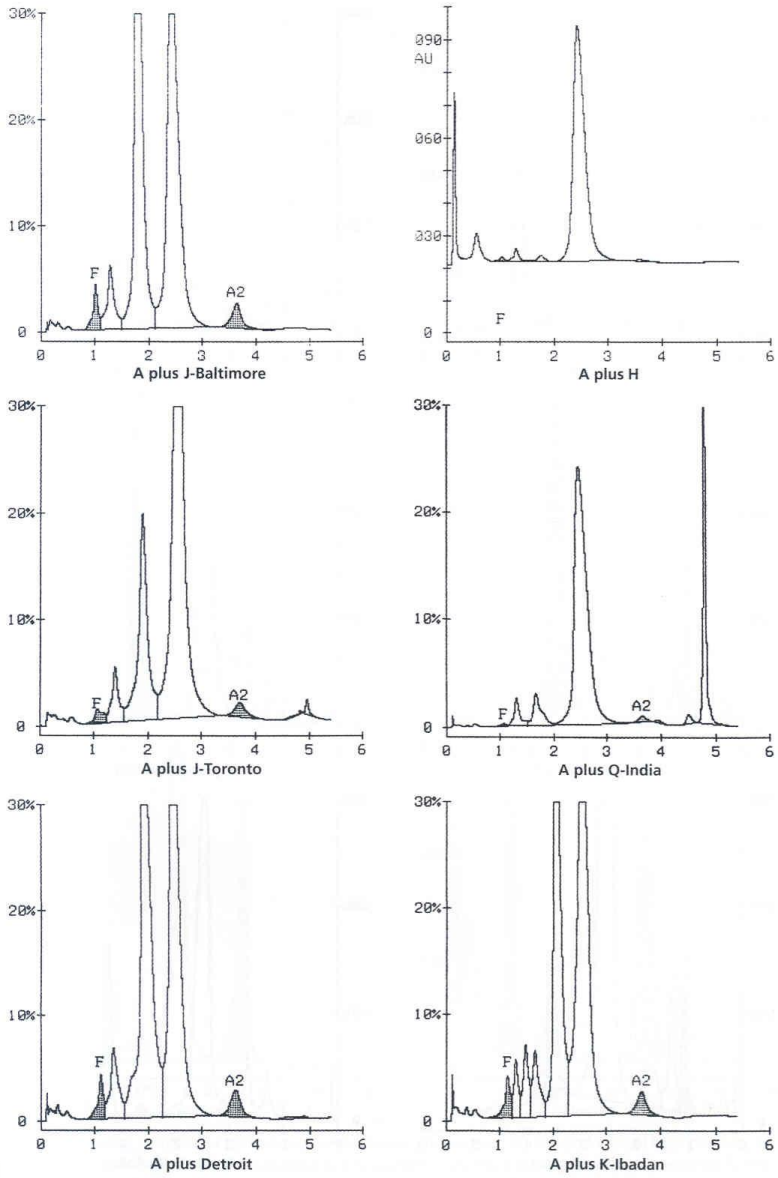
(a)

Fig. 2.13 Typical elution patterns for normal and variant haemoglobins with the Bio-Rad variant high performance liquid chromatography (HPLC) system. Unless specified, heterozygosity is illustrated: (a) some clinically relevant haemoglobins; (b) some haemoglobins that have the same mobility as haemoglobin S on cellulose acetate electrophoresis at alkaline pH but can be distinguished by HPLC; (c) some variant haemoglobins that are 'fast' on cellulose acetate electrophoresis at alkaline pH; (d) miscellaneous variant haemoglobins.



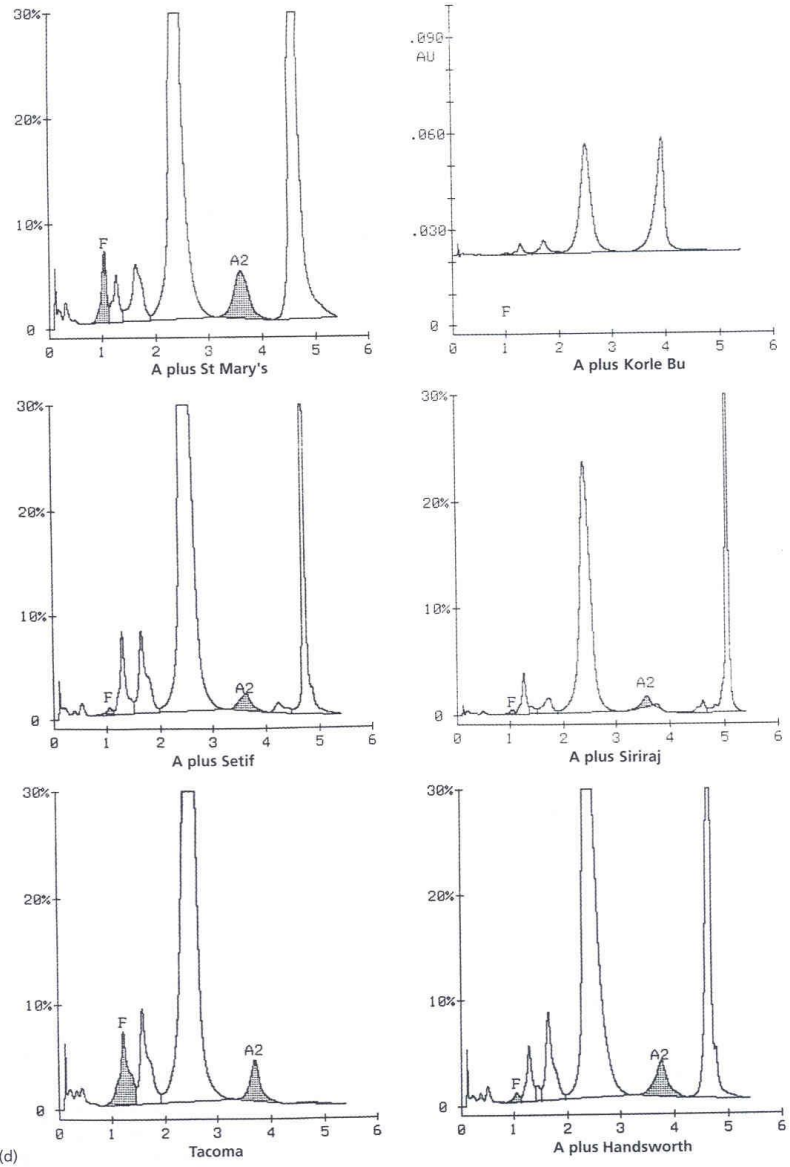
(b)

Fig. 2.13 *Continued*



(c)

