DISSOCIATION OF HEMOGLOBIN: MOLECULAR SIEVE CHROMATOGRAPHY OF A RAPIDLY INTERACTING EQUILIBRIUM SYSTEM

BY

NAILA K. ABED

submitted in partial fulfillment for the requirements
of the degree Master of Science
in the Department of Chemistry
American University of Beirut
Beirut, Lebanon
June, 1965

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ABSTRACT

Molecular sieve chromatography has recently become a technique for the determination of molecular weight. In the present work, molecular sieve chromatography has been used in the investigation of the dissociation of human oxyhemoglobin in buffers of high and low ionic strengths. Hemoglobin was found to have a weight average molecular weight of 35,800 at I = 2.2, 42,500 at I = 1.2, 51,100 at I = 0.2, 47,300 at I = 0.02, and 44,200 at I = 0.002 for a hemoglobin concentration of 1.75×10^{-3} moles/liter.

The following conclusions were drawn:

1. Human oxyhemoglobin dissociates to a large extent in media of high as well as low ionic strengths.

2. The dissociation is a single dimerization reaction involving a symmetrical split of the hemoglobin tetramer molecule, represented by

$$\alpha_2\beta_2 \rightleftharpoons 2\,\alpha\beta$$

The dissociation at high ionic strength may be due to the weakening of the electrostatic forces holding the molecule because of the shielding effect of the salts. Dissociation at low ionic strength must be due to another reason, probably due to the small number of foreign ions that hold the molecule together as a result of weak binding forces.

Due to theoretical difficulties inherent in narrow band chromatography, it was not possible to calculate exact dissociation constants. However, it was possible to calculate dissociation constants giving a relative order of magnitude.

It is thus obvious that hemoglobin in solution should be regarded to be in a state of dynamic equilibrium with its subunits under all solvent conditions. This evidence is in favor of the association-dissociation formulations for explaining the classical Bohr effect.

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LIST OF SYMBOLS

BD	:	Blue Dextran 2000
BGG	:	Bovine gamma globulin
HSA	:	Human serum albumin
Hb	:	Hemoglobin
Mb	:	Myoglobin
Cyt. c	:	Cytochrome c
V_e	:	Weight average elution volume
M_w	:	Weight average molecular weight
M_2	:	Molecular weight of hemoglobin dimer ($\alpha\beta$)
M_4	:	Molecular weight of hemoglobin tetramer ($\alpha_2\beta_2$)
C_0	:	Initial total concentration expressed in moles of iron per liter
I	:	Ionic strength
mM	:	Millimolar
K_d	:	Molecular sieve distribution coefficient
K_{diss}	:	Equilibrium constant of the dissociation $\alpha_2\beta_2 \rightleftharpoons 2\alpha\beta$

INTRODUCTION

The structure of hemoglobin has been investigated both by X-ray analysis and by chemical methods. Braunitzer et al.[12] have determined the amino acid sequence of the peptide chains of normal human hemoglobin. Perutz[33], on the other hand gives a detailed description of the X-ray results on horse methemoglobin. There is a large measure of agreement between X-ray and chemical results.

Mammalian hemoglobin contains four polypeptide chains of roughly equal length, which are identical in pairs: the α chains and the β chains. Thus hemoglobin may be represented by the formula $\alpha_2\beta_2$. The surface contours of one pair of chains match those of the other pair so that there is a large area of contact between the individual polypeptide chains. In its overall shape the molecule resembles a spheroid, with dimensions of 64 x 55 x 50 A.[33] The heme groups lie in four separate pockets on the surface of the molecule. Each pocket is formed by the folds in one of the polypeptide chains, which appears to make contact with the heme group at four different points at least. The closest distance between any two iron atoms is 25 A. Thus the distance between one iron atom and another is too great for heme-heme interaction to be possible in a simple direct manner. The two chains have histidine residues in the position which corresponds to the heme-linked histidine[13] in myoglobin found by

