Chromatography

- Intro – basic terminology – types
- Partition and Adsorption C
- Ion-Exchange C
- Gel Filtration (aka Exclusion or Molecular Sieve) C
- Affinity C

- Extremely varied and widely used methodology for separation and analysis
- Based on early expts of Tsvet (1903-6) on plant pigments
- Martin & Synge started method in 1944 (Nobel)

Terminology & Set-up for columns

- Pack column with stationary phase = sorbent (solid or liquid) and a mobile phase = solvent or eluent (liquid)

- Stationary phase is held in place by a support or matrix (inert)

- Sample (solute) is layered on top and flows through under gravity or high pressure (HPLC) – separates into components based on interactions between two phases

Variations

1. Partition
2. Adsorption
3. Ion-exchange
4. Exclusion
5. affinity

1. Column
2. Paper
3. Thin layer
4. gas

Chromatography

Short video clip
Second Video

3 levels of understanding

1. Basic physics – not known, too complex – involves hydrodynamics, solution theory, porous media, mass transfer kinetics, surface chemistry

2. Descriptive evaluation of concentration profile and its dependence on lab parameters (T, P, flow rate, etc.)

3. Procedures and optimization

- We'll discuss a bit of 2 & 3 for column chromatography using each of the methods

Partition & Adsorption Chrom.

- Solute partitions (distributes) itself between 2 phases in a characteristic way given by the partition coeff. K

$$K = \frac{c(stationary)}{c(mobile)}$$

- Molecular kinetic approach –
  - A single solute molecule either flows w/ solvent or is immobilized w/ stationary phase
  - Molecule hops in and out between 2 phases
  - When it becomes "sorbed" it stays in stationary phase for an average time $<t> = t_d$ = desorption time
  - When it flows, it does so for an average time $<t> = t_a$ = absorption time
  - The molecule spends an average time $\dfrac{t_a}{t_a + t_d}$ in the mobile phase
  - So therefore R = ratio of "zone" velocity to mobile phase velocity is $R = \dfrac{t_a}{t_a + t_d}$ where R = retension ratio, or retardation factor

Simulation of 512 molecules of 2 types: half (bold) distribute 1:1 between mobile (m) and stationary (s) phase while other half distribute 1:3 m:s