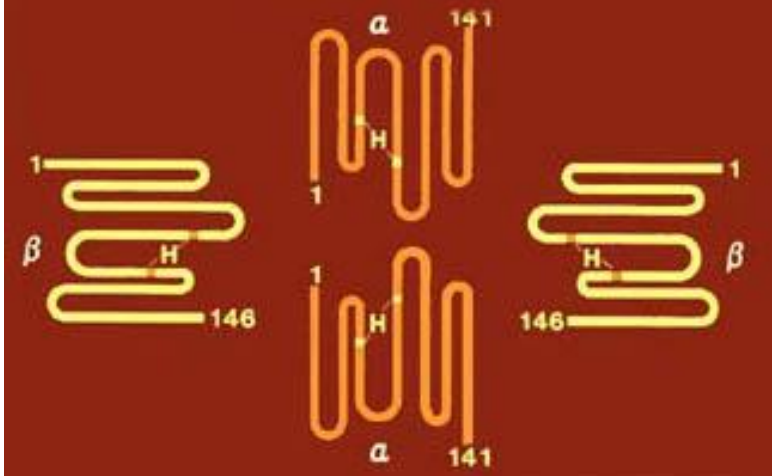
Fig. 2.13 *Continued*



(d)

g. 2.13 *Continued*

HEMOGLOBIN MOLECULE



Three normal hemoglobins

Hb A	$\alpha_2\beta_2$	96%
Hb A_2	$\alpha_2\delta_2$	3%
Hb F	$\alpha_2\gamma_2$	1%

GLOBIN CHAIN GENES

GENE	NUMBER	CHROMOSOME
α	2/2	16
$^G\gamma$	1/1	11
$^A\gamma$	1/1	11
δ	1/1	11
β	1/1	11

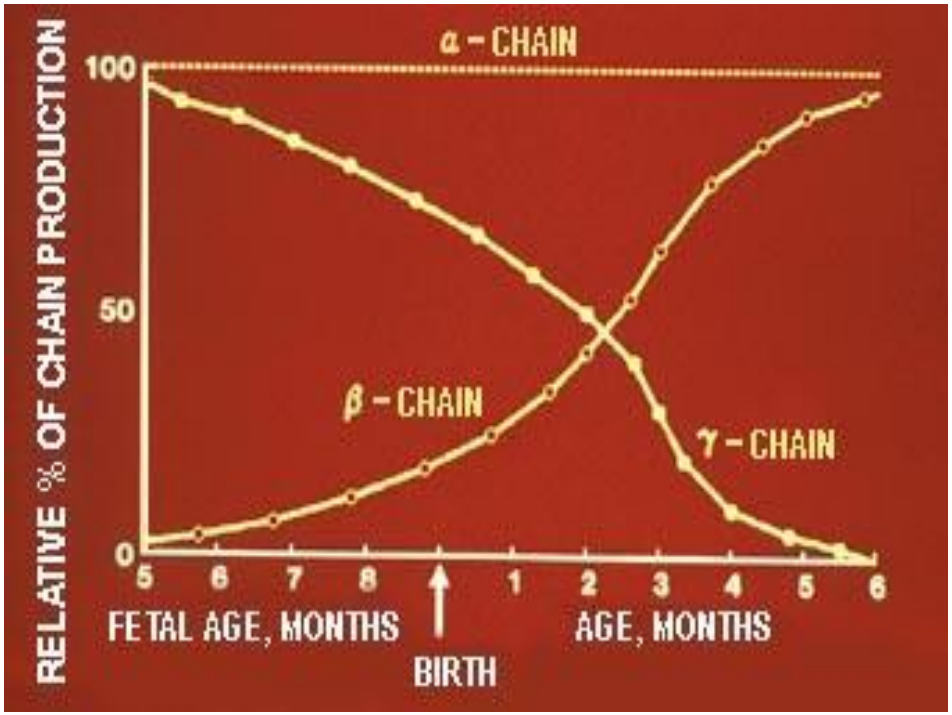
NORMAL HEMOGLOBINS

	Newborn(%)	Adult(%)
Hb A $\alpha_2\beta_2$	25	97
Hb A_2 $\alpha_2\delta_2$	<1	3
Hb F $\alpha_2\gamma_2$	75	<1

Hemoglobin molecule is a tetramer

Subunits: α, β, γ, δ, ζ, ε

Hg A(α2β2), Hg A2(α2δ2), F(α2γ2), Gower 1(ζ2ε2), Gower 2 (α2ε2), Portland (ζ2γ2)



The switch in percentages occurs as a result of an **increase in beta chain production** and a **decrease in gamma chain production** beginning at the 6th month of fetal life.

Delta chain production is minimal at birth and reaches normal levels (about 3% of total) at about one year of life.

This list shows some of the commoner tests used to investigate the hemoglobinopathies.

Blood count
Hemoglobin electrophoresis: Cellulose acetate pH 8.4,
Citrate agar pH 6
Solubility tests
Quantitation: Hb A2, Hb F, Hb Barts
Tests for unstable hemoglobins
Gene analysis

Hb A	$\alpha_2\beta_2$	96%
Hb A_2	$\alpha_2\delta_2$	3%
Hb F	$\alpha_2\gamma_2$	1%

Beta Thalassemia	Delta-Beta Thalassemia	Alpha Thalassemia
$\alpha_2\beta_2\downarrow$	$\alpha_2\beta_2\downarrow$	$\downarrow\alpha_2\beta_2$
$\alpha_2\delta_2$	$\alpha_2\delta_2\downarrow$	$\downarrow\alpha_2\delta_2$
$\alpha_2\gamma_2$	$\alpha_2\gamma_2$	$\downarrow\alpha_2\gamma_2$