X-ray analysis.[33] The residue immediately following histidine in the β Chain of human hemoglobin is cysteine. The side chain of this cysteine residue lies near the surface of the hemoglobin molecule and points away from the heme groups, in a direction opposite to the heme-linked histidine.[33] The sulfhydryl group may interact with a loop of the α chain coming in contact with it. There are of course two such cysteines, one in each of the β chains, forming a symmetrical pair. The blocking of this pair drastically reduces heme-heme interaction which suggests that they play a part in the mechanism.[33]

The oxygen equilibrium reaction of ferrohemoglobin has been studied extensively because of the physiological importance. In principle, the equilibrium between hemoglobin and oxygen should not require any complicated treatment, since it is of the type:

$$pA + nB \rightleftharpoons qAB.$$

But in 1903 Bohr[11] showed that when the fraction of oxygenated hemoglobin was plotted against the partial pressure of oxygen, the curve had a sigmoid shape rather than the hyperbolic form which should result from a reaction such as:

$$Hb + O_2 \rightleftharpoons HbO_2.$$

In order to explain this, other theoretical treatments were developed, the best known of which are those of Adair[3] and of Hill.[25]

Adair[3] assumed that the combination with oxygen takes place in a set of sequential addition reactions:

$$Hb_4 + O_2 \xrightleftharpoons{K_1} Hb_4O_2$$

$$Hb_4O_2 + O_2 \xrightleftharpoons{K_2} Hb_4O_4$$

$$Hb_4O_4 + O_2 \xrightleftharpoons{K_3} Hb_4O_6$$

$$Hb_4O_6 + O_2 \xrightleftharpoons{K_4} Hb_4O_8$$

and by assuming that the values of K_1, K_2, K_3, and K_4 are of increasing magnitude. The resulting equation from this treatment has been fitted to the experimental curve of the Bohr effect. Since these equilibrium constants are unequal, the hypothesis of heme-heme interaction was postulated. This postulate implies that the combination of any one of the iron atoms with oxygen increases the rate of combination with oxygen of at least one of the remaining three.

Hill,[25] on the other hand assumed the following two reactions:

$$nHb \rightleftharpoons Hb_n$$

$$Hb_n + nO_2 \rightleftharpoons Hb_nO_{2n}$$

From which he derived an equation in terms of n, which fits the sigmoid curve. This number n roughly represents the degree of polymerization of hemoglobin. In 1912 Douglas et al.[16] published an analysis of the oxygen equilibrium curve of hemoglobin in terms of aggregation - disaggregation equilibrium. This treatment has been revived recently by Schejter et al.[37] who write the oxygen reaction as:

$$Hb_4O_8 \rightleftharpoons 2Hb_2 + 4O_2$$

which is an expression of an overall thermodynamic cycle including an

equilibrium between the dimer and tetramer forms of hemoglobin in both the oxygenated and deoxygenated states. The authors derived an algebraic expression which fits the experimental data based on this association - dissociation assumption. The authors report some observations in support of their hypothesis. In dilute solutions of hemoglobin the oxygen dissociation curve tends to be hyperbolic rather than sigmoid. This may be expected since hemoglobin tends to dissociate in dilute solutions of hemoglobin. Another observation[37] is that the hemoglobin of lamprey is a single chain molecule with one iron atom per chain. The oxygen dissociation curve is hyperbolic. However, when the concentration of this single chain hemoglobin is increased experimentally to a very high value, the shape of the oxygen dissociation curve changes to sigmoid. Simultaneously, there is an increase in the sedimentation constants which indicates aggregation.