After 20 transfers
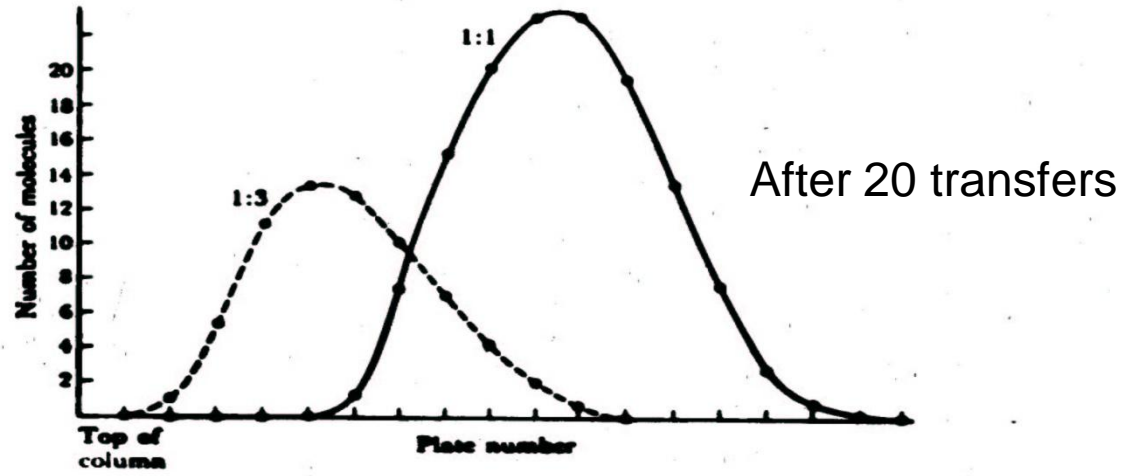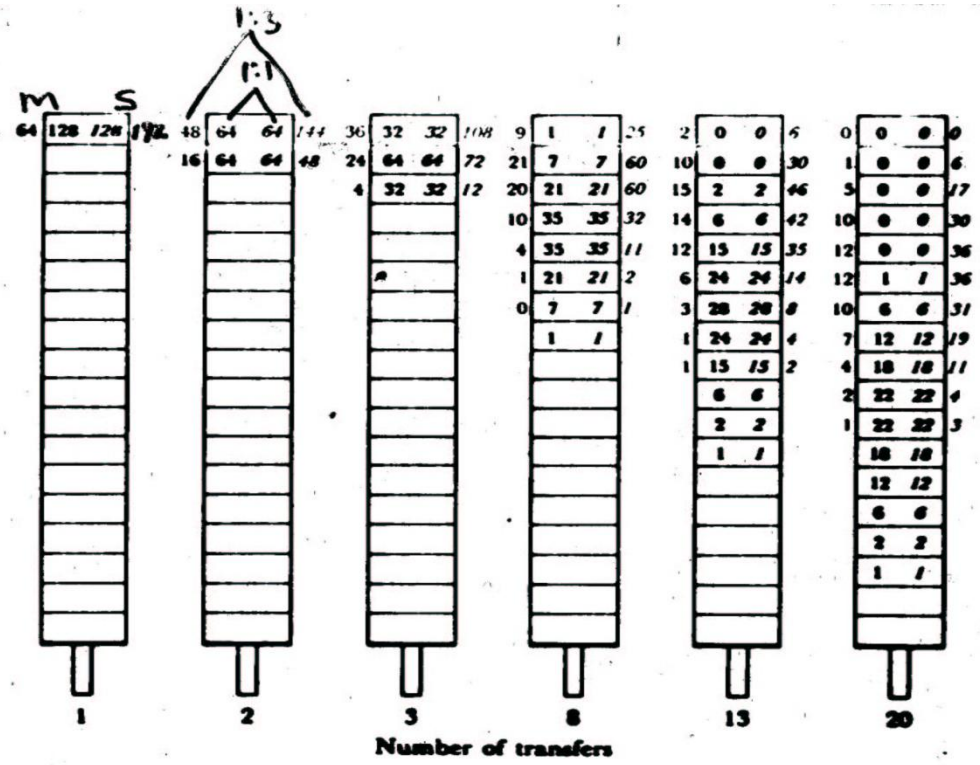
Partition C con't

- Bulk or equilibrium approach
 - Fraction of solute in mobile phase is

$$R = \frac{c_m V_m}{c_m V_m + c_s V_s} = \frac{V_m}{V_m + \left(\dfrac{c_s}{c_m}\right) V_s} = \frac{V_m}{V_m + KV_s}$$

- Common support materials are silica gel, cellulose, or (cross-linked) dextrans
- Common stationary phases are either hydrophobic (benzene) to separate non-polars or hydrophilic (alcohols) to separate polars – stationary phase is held in matrix by adsorption
- Mobile phase is typically alcohols for non-polars or water for polars
- Partition C is primarily for small molecules – rapid separation w/ narrow initial zones to minimize zone spreading by diffusion
- Adsorption C is the oldest form – the solute actually adsorbs to the stationary material – otherwise it is similar to Partition C

sample molecules carried by mobile phase

sample molecules held in sorbed solvent
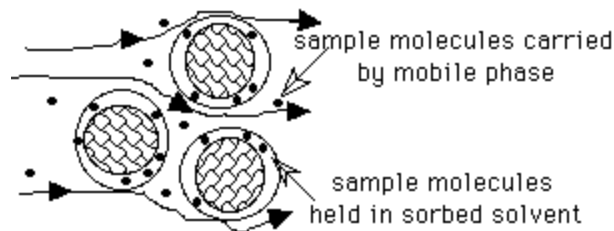
Ion-Exchange Chromatography

- Separation based on electric charge
- Ion-exchanger = solid w/ chemically bound charged groups – usually in the form of a resin, or cross-linked matrix