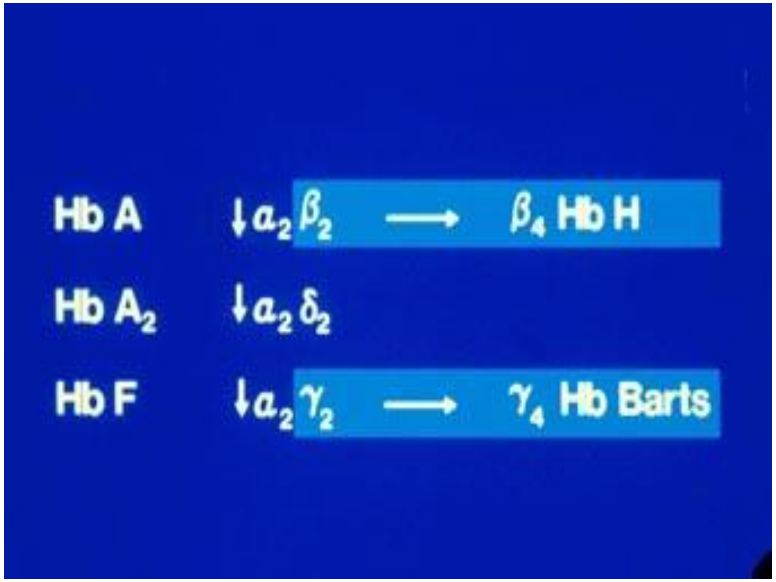
THALASSEMIA

MAJOR - Lifelong transfusion requirement

INTERMEDIA - Moderate anemia
Minimal or no transfusion need

MINOR - Slight anemia at worst

"SILENT" - Detectable only by:
Family studies
Gene analysis

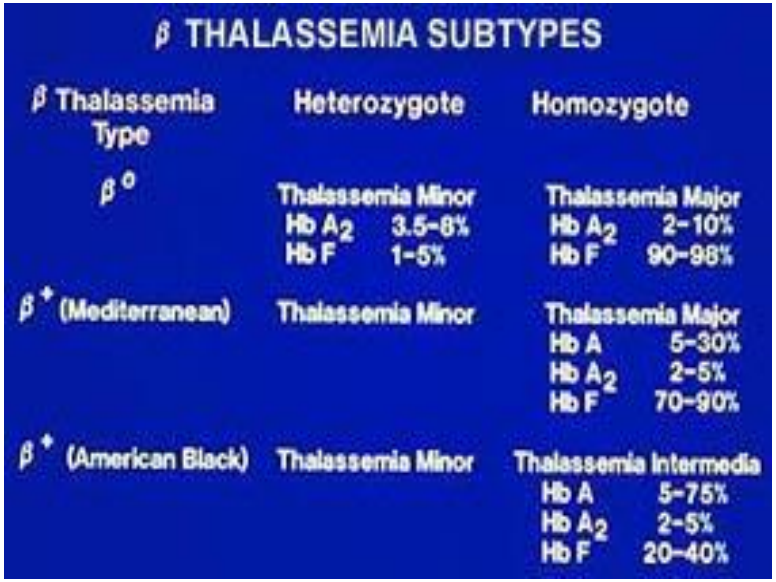


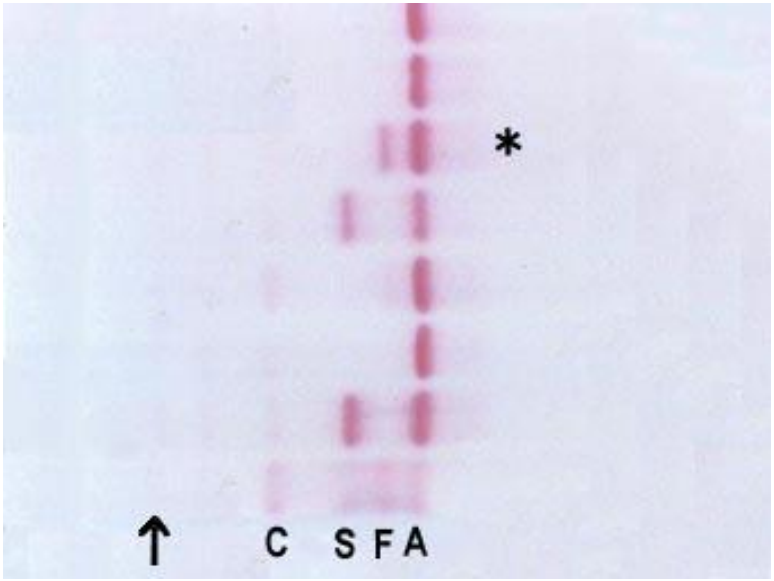
ALPHA THALASSEMIAS

Alpha Genes Deleted	Clinical Disorder	Hemoglobin Abnormalities Newborn	Adult
		Hb Barts	Hb H
One	None	1-3%	0%
Two	Thalassemia Minor	4-10%	0%
Three	Hb H Disease	15-25%	10-25%
Four	Fetal death	100%	-

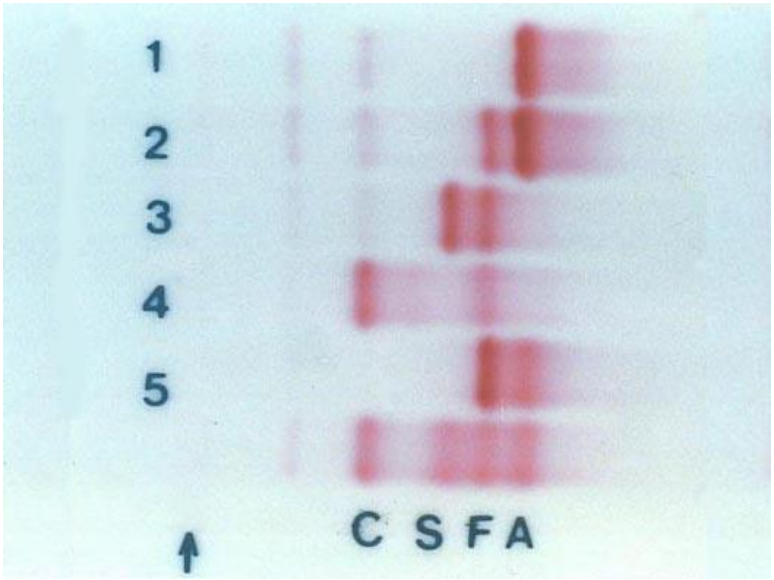
Electrophoresis of Hb Barts and Hb H
Cellulose acetate pH 8.4

1. Hb Barts with Hb A and HbF and albumin in newborn
2. Hb H, Hb A and albumin in an adult
3. Hb J and Hb A in an adult.


β THALASSEMIA SUBTYPES
β Thalassemia Type
Heterozygote
Homozygote
β°
Thalassemia Minor
Hb A2 3.5–8%
Hb F 1–5%
Thalassemia Major
Hb A2 2–10%
Hb F 90–98%
β+ (Mediterranean)
Thalassemia Minor
Thalassemia Major
Hb A 5–30%
Hb A2 2–5%
Hb F 70–90%
β+ (American Black)
Thalassemia Minor
Thalassemia Intermedia
Hb A 5–75%
Hb A2 2–5%
Hb F 20–40%



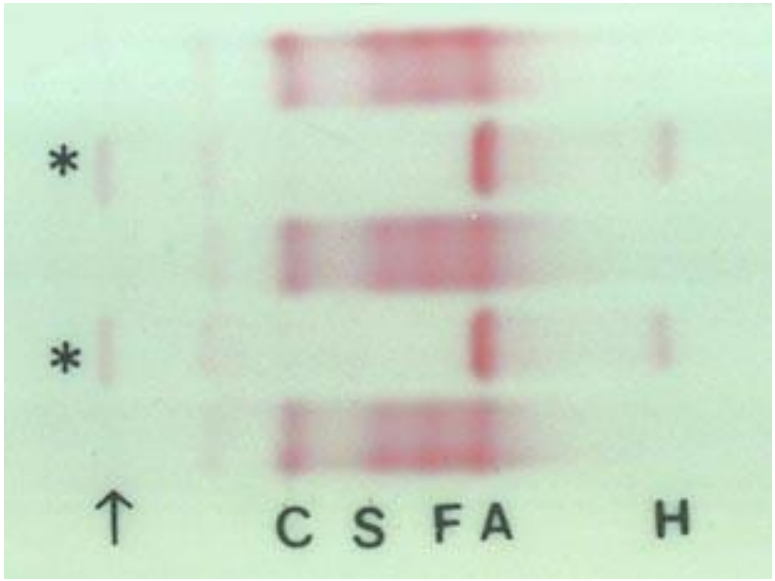
Healthy 25 year old African-American man.
Blood count :
Hb 15.0g/dl
RBC 5.5 10^0/l
MCV 82 micro
RDW 13.1
Hb electrophoresis, cellulose acetate pH 8.4
Diagnosis : HPFH (heterozygote)
There are also 2 examples of sickle cell trait on this plate.