The value of 68,000 for the molecular weight of hemoglobin which was generally accepted since the classic work of Adair[4], who used osmotic pressure measurements, is today refined to 64,458 as a result of the amino acid sequence determination by Braunitzer et al.[12] For a long time it has been known from the work of Steinhardt,[38] Wu and Yang,[43] Gutter et al.,[24] and others that the hemoglobin tetramer may undergo dissociation as a result of the action of denaturing agents such as urea, acetamide, formamide, mercaptoethanol, and furthermore, that the susceptibility of hemoglobin towards dissociation varies considerably with the species. Partial disaggregation of the hemoglobin tetramer in solutions containing high salt was observed by Adair[4], Suedberg and Pedersen[39], Benhamou et al.[10] and Benesch et al.[9]

Added interest has recently been provided by the work of Rossi-Fanelli et al.[34,35,36] who pointed out that the high value of Hill's constant $n \approx 3$ characteristic of the oxygen equilibrium under normal conditions persists unchanged when the molecule dissociates in strong salt solutions. They pointed out that this creates a paradox, for in 2M NaCl solution, the hemoglobin tetramer undergoes dissociation essentially into the dimer, which they measured by light scattering. From equilibrium sedimentation measurements by the Archibold method, Benesch et al.[8] have reported that oxyhemoglobin A dissociates to a slightly but significantly greater extent than deoxyhemoglobin A and that hemoglobin H (β_4) dissociates less than hemoglobin A ($\alpha_2\beta_2$). Kirschner and Tanford[28] have investigated the dissociation of human carbomonoxy hemoglobin and bovine ferrihemoglobin in solutions containing high concentrations of NaCl, $CaCl_2$, $MgCl_2$ and $(NH_4)_2SO_4$ by sedimentation velocity.

During the last few years, molecular sieve chromatography has emerged as a tool for the determination of molecular weight. Before the present work was started there were no reports on the chromatographic behaviour of rapidly interacting equilibrium systems on molecular sieve columns. Since then, Winzor and Scheraga[41,42] have studied the aggregation equilibrium of α-chymotrypsin and bovine thrombin on sephadex G-100. More recently, Ackers and Thompson[2] have elaborated some of the theoretical aspects and have reported equilibrium constants for the dissociation of human CO-hemoglobin in 0.2M phosphate buffer.

The present work is an investigation of the dissociation of normal human oxyhemoglobin in low and high ionic strength buffer of pH 7.0 at room temperature using sephadex G-75.

MOLECULAR SIEVE CHROMATOGRAPHY

Separation on the basis of molecular size differences is important in the study of macromolecules. Dialysis has been used for a long time in separating macromolecules from low molecular weight substances. Craig et al.[14] have determined the 50% escape times of a number of solutes through cellophane membranes. The order of increasing times was roughly in the order of increasing molecular weights. Separation on molecular weight basis was observed in connection with the passage of solutions containing proteins through beds of uncharged granular materials. Zeolites and granulated agar gels are two such examples. Size effects and sorption are also factors in ion-exchange chromatography. The solutes appear in the effluent in the order of decreasing molecular weight. In 1955 Lathe and Ruthven[29] described the graded penetration into starch granules of solutes differing in size. Prior to that time starch had been used as an inert support in partition chromatography and it was assumed that starch grains are uniformly permeable to all solutes. But Lathe and Ruthven demonstrated that solutes vary greatly in their penetration ability and that the extent of penetration is determined primarily by molecular size. The order of elution of the solutes is in the order of decreasing molecular weights. Several cross-linked hydroxylic substances have been synthesized and tested for their molecular sieve properties. Partially hydrolyzed dextran is cross-

linked with epichlorohydrin and is commercially synthesized by "AB Pharmacia" (Uppsala, Sweden) under the trade name "Sephadex".

Properties of Sephadex Gels

Sephadex, in the dry form, is a hard and tough powder with a density of 1.64 - 1.65. When wetted with solvent the particles swell and form a gel. In the early production of the substance, the dextran and epichlorohydrin reaction mixture were allowed to polymerize and to form a solid block which upon disintegration yielded particles of irregular shape. This irregularity would result in more elasticity in the column thus decreasing the flow rate and eventually clogging the column. Later, this was avoided by dispersing the original reaction mixture into drops in an inert dispersion medium and thus forming uniform spherical gel particles. The spherical particles or beads offer considerably lower resistance to the flow of eluant in a packed column and hence little or no deformity due to increased pressure is obtained. When the original non-beaded form was used it had to be backwashed thoroughly to remove all the fine particles and to make the particle size more uniform.