Charge Properties of Ion Exchangers

DEAE Anion exchanger

CM Cation exchanger
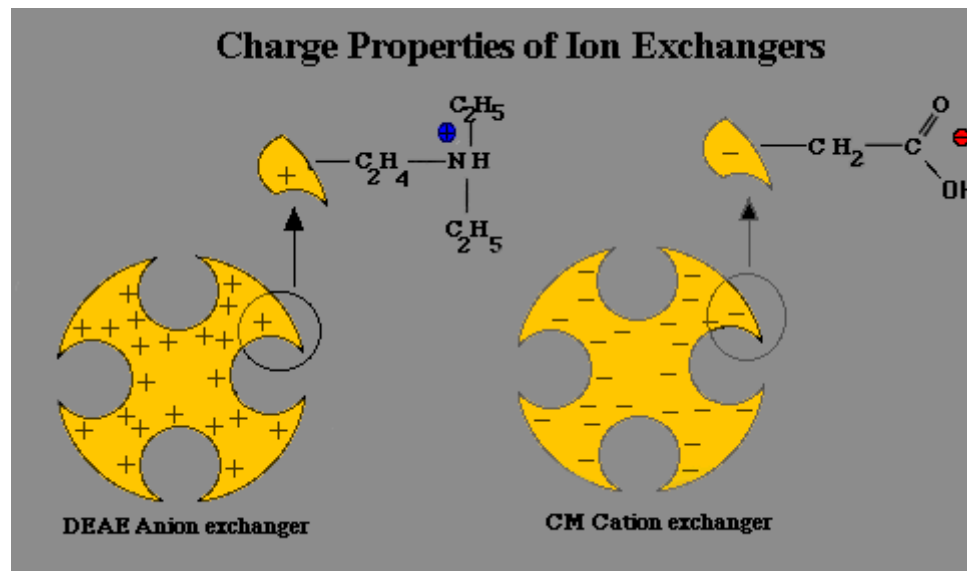
- Run sample through column and charges bind electrostatically to exchanger

Ion-Exchange C. II

- Porosity of matrix also affects resolution
- How to choose ion exchanger?
  - Anionic or cationic?
    - If only 1 charge it's easy
    - If stable above pI use anionic R+ and pH>pI
    - If stable below pI use cationic R- and pH<pI
  - Also need to choose between strong or weak exchanger – usually weak used for proteins
- Columns are re-usable – wash and keep in cold (bacteria)
- Amino acid analyzers use this method – need ~ 5 nM aa to detect

TABLE 8-3
Properties of various ion exchangers.

Matrix	Exchanger*	Functional group	Trade name
Dextran	SC	Sulfopropyl	SP-Sephadex
	WC	Carboxymethyl	CM-Sephadex
	SA	Diethyl-(2-hydroxypropyl) aminoethyl	QAE-Sephadex
	WA	Diethylaminoethyl	DEAE-Sephadex
Cellulose	C	Carboxymethyl	CM-cellulose
	C	Phospho	P-cel
	A	Diethylaminoethyl	DEAE-cellulose
	A	Polyethyleneimine	PEI-cellulose
	A	Benzoylated-naphthoylated, diethylaminoethyl	DEAE(BND)-cellulose
	A	p-Aminobenzyl	PAB-cellulose
Styrene-divinyl-benzene	SC	Sulfonic acid	AG 50
Acrylic	WC	Carboxylic	Bio-Rex 70
Phenolic	SC	Sulfonic acid	Bio-Rex 40
Epoxyamine	WA	Tertiary amino	AG-3

*C, cationic; A, anionic; S, strong, W, weak.

Exclusion or Mol. Sieve or Gel Filtration Chromatography

- Separation based on size
- Column is prepared with inert small gel-like molecules with pores
- Small enough molecules diffuse into pores and get trapped (not adsorbed) and so larger molecules elute first
- By calibration of the column, can get M
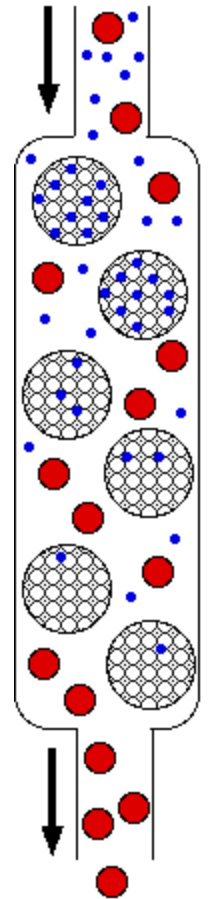
Semi-Quantitative Analysis

- $V_{total} = V_o$ (void volume external to gel)

  $+ V_{gel}$ (solid gel volume)

  $+ V_i$ (internal pore volume)