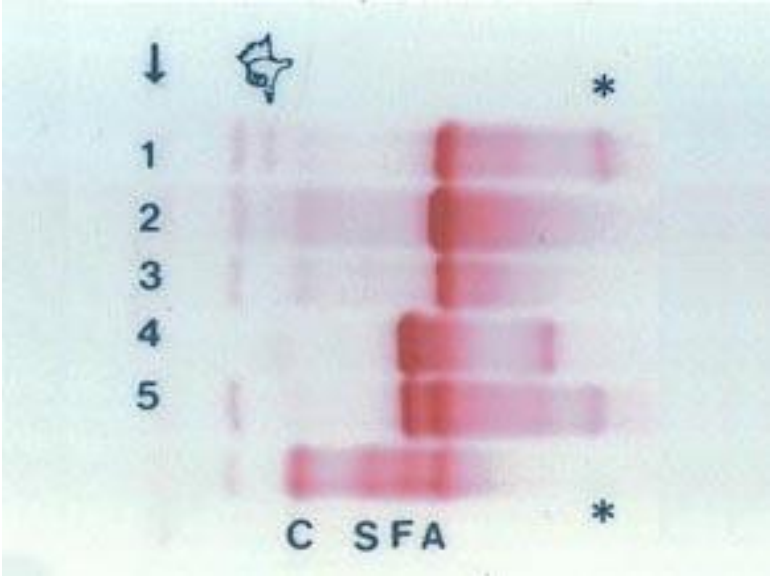
Other examples of HPFH

Hb electrophoresis. cellulose acetate pH 8.4

1. Normal adult
2. HPFH (heterozygote)
3. Hb S--HPFH
4. Hb C--HPFH
5. Normal newborn



A 32 year old oriental lady with a lifelong history of anemia had the following blood count:
Hb 7.9 g/dl RBC 6.4 10^{12}/l MCV 67 microns RDW 32.6
Hemoglobin electrophoresis on cellulose acetate at pH 8.4.
Patient shown by *
Comment. A large band of Hb A and a small band of Hb H are seen. The history and findings are typical of Hb H disease, usually due to the inheritance of a total of three deleted alpha chain genes. Hb H is an unstable hemoglobin which causes a hemolytic anemia

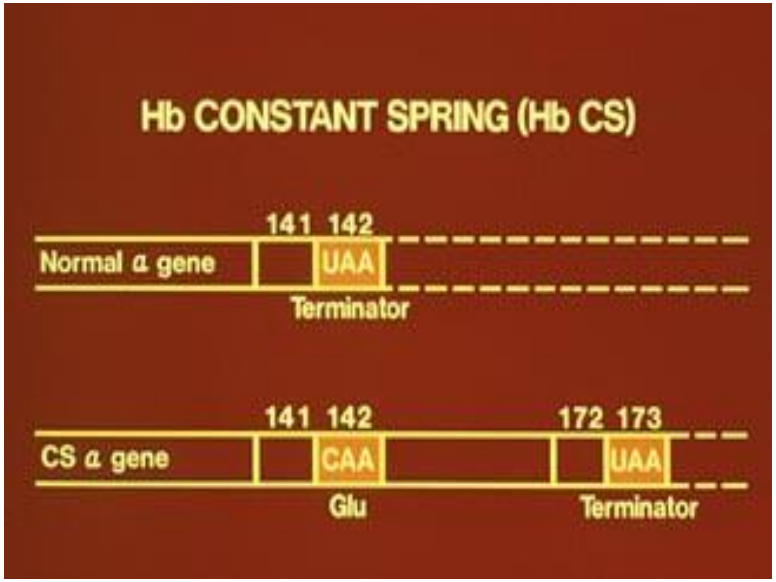


This hemoglobin electrophoresis on cellulose acetate at pH 8.4 contains the following:

1. Patient 2. Patient's mother.3. Patient' s father
4. Cord blood with Hb Barts.
5. 5 month old with Hb Barts and Hb H

All were applied heavily so that the minor bands could be seen.

Comment : The patient (#1) shows Hb A, Hb H(*) and a faint band ahead of the point of application marked with the hand. This represents **Hb Constant Spring** a common abnormal hemoglobin in southeast Asia.



This diagram shows the abnormality in the alpha chain of Hb Constant Spring.
In the normal alpha gene the 142nd "message" is a terminator.
In the Constant Spring alpha gene this codon has been mutated to a codon for glutamine.
This is followed by 29 codons for various amino acids before another terminator is arrived at.
Thus the alpha chain of lib Constant Spring has 172 amino acids instead of 141.

This abnormal hemoglobin occurs in 5% to 10% of some populations in southeast Asia.

When one of the four alpha genes is programmed for Hb Constant Spring one would expect to find about 25% of the hemoglobin to be Hb Constant Spring but this hemoglobin is difficult to manufacture and in such a person only about 1.5% is abnormal (when two alpha genes are affected then only about 3.0% of the total hemoglobin is Hb Constant Spring). Thus this hemoglobin is very similar to a deletion of an alpha gene and when an individual inherits two alpha gene deletions from one parent and a Hb Constant Spring gene from the other he develops Hb H disease.

Other elongated alpha chains. The mutation of the terminator codon in Hb Constant Spring is only one of four that have been described.

This list shows the 4 possibilities (in addition to normal Hb A) that have been described. Hb Constant Spring is the only one that is common

HEMOGLOBINOPATHIES

1. Quantitative defects (the thalassemia syndromes)
   imbalance of globin chain production

2. Qualitative defects
   Substitution, addition or deletion
   of one or more amino acids

3. Hereditary persistence of fetal hemoglobin (HPFH)

Nine most important hemoglobinopathies (In order of world wide prevalence) are: S, E, C, D-Los Angeles, G-Philadelphia, O-Arab, H, Lepore, and Koln

Clinical and hematologic manifestations of hemoglobinopathies

- Normal health, nl hem parameters
- Sickling disorders (S, C, D, O)
- Thalassemia syndromes (E, Lepore)
- Life-long cyanosis (Kansas, Freiburg, M-Chicago)
- Hemolytic anemia (H, Koln)
- Erythrocytosis (three dozens of Hg, high O2 affinity, example - Malmo)

-Mutation could occur either in the beta or alpha chains

- S, C, E, D are beta chain variants

- G and J may be either alpha or beta variants

STRUCTURAL ALTERATIONS

Amino acid substitutions

e.g. Hb S $\alpha_2\beta_2^{6\ glu \rightarrow val}$

Amino acid deletions

e.g. Hb Leiden $\alpha_2\beta_2^{6\ glu\ (or\ 7\ glu)\ deleted}$

Amino acid additions

e.g. Hb Constant Spring $\alpha_2^{141-172}\beta_2$

Fusion chains

e.g. Hb Lepore $\alpha_2(\delta\beta)_2$

Hemoglobin S: β 6(A3)Glu→Val

- 8% of American Blacks Hg AS
- 1 in 500 newborn AB Hg SS
- Hg S also in Italians, Turks, Greeks, Arabs and Asian Indians

Hemoglobin C: β 6(A3)Glu →Lys

- About 2% AB have C trait (Hg AC)
- Some areas of Africa up to 20%, also Italians , Greeks, Arabs
  - Clinically entirely well
  - A:C=60:40
- Homozygotes (Hg CC): mild hemolytic anemia, abundant targets, no Hg A
- Hg SC (more often than CC): moderate to severe sickle cell anemia