The degree of cross-linking determines the pore size of the gel. The higher the cross-linking the smaller the pore size which in turn governs the excluded molecular size. The degree of cross-linking depends on the concentration of epichlorohydrin, the cross-linking agent. Types of sephadex with different pore sizes have been manufactured and graded according to an estimated average exclusion limit. The exclusion limit means the maximum molecular weight to pass through the pores. These limits are not clear cut, but they help in deciding which grade to use

for what molecular weight range. For example, in the present work, G-75 which has an exclusion limit of 50,000 was used to separate molecular weights ranging from 13,000 for cytochrome c to 64,500 for human serum albumin. The following exclusion limits are given by the manufactures for the various grades of sephadex:

G-10	2,500
G-25	5,000
G-50	10,000
G-75	50,000
G-100	100,000
G-150	150,000
G-200	200,000

The swelling of sephadex gels in contact with solvents depends both upon the degree of cross-linking and on the solvent-gel interaction. The gels are polar and hence swell only in polar solvents. On the other hand less polar water-miscible solvents are used to dehydrate the swollen gels. A measure of the swelling is obtained by suspending a weighed amount of dry sephadex in a graduated cylinder and reading the volume of the swollen sediment. This is useful in determining the weight of dry sephadex needed to pack a column of certain dimensions.

Theoretical Aspects of Sephadex Chromatography

Flodin[18] has defined for gel filtration a parameter, K_d, the distribution coefficient which is the ratio between the concentrations of a solute in the mobile and stationary phases. The stationary phase is the water imbibed in the gel particles and the mobile phase is the water moving in the void space between the particles. The molecular-

sieve theory states that each molecular species can only diffuse in a sephadex column up to a forbidden region surrounding each cross-linking site. The magnitude of this forbidden region and hence minimum distance of approach depends on the size and steric properties of the solutes. In terms of this picture the distribution coefficient is the volume of the imbibed water which is available to the solute.

In terms of the distribution coefficient the elution volume of a solute is:

$$V_e = V_0 + K_d \cdot V_i$$

where V_0 is the void volume

V_i is the volume of water imbibed by the gel particles, and

V_e is the elution volume of the solute.

Thus $K_d = \frac{V_e - V_0}{V_i}$

which can be obtained from experimental data by assuming that a very small molecule is totally accessible and assigned a K_d of 1, while a big molecule much bigger than the exclusion limit is totally excluded from the gel particles and hence has a K_d of 0.

It follows that:

$$1 = \frac{V_s - V_0}{V_i}$$

$$V_s = V_i + V_0$$

where V_s is the elution volume of the very small particle.

on the other hand:

$$0 = \frac{V_b - V_0}{V_i}$$

Therefore, $V_b = V_o$

where V_b is the elution volume of the large molecule.

In a separation governed entirely by a molecular sieve mechanism, the K_d value cannot exceed unity. When greater K_d values are found sorptive forces are operative as has been found for aromatic compounds.

Laurent and Killander[31] define a molecular sieve coefficient K_{av} given by:

$$K_{av} = \frac{V_e - V_o}{V_x}$$

where V_x is the total volume of column minus the void volume. V_x differs from V_i of Flodin by the material volume of the fibers in the hydrated gel particles. Laurent and Laurent[32] have used an analog computer to investigate the behaviour of molecular sieve columns.