- Solute partitions between V_o and V_i with partition coefficient

$$\sigma = \frac{V_{penetrable\ by\ solute}}{V_{i,total}}$$

  - If $\sigma = 0$, solute in void volume

  - If $\sigma < 1$ solute less likely in pores than in bulk

  - If $\sigma = 1$, partitions equally between pores and external volume

  - If $\sigma > 1$ preferentially attracted to pores

Typical Procedure

1. Measure elution volume – volume of solvent that flows before any solute exits

   – A solute with $\sigma = 0$ must displace the entire void volume, but a solvent that can enter pores must displace an extra volume $V_p = \sigma V_i$

   So elution volume $V_{elution} = V_o + \sigma V_i$

2. Can separately measure $(V_i + V_o)$ by weight of solvent taken up by dry gel

3. V_o can be determined by measuring $V_{elution}$ for a solute much larger than pores, so then V_i is also known

4. All together then σ is found where $\sigma = \dfrac{V_{elution} - V_o}{V_i}$

5. But σ can be empirically related to M

   $\sigma = $ -A logM + B

- Gels are either dextran – (x-linked to different pore sizes) supplied as dry beads = Sephadex, or

agarose – H-bonded, so concentration determines pore size – used for large proteins and DNA (Sepharose or Biogel) or
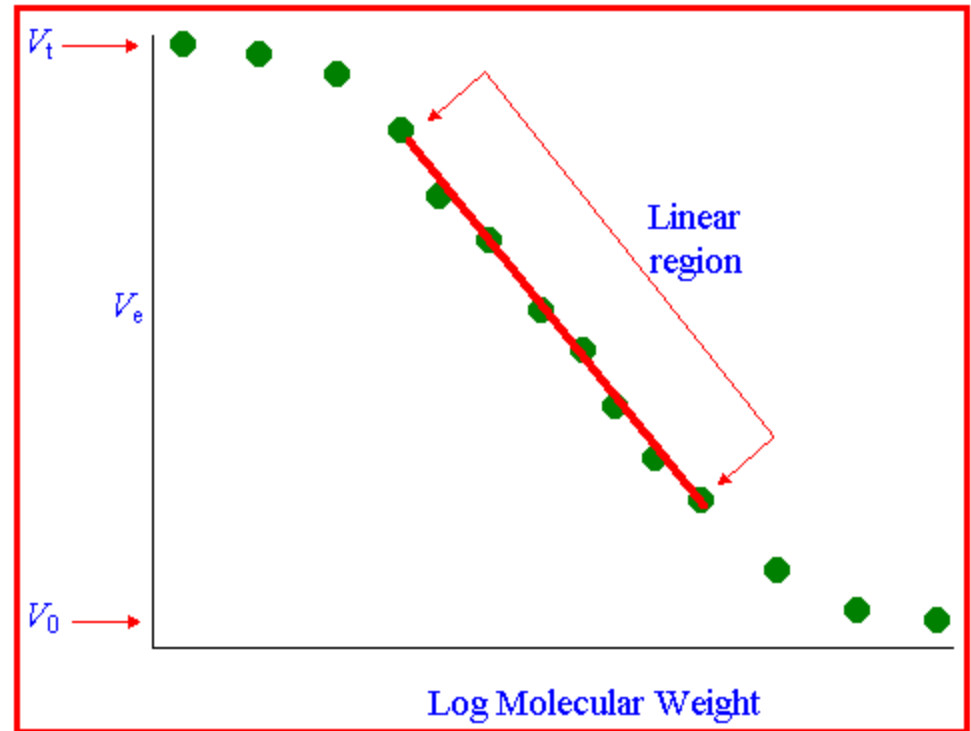
polyacrylamide (x-linked)

V_t

V_e

V_0

Linear region

Log Molecular Weight

TABLE 8-2

Materials commonly used in
gel chromatography.