Hemoglobin E: β 26(B8)Glu→Lys

- South East Asians
- Hg AE: A- 70%, E- 30%
  - Inocuous, no anemia, slight microcytosis, mildly thalassemic blood picture
- Hg EE: no A, E – 99%, about 1% F
  - Not a serious disorder, marked hypochromia and microcytosis
- E/β-thal: severe thalassemia similar to classic β-thal major

Hemoglobin D (D-Los Angeles, D-Punjab): β 121(DH4)Glu→Gln

- English, Irish, Scotch ancestry
- Uncommon in N.America (AD < 1:5000)
- India & Pakistan (Punjab) – 3% D trait
- AD (A:D= 50:50) : entirely well, hematologically normal,
- DD: very rare, not disabling Dz
- S/D: severe sickling disorder

Hemoglobin G (G-Philadelphia): α68(E17)Asn→Lys

- The only alpha chain variant common in US (AB and African Blacks, not in other ethnic groups)
- AG (A:G=75:25): no physical or hem abn
- GG: ??
- S/G-Phil: clinically well, no hem abn
  - Three major bands: 1)A, 2)S+G, 3)SG (in A2 position)

Hemoglobin O (Arab): β 121(GH4)Glu→Lys

- First described in an Arab indiv, most common in BA (trait in 0.4%), also Bulgaria
- Trait (Hg AO) innocuous, no hem abn
- Homozygotes very rare: hypochromia, microcytosis, but no disability
- S/O-Arab: severe sickling disorder

Hemoglobin H: β4 tetramer

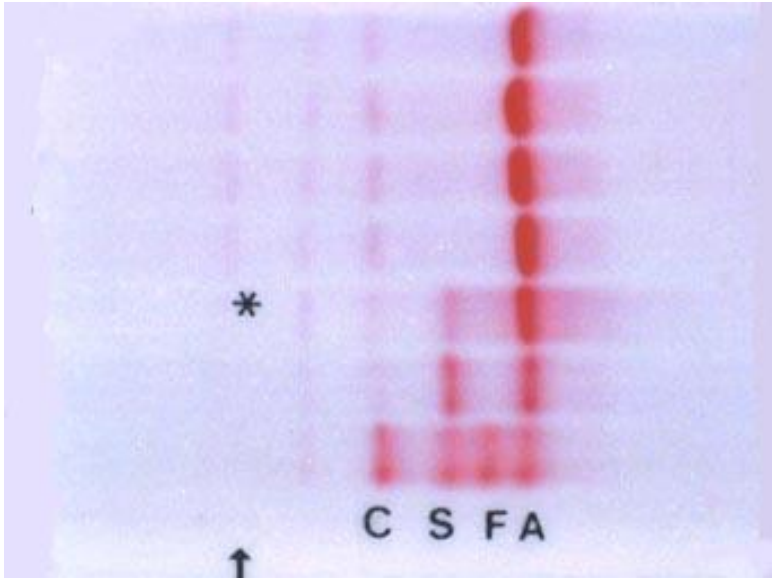
- Deletion of 3 of 4 $\underline{\alpha}$ genes (S.E.Asia)
- Unstable Hg
- Moderate to severe anemia, jaundice, splenomegaly
- Blood: microcytosis, hypochromia, target cells, polychromasia

Hemoglobin Lepore-Boston:δ(1-87) β(115-146)

- Fusion Hb, nonhomologous crossing-over
- Mainly Mediterranian ancestry
- Trait: mild thalassemia minor (mild microcytosis and mild anemia)
- pH 8.6 at S position (10-15% of total Hg)
- A2↓ F (2-10%) like δβ-thal
- Lepore homozygotes or Lep/ β-thal: thalassemia major-like disorder

Hemoglobin Koln: β98 (FG5)Val→Met

- Unstable Hg
- Nothern Europeans
- Mild congenital hemolytic anemia (AD, maybe mistaken for hereditary spherocytosis)
- Hypochromia, **macro**cytosis
- Broad smudge in the S position
- Homozygotes not reported



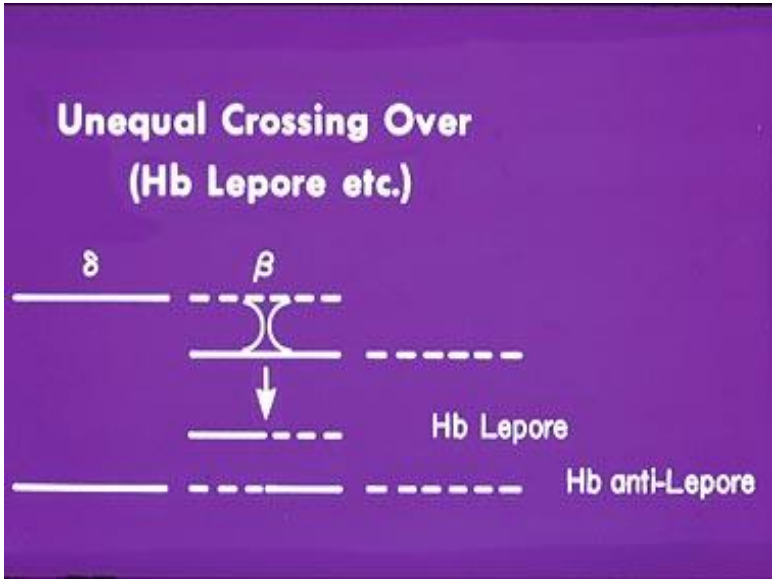
Healthy 5 year old with the following blood count :
Hb 11.9g/dl
RBC 6.3 10^{12}/l
MCV 63 microns
• A typical thalassemia minor blood count
Hemoglobin electrophoresis on cellulose acetate pH 8.4 *
Patient with four normal adults and one sickle trait on either side

Comment :

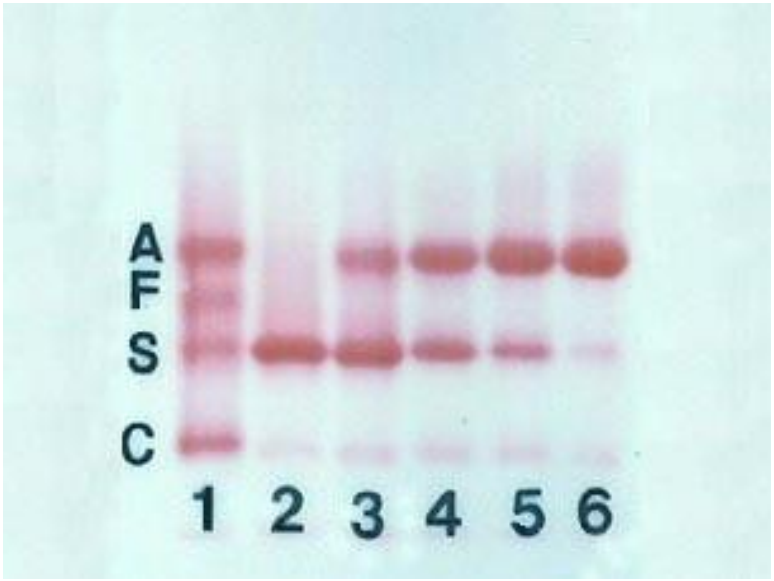
Approximately 10% of a hemoglobin migrating like Hb S In an untransfused patient (a most important part of the history) this small amount of Hb S is never found. Hemoglobin electrophoresis in acid agar would show this abnormal hemoglobin migrating as Hb A.