In practice ideal conditions for column operations are never attained and there are always some distortions of the ideal profiles. Three factors contribute: (1) local non-equilibrium, (2) longitudinal diffusion, and (3) perturbations due to imperfect packing. The first factor arises because the particle is diffusing in and out and hence there is no instantaneous equilibration. For such cases the flow rates have to be small enough to allow for instantaneous equilibration. Longitudinal diffusion is of little importance in the time required for a gel filtration experiment. Imperfect packing leading to irregular flow is practically the only important cause of deviation from ideal behaviour. Glueckauf[21] has derived an expression for the column behaviour of solutes in ion-exchange resins. This should be valid also for gel filtration since only the distribution coefficients, diffusion

coefficients, the flow rate and the particle size are represented in the equations. It was shown that the EHTP (equivalent height of a theoretical plate) depends on three terms, one representing imperfections in packing and longitudinal diffusion and the other two, to film diffusion and gel diffusion, i.e., to local non-equilibrium. According to Glueckauf the number of theoretical plates N is given by

$$N = 8\left(\frac{V_e}{\beta}\right)^2$$

where β is the width of the elution curve at the height $C_{max}/2.72$ over the baseline. The EHTP is obtained by dividing the column length (l) by the number of theoretical plates obtained

$$EHTP = \frac{l}{N}$$

EHTP values on sephadex columns have been determined in relation to variations in elution rate and particle size by Flodin.[18] The most drastic effect on EHTP is the variation of the particle size. Experiments should be performed with as small particles as possible.

In the present work, Flodin's K_d values were found to be constant for a certain substance if the packing, the column dimensions and the flow rate were reproducible. Thus K_d values cannot be used for general identification of substances on sephadex columns of a certain grade.

G. Achers[1] accepts Flodin's mechanism of gel filtration which presumes the existence of microregions of excluded volume which are greater for the larger molecules. By this mechanism equilibrium partitioning occurs between mobile exterior liquid and the fractional regions of non-excluded volume within the bed.

For this model $K_d = \frac{V_p}{V_i}$

where V_p is the nonexcluded volume, and

V_i is the volume of unbound solvent internal to the gel phase. From measurements of equilibrium solute partitioning, it is possible to calculate K_d values which should be identical with Flodin's K_d values obtained under non-equilibrium conditions. Thus, for a system of total volume V_t at equilibrium, consisting of a gel phase and an external liquid phase of volume V_l, the penetrable volume is given by:

$$V_p = \frac{Q_p}{C_p} = \frac{Q_t - Q_l}{C_p} = \frac{Q_t - C_o V_l}{C_p}$$

where Q_p is the amount of solute partitioned into the gel phase,

Q_t is the total amount of solute in the system,

Q_l is the amount of solute in the liquid phase external to the gel,

C_p is the concentration of solute within the pores of the gel, and

C_o is the concentration of solute in the external liquid. Hence:

$$K_d = \frac{Q_t - C_o V_l}{C_p V_i}$$

In order to relate this expression to experimentally measurable quantities, it is assumed that $C_p = C_o$, then

$$K_d = \frac{Q_t/C_o - V_l}{V_i}$$

Ackers found that his and Flodin's K_d values compare for columns of G-75 and G-100 while they do not agree for columns of G-200. It was therefore, concluded that whereas G-75 and G-100 obey the molecular exclusion mechanism, G-200 does not and hence has another mechanism of

operation.

Since the mechanism of action of gel filtration is in direct correlation with molecular size, it was thought that properties on sephadex columns may lead to a knowledge of molecular weight and size. Andrews[5] found a linear relationship over a certain molecular weight range between the elution volume on a certain column with the logarithmic scale of the molecular weight. For G-75 the minimum and maximum molecular weights at which the V_e - log M_w curve has any slope are 2,000 and 110,000 respectively and any values below or above the limiting range lie outside the linear relationship. He also calculated the molecular weight of certain proteins and his values were found to check with other values found in the literature. Some consideration and evaluation of possible errors in molecular weight estimated by gel filtration is given. The acidic groups in sephadex gels cause retention of strongly basic proteins on the gels and exclusion of strongly acidic macromolecules from the inner cavities. These effects may be overcome by certain conditions of ionic strength and pH. Such effects may lead to an increase in V_e and hence an increase in the apparent molecular weight. A more likely error in molecular weight estimation is the inaccurate estimation of elution volumes, differences in shape between one protein and another and differences in density between solvated protein molecules, resulting in different ratios between size and molecular weight. The greatest deviation in molecular weight occurred with Pseudomones cytochrome c-551 for which the deviation was 10%.

Whitaker[40] found a linear correlation between the logarithm of the molecular weight of a protein and the ratio of its elution volume

V_e to the void volume of the column. He found that no advantage is gained when K_d values were used instead of this simple ratio. These ratios were rather constant for each protein regardless of column size. His plots and molecular weight determinations agree very well for a number of proteins on both sephadex G-75 and G-100.

Systems in Rapid Dissociation Equilibrium

The problem of the sedimentation of systems in rapid association - dissociation equilibrium has been solved by Gilbert.[19,20] By analogy with chromatography Gilbert used arguments to describe the behaviour of solutes in sedimentation, similar to those which De Vault[15] used for idealized chromatography. He derived an equation for the schlieren patterns of associating molecules. The Gilbert theory predicts that for dimerization reactions, the schlieren pattern would have one peak, but the boundary would be diffuse. In the case of more complicated equilibria, multiple boundaries may appear.

Winzor and Scheraga[41,42] have demonstrated that molecular sieve chromatography possesses all of the qualitative features predicted by Gilbert. The authors studied the polymerization of α-chymotrypsin and bovine thrombin by passing these proteins through columns packed with sephadex G-100. They used the continuous feed technique* and observed

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* Continuous feed may be defined as the case when the sample volume is large as compared with the width of the elution band. In such a situation the elution profile will exhibit a plateau region between the leading and the trailing edges.

that the trailing edge is more diffuse than the advancing edge. Analogies to Gilbert's schlieren patters[5] were obtained from the elution profiles by plotting the first derivative of the trailing edge against the volume.

Recently, Ackers and Thompson[2] have developed a quantitative theory for molecular sieve columns applicable to conditions of continous feed. The derivation of Ackers and Thompson is related to the theory of Gilbert for the sedimentation velocity of a rapidly equilibrating, monomer-n-mer system. The parameter of molecular sieve chromatography is the molecular sieve coefficient, σ, defined by

$$\sigma = K_d = \frac{Q}{bC}$$

where Q is the amount of solute within the gel per unit column length,

C is the solute concentration within the void volume, and

b is the internal gel volume per unit column length.

The discussion of De Vault shows that the conservation of mass requires the following continuity equation*

$$\left(\frac{\partial C}{\partial x}\right)_V - \left[a + b\sigma\right]\left(\frac{\partial C}{\partial V}\right)_x = 0 \qquad (1)$$

where V is the volume of liquid that has passed into the column,

---

* The equation is written by Ackers and Thompson with a positive sign, namely

$$\left(\frac{\partial C}{\partial x}\right)_V + \left[a + b\sigma\right]\left(\frac{\partial C}{\partial V}\right)_x = 0 \qquad (1a)$$

On a purely formal basis equation (1a) does not lead to equation (3), whereas equation (1) does. There may be an implicit sign convention in equation (1a) which is not clearly apparent, in which case equation (1) and equation (1a) would be identical.

x is the distance from the top of the column, and

a is the void volume per unit column length.

For the instantaneous equilibrium

$$M_n \Longleftrightarrow nM$$

The equilibrium constant K is given by

$$K = \frac{C_m^n}{C_p} \tag{2}$$

where C_m and C_p are the concentrations of monomeric and polymeric species.

Straightforward integration of the partial differential equation (1) with proper insertion of the concentration relation given by equation (2) leads to

$$C_T = \left[1 + \frac{1}{n} \cdot \frac{\phi}{1-\phi}\right] \left[\frac{K}{n} \cdot \frac{\phi}{1-\phi}\right]^{1/(n-1)} \tag{3}$$

where C_T is the total concentration $(C_m + C_p)$, and

ϕ is a function of σ and V.

Equation (3) is formally identical with Gilbert's equation for sedimentation. The analogy to sedimentation holds for the trailing edge only. Differentiation of equation (3) w.r.t. V gives the schlieren pattern analogues. Equation (3) can be used to find the value of n.

The leading boundary can give information concerning the degree of dissociation, α, by means of the relation

$$\alpha = \frac{\bar{v} - \sigma_p}{\sigma_m - \sigma_p} \tag{4}$$

where $\bar{v}$ is the centroid position of the leading edge, and

σ_m and σ_p are the molecular sieve coefficients for the monomer and

the polymer.

Once n is known, the equilibrium constant can be calculated from the leading edge using equation (4) to obtain α and the relation

$$K = \frac{\alpha^{n} C_{o}^{n-1}}{1 - \alpha} \qquad (5)$$

where C_o is the total solute concentration in the plateau region.

The equations of Ackers and Thompson are not strictly valid for analysis of narrow band elution profiles, but may still be applied in a meaningful approximate manner.

EXPERIMENTAL

A. Materials

Sephadex G-75. Sephadex G-75 bead polymerized fine grain (Pharmacia, Uppsala, Sweden) was used without further treatment. As quoted by the manufacturers, the water regain is 7.5 ± 0.5 grams of water imbibed per gram dry gel, the particle size is 40 - 120 u, and the bed volume per gram dry gel is 12 - 15 ml.

For each chromatographic run 20 g of dry sephadex were added to 500 ml of the desired buffer. The gel was allowed to swell for at least 24 hours before packing.

Blue Dextran 2000. The spectrum of a 0.12% solution of Blue Dextran 2000 (Pharmacia, Uppsala, Sweden) was found to have an absorption maximum at 262 mu with an extinction coefficient of 6.5×10^6 (Figure 1).

Solutions of 0.25% BD in the desired buffer were used as samples in the chromatographic runs.

Human Serum Albumin. Human serum albumin (Biochemical Corporation, California) was found to contain both the dimer and monomer as evidenced by chromatography on sephadex G-75 (Figure 2). The material was first passed through a sephadex G-75 column equilibrated with 0.1M phosphate buffer pH 7.00. Rechromatography of the monomer fraction showed no re-equilibration with the dimer. For this purpose a solution containing 50 mg per ml was chromatographed and the monomer fractions were collected and stored at $4^{\circ}C$.

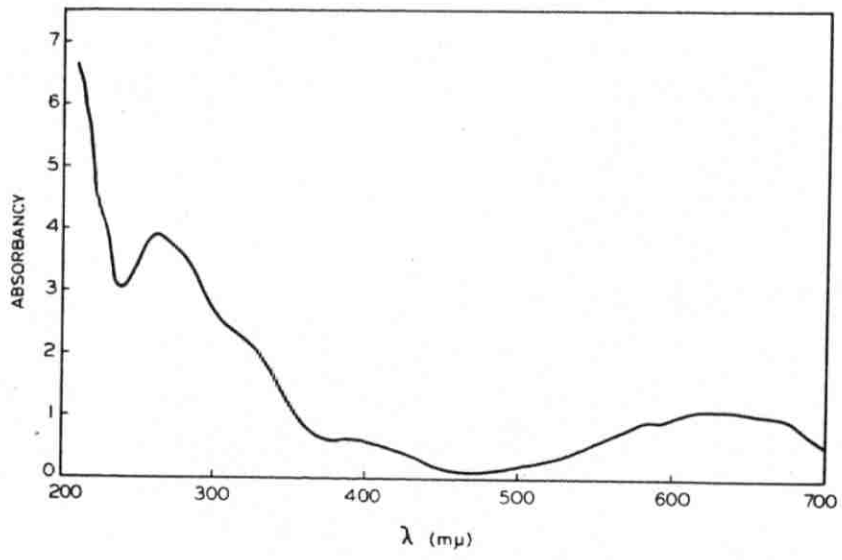

Figure 1 - Spectrum of Blue Dextran 2000

0.12% BD in 0.1M phosphate buffer pH 7.0

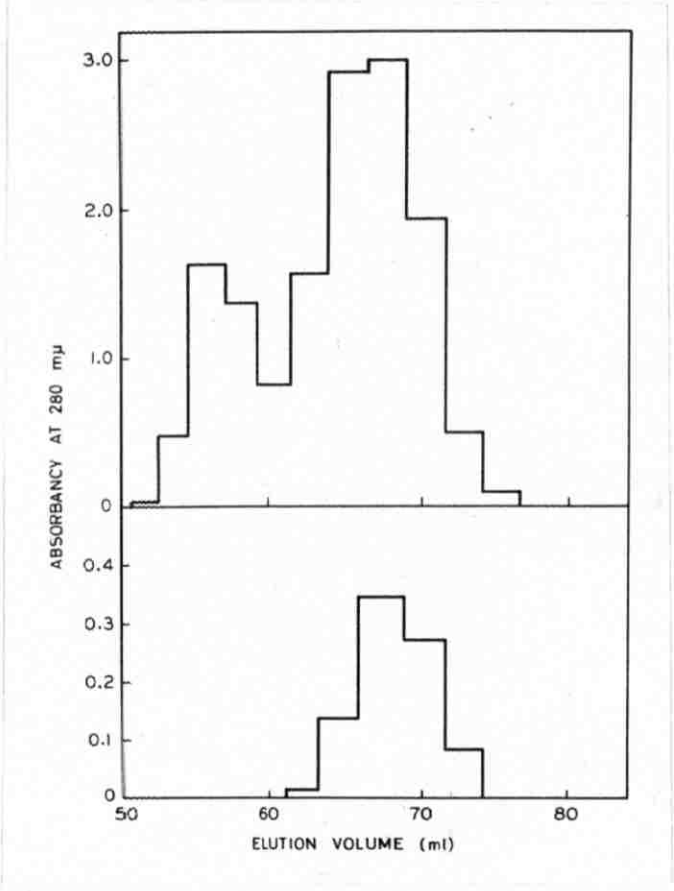

Figure 2 - Elution Profile of Human Serum Albumin on Sephadex G-75
Column dimensions 41.5 x 2.5 cm
In 0.1M phosphate buffer pH 7.0
Flow rate = 5.0 ml/hr.

(a) Elution profile of the monomer-dimer mixture

(b) Rechromatography of the monomer fraction

Myoglobin. Sperm whale skeletal muscle ferrimyoglobin, salt-free, lyophilized, Batch II, purchased from Seravac Laboratory (Maidenhead, Berks, England) was used without furtherpurification. Work in this laboratory showed that the sample contained 5% hemoglobin, the iron content was 0.307% on dry weight basis, and the absorbancy ratio $A_{410\ m\mu}$ / $A_{280\ m\mu}$ was 5.18.

Myoglobin stock solution approximately 1.5×10^{-3}M was prepared immediately before use.

Cytochrome c. Lyophilized, equine cytochrome c (Nutritional Biochemical Corporation, Cleveland, Ohio) was found to contain a number of polymeric forms in addition to the monomer (Figure 3). The monomer was isolated by chromatographing 50 mg of Cytochrome c dissolved in 1 ml of 0.1M phosphate buffer pH 7.00 on sephadex G-75. Rechromatography of the monomer fraction showed no re-equilibration with the polymers. Fractions containing the monomer were stored at 4^{0}C.

Hemoglobin. Human oxyhemoglobin stock solution was prepared from citrated normal adult blood by the method of Drabkin[17] as modified by Huisman and Prins.[26] 10 ml of human blood were centrifuged at 3,000 rpm at 4^{0} C for 10 minutes. The supernatant was sucked off and the blood cells were washed 4 times with 0.9% NaCl (physiological saline). The volume was brought to 10 ml with 0.9% NaCl. The blood cells were then hemolyzed by adding 10 ml of water and 4 ml of toluene. The mixture was shaken well and left for 8 hours at 4^{0}C. A small amount of NaCl was added and the mixture was centrifuged at 4,000 rpm. The upper layer containing toluene and some stroma was sucked off. The hemoglobin solution was filtered and dialyzed twice versus 0.9% NaCl. The