Material and trade name	Fractionation range* (molecular weight)
Dextran	
Sephadex G-10	700
Sephadex G-25	1,000–5,000
Sephadex G-75	3,000–70,000
Sephadex G-200	5,000–800,000
Polyacrylamide	
Bio-gel P-2	200–2,000
Bio-gel P-6	1,000–6,000
Bio-gel P-150	15,000–150,000
Bio-gel P-300	60,000–400,000
Agarose	
Sepharose 2B	$2 \times 10^6 - 25 \times 10^6$
Sepharose 4B	$3 \times 10^5 - 3 \times 10^6$
Bio-gel A-0.5 M	30,000–500,000
Bio-gel A-15 M	$30,000 - 15 \times 10^6$
Bio-gel A-150 M	$5 \times 10^6 - 150 \times 10^6$

*Globular proteins or polynucleotides

Advantages of Gel Exclusion

1. Separations can be done over large range of pH, T, I, and solvents

2. Virtually no adsorption or loss of material or denaturation

3. Less zone spreading than with most other methods

4. Elution volume related to M in simple way

Applications

1. De-salting – use low MW gel column; protein in void volume – salt later

2. MW determinations - +/- 10% - protein still in native form

3. Study binding of small molecules – ligands – use column equilibrated with small molecule ligand; then put protein through column and monitor elution profile – see ligand peak and can measure binding constant

Affinity Chromatography

- The goal of affinity chromatography is to separate all the molecules of a particular specificity from the whole gamut of molecules in a mixture such as a blood serum. For example, the antibodies in a serum sample specific for a particular antigenic determinant can be isolated by the use of affinity chromatography.

- **Step 1.** An **immunoadsorbent** is prepared. This consists of a solid matrix to which the antigen (shown in blue) has been coupled (usually covalently). Agarose, sephadex, derivatives of cellulose, or other polymers can be used as the matrix.

- **Step 2.** The serum is passed over the immunoadsorbent. As long as the capacity of the column is not exceeded, those antibodies in the mixture specific for the antigen (shown in red) will bind (noncovalently) and be retained. Antibodies of other specificities (green) and other serum proteins (yellow) will pass through unimpeded.

- **Step 3. Elution.** A reagent is passed into the column to release the antibodies from the immuno-adsorbent. Buffers containing a high concentration of salts and/or low pH are often used to disrupt the noncovalent interactions between antibodies and antigen. A denaturing agent, such as 8 M urea, will also break the interaction by altering the configuration of the antigen-binding site of the antibody molecule.

- Another, gentler, approach is to elute with a soluble form of the antigen. These compete with the immunoadsorbent for the antigen-binding sites of the antibodies and release the antibodies to the fluid phase.

Antigen

Antibody specific for the antigen

Antibody of different specificity

Inert matrix

Nonantibody protein
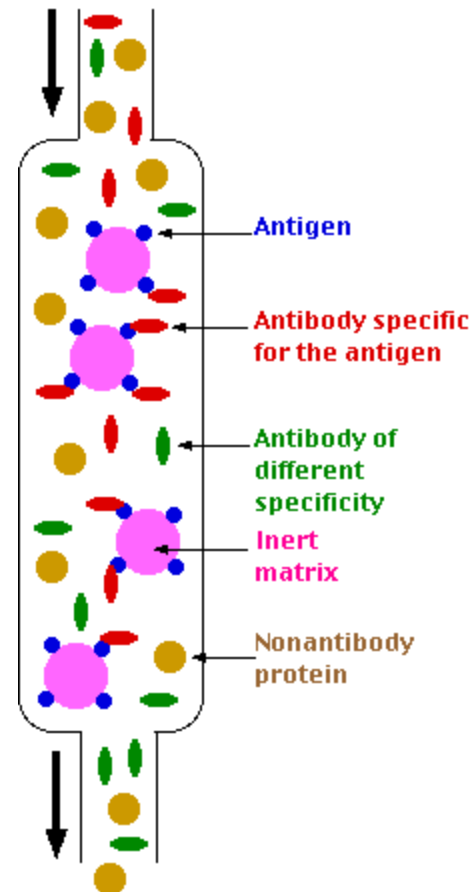
video

Affinity Gel Details

- **The Matrix**: Sepharose is a bead-formed of agarose gel. The hydroxyl groups on the sugar residues can be easily derivatized for covalent attachment of a ligand. Sepharose 4B is the most favored and widely-used matrix. The open-pore structure Sepharose 4B is vary large (Exclusion limits of MW 20×10^6) and exhibits extremely low non-specific adsorption

- **The Ligand**: Selection of the ligand for affinity chromatography is influenced by two factors: want specific and reversible binding affinity for the substance to be purified and chemically modifiable groups which allow it to be attached to the matrix without destroying its binding activity.
  Spacer Arms: The active site of a biological substance is often located deep within the molecule and adsorbents prepared by coupling small ligands (e.g. enzyme cofactors) directly to Sepharose can exhibit low capacities due to steric interference between the matrix and substances binding to the ligand. In these circumstances a "spacer arm" is interposed between the matrix and ligand to facilitate effective binding.