Diagnosis : Hb Lepore

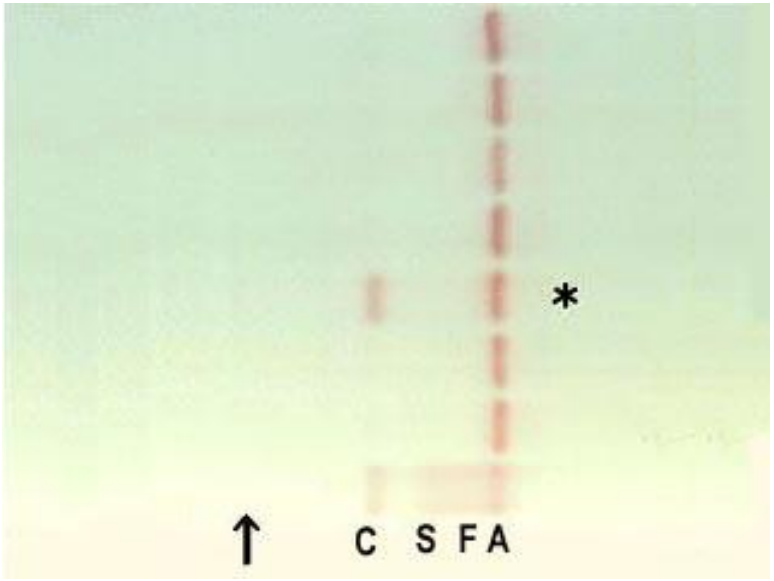
Hb Lepore has an abnormal "beta" chain made up of the beginning of the delta chain and the end of the beta chain. This arises from a cross over between the two chromosomes 11 as shown in the diagram.

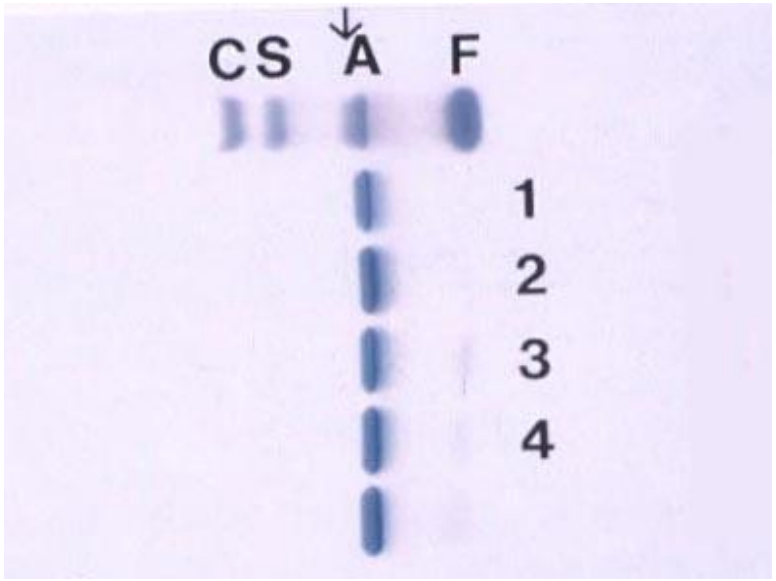


The delta-beta chain is difficult to manufacture and instead of the expected 50% in the heterozygote there is only 10%. This imbalance explains the thalassemic blood count.



- 1. is the control
- 6. is an example of Hb Lepore trait (see Case 10)
- 5. is an example of Hb S with alpha thalassemia, There is significantly more Hb A than Hb S. A typical finding when a beta chain abnormality (e.g Hb S or Hb C) is coinherited with alpha thalassemia.
- 4. is an example of sickle cell trait (heterozygous Hb S) where there is almost equal amounts of Hb A and Hb S.
- 3. is an example of Hb S with beta thalassemia. There is significantly less Hb A than Hb S plus a band of Hb F. The beta thalassemia gene is in this case beta+: beta gene activity is reduced but not absent as in beta-O. hence the presence of some, but not a normal amount of Hb A.
- 2. is an example of sickle cell anemia (homozygous Hb S) with no Hb A. It could just as well be a double heterozygote for Hb S and beta-O thalassemia where the patient is unable to produce any beta-A chains and therefore no Hb A.

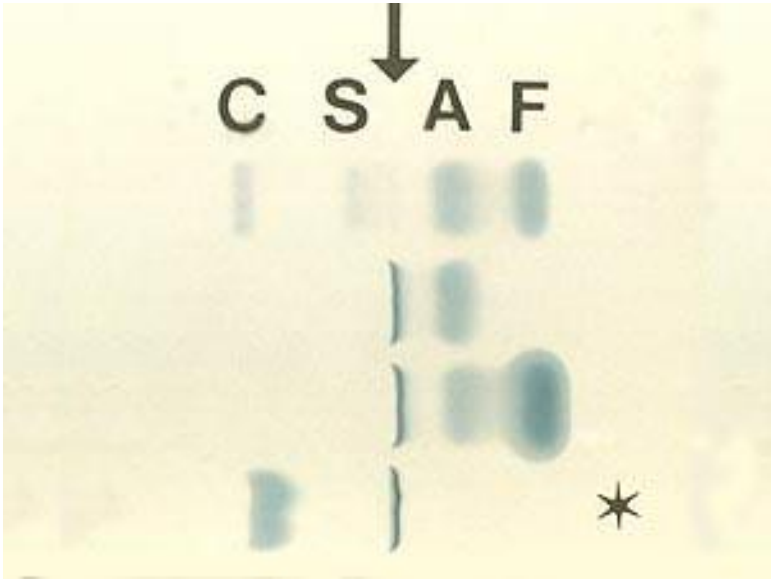




The abnormal hemoglobin migrates as Hb C on cellulose acetate and as Hb A in acid agar.

Diagnosis : Hb E trait (heterozygote for Hb E)

A healthy African American with a normal blood count
Hemoglobin electrophoresis on cellulose acetate at pH 8.4
1. Control
2. Patient
3. Hb C trait (HbAC)

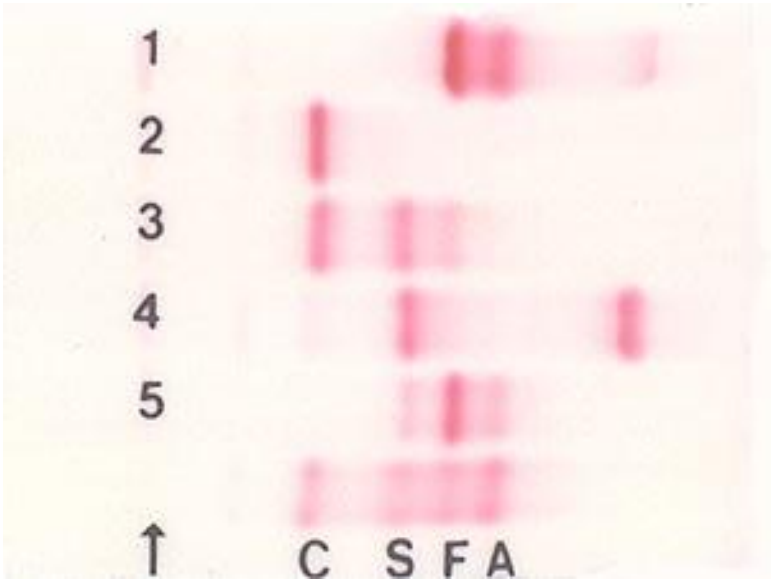


Hemoglobin electrophoresis in acid agar at pH 6.0
* marks the patient
The other two electrophoreses are from :
a mother with Hb O Arab trait (heterozygote for Hb O)
her newborn son also with Hb O trait

Diagnosis. Hb CO (double heterozygote for Hb C and Hb O)

COMMON HEMOGLOBINS MIGRATING AS Hb C

Hb C	β^{6}	glu → lys
Hb E	β^{26}	glu → lys
Hb O	β^{121}	glu → lys



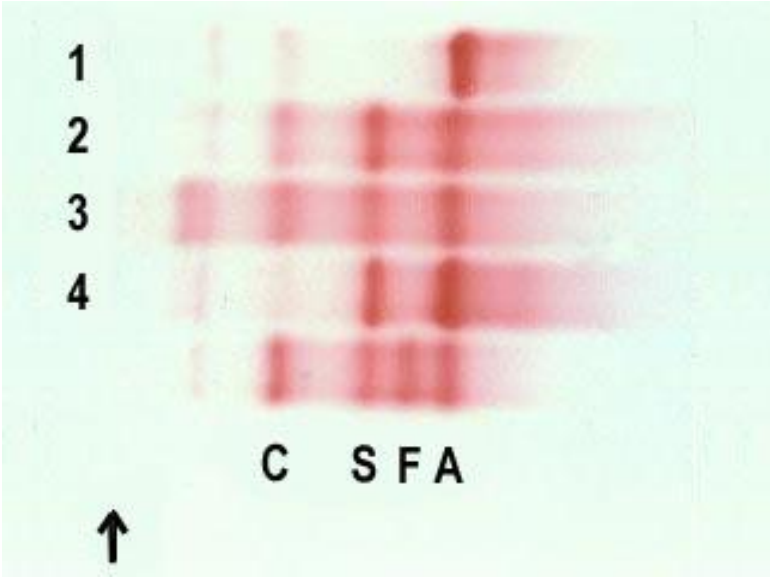
An African American woman with a history of intermenstrual bleeding. Her gynecologist ordered a blood count which showed a **Hb 20.0 g/dl**, normal white cell count and platelet count and normal morphology.
Hgb electrophoresis on cellulose acetate at pH 8.4
4. The patient.
1. Normal newborn with Hb Barts
2. Hb C disease
3. Hb SC
5. Hb S trait in newborn

Diagnosis : Hb SN, double heterozygote for Hb S (the solubility test was positive) and Hb N Baltimore.

Comment : There are equal amounts of Hb S and the fast Migrating Hb N (about the same speed as Hb Barts)
Hb N has a beta chain abnormality.
Hb N acts like normal Hb A. therefore this combination is similar to Hb S trait.

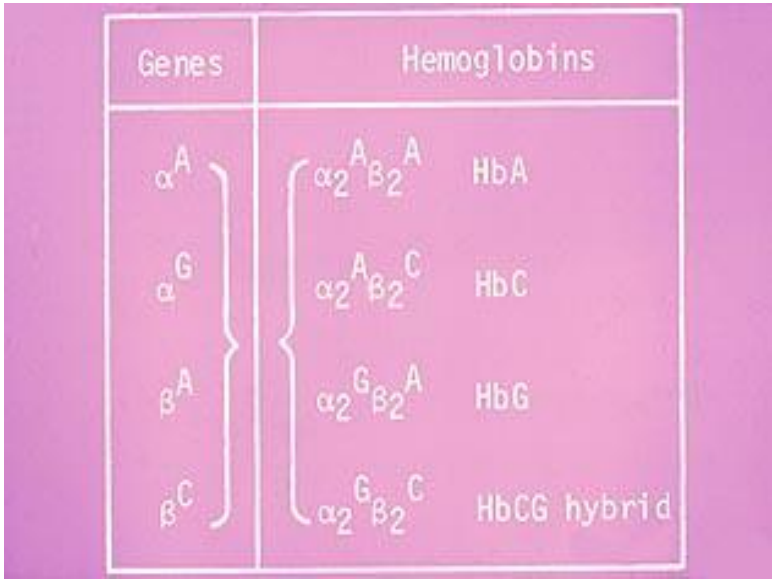
RELATIVE CLINICAL SEVERITY

0	SG, SN, CO, and heterozygotes
1	OO, EE
2	CC
3	SC, SD
4	SS, SO



Hemoglobin electrophoresis on cellulose acetate pH 8.4
1 . Normal adult
2. Case 15
3. Case 14
4. Hb AS (sickle cell trait)

Diagnosis :Case 14 Hb CG Philadelphia (double heterozygote Hb C and Hb G)
Case 15 Hb SG Philadelphia (double heterozygote Hb S and Hb G)



In this diagram the possible combinations in Case 14 are listed 4 different hemoglobins can be produced :

Hb A

Hb C

Hb G

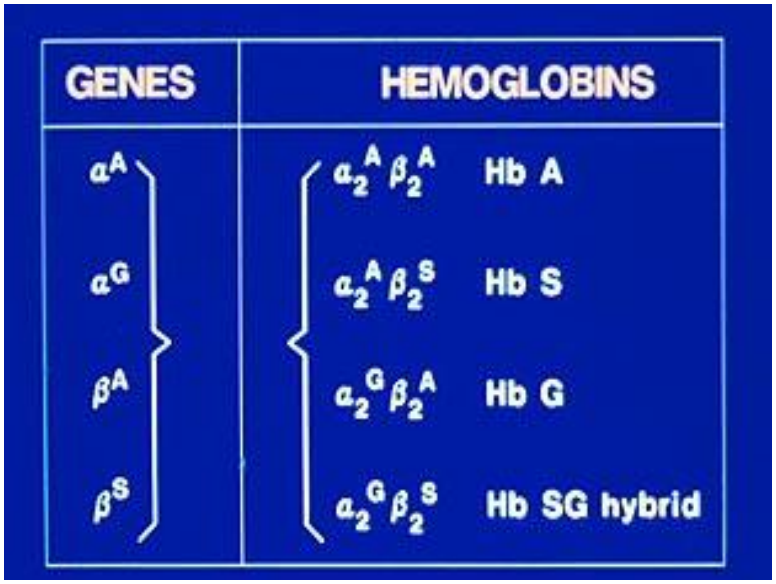
Hb CG hylrid

Hb A migrates as Hb A

Hb C migrates as Hb C

Hb G migrates as Hb S

The hybrid Hb CG, adding the slow migration of Hb C to that of Hb G,migrates even slower, adding the distance from Hb A to Hb G to the distance from HbA to HbC.



In this diagram the possible combinations in Case 15 are listed 4 different hemoglobin are again produced but only 3 bands :

Hb A

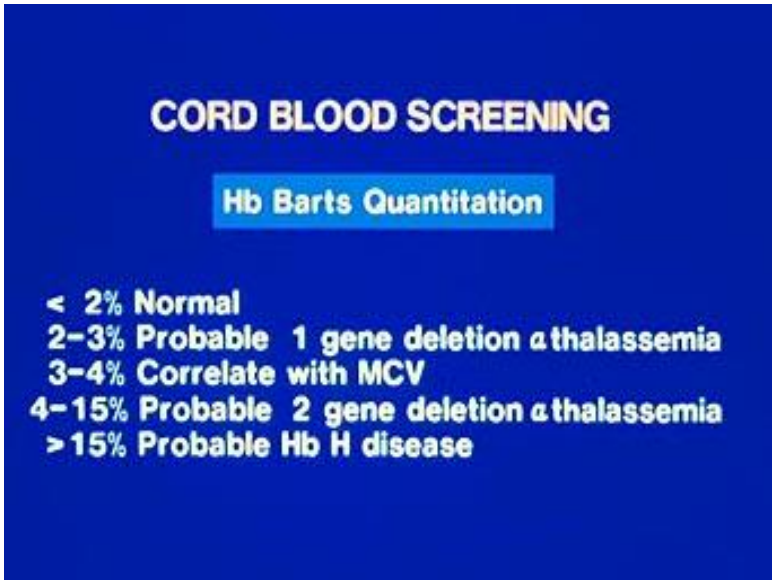
Hb G and Hb S migrating together (as a thick band)

Hb SG hybrid

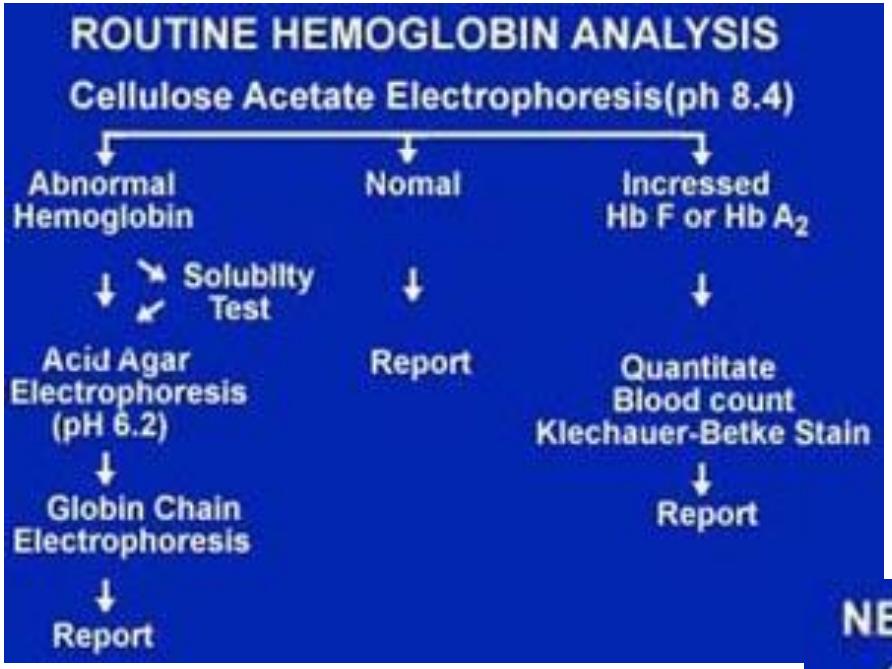
Comments : The hybrid Hb SG, adding the slow migration of Hb S to that of Hb G, migrates as Hb C.

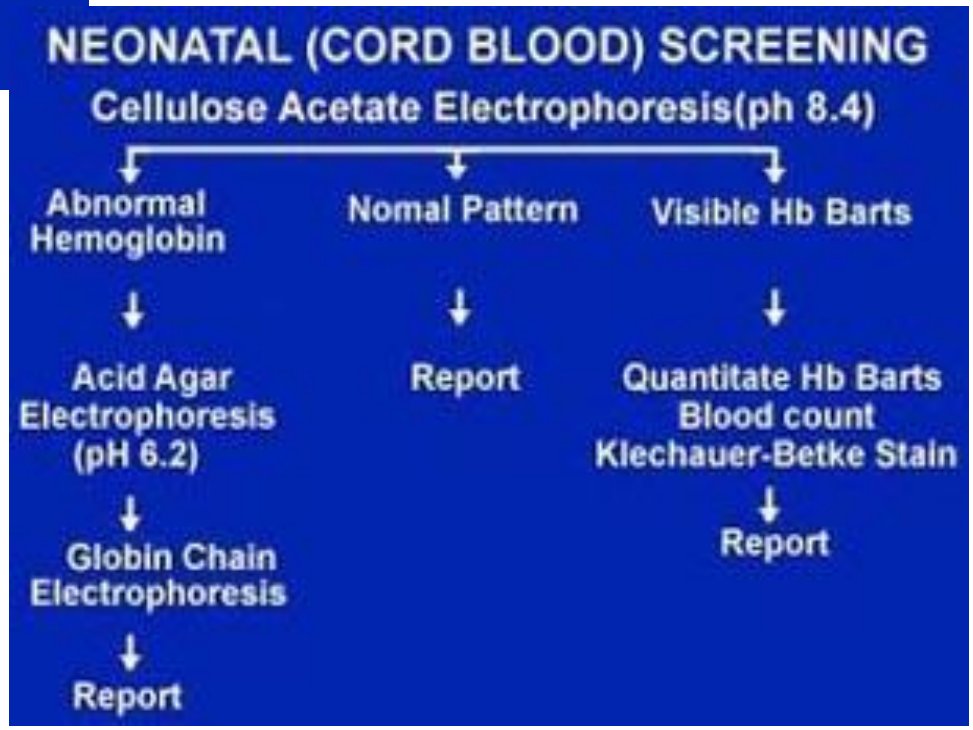
Screening of newborn (cord blood)

- The normal newborn has about 70% Hb F
- The amount of an abnormal hemoglobin, such as Hb S in sickle cell trait, will only be about 15%
- Therefore more lysate must be used in the electrophoresis
- There is virtually no Hb A2 in cord blood. If present it indicates the admixture of maternal blood and the electrophoresis cannot be interpreted correctly.
- The solubility test cannot be relied on since the maximum amount of Hb S, in a homozygote, would be about 30% and in the presence of a lot of Hb F would not give a positive result.



Making a diagnosis of alpha thalassemia minor (two gene deletion type) on the basis of a high level of Hb Barts in the newborn is very useful, because in later life he will have a typical thalassemia minor blood count but no positive diagnostic finding to suggest alpha (as opposed to beta) thalassemia.


ROUTINE HEMOGLOBIN ANALYSIS
Cellulose Acetate Electrophoresis(ph 8.4)
Abnormal Hemoglobin
Nomal
Incressed Hb F or Hb A2
Solubility Test
Acid Agar Electrophoresis (pH 6.2)
Report
Quantitate Blood count Klechauer-Betke Stain
Globin Chain Electrophoresis
Report
Report


NEONATAL (CORD BLOOD) SCREENING
Cellulose Acetate Electrophoresis(ph 8.4)
Abnormal Hemoglobin
Nomal Pattern
Visible Hb Barts
Acid Agar Electrophoresis (pH 6.2)
Report
Quantitate Hb Barts Blood count Klechauer-Betke Stain
Report
Globin Chain Electrophoresis
Report