- **Coupling Gels**: Methods are available for immobilizing ligands quickly, easily and safely through a chosen functional group. The correct choice of coupling method depends on the substance to be immobilized. The following derivatives of Sepharose allow the convenient immobilization of ligands without the need for complex chemical synthesis or special equipment:

- CnBr-activated Sepharose 4B enables ligands containing primary amino groups to be safely, easily and rapidly immobilized by a spontaneous reaction.

- AH-Sepharose 4B and CH-Sepharose 4B both have a six-carbon long spacer arm and permit coupling via carboxyl and amino groups respectively.

- Activated CH-Sepharose 4B provides a six-carbon spacer arm and an active ester for spontaneous coupling via amino groups.

- Epoxy-activated Sepharose 6B has a long hydrophilic spacer arm and provides a method for coupling through hydroxyl, amino or thiol groups.

- Activated Thiol-Sepharose 4B has a gluthathione spacer arm and provides a method for reversibly coupling proteins through free thiol groups.

- Thiopropyl-Sepharose 6B has a short hydrophilic spacer arm and provides a method for reversibly coupling proteins and small thiolated ligands through thiol groups; it also reacts with heavy metal ions, alkyl and aryl halides and undergoes additonal reactions with compounds containing C=O, C=C, and N=N bonds.

Paper/thin-layer chromatography

- Paper/or thin-layer – detect with stain, fluorescence, radioactivity – 2 dimensional with different solvents –

- Fingerprinting of proteins – technique that uses paper chromatography in one dimension and paper electrophoresis in another to map all amino acids in proteins

Fingerprint of Hemoglobin: Normal (HbA) vs. Sickle Cell (HbS)
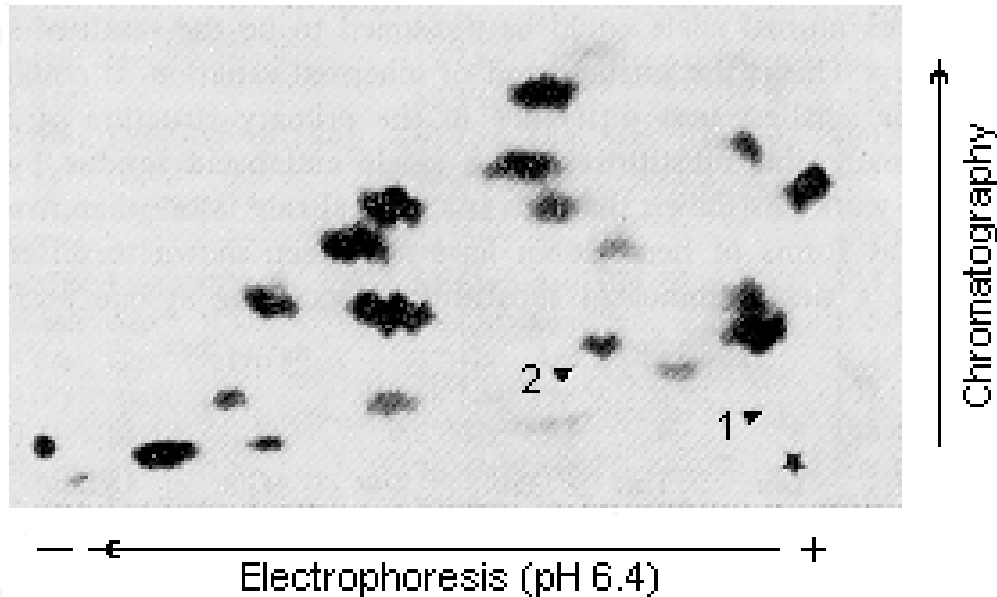
'Fingerprint' of HbS. The peptides obtained by digestion of the hemoglobin have been separated on filter paper first by electrophoresis (horizontal) and then by chromatography (vertical). Peptide spots were then developed with ninhydrin. **1**: position of peptide present in HbA but not in HbS. **2**: position of peptide present in HbS but not in HbA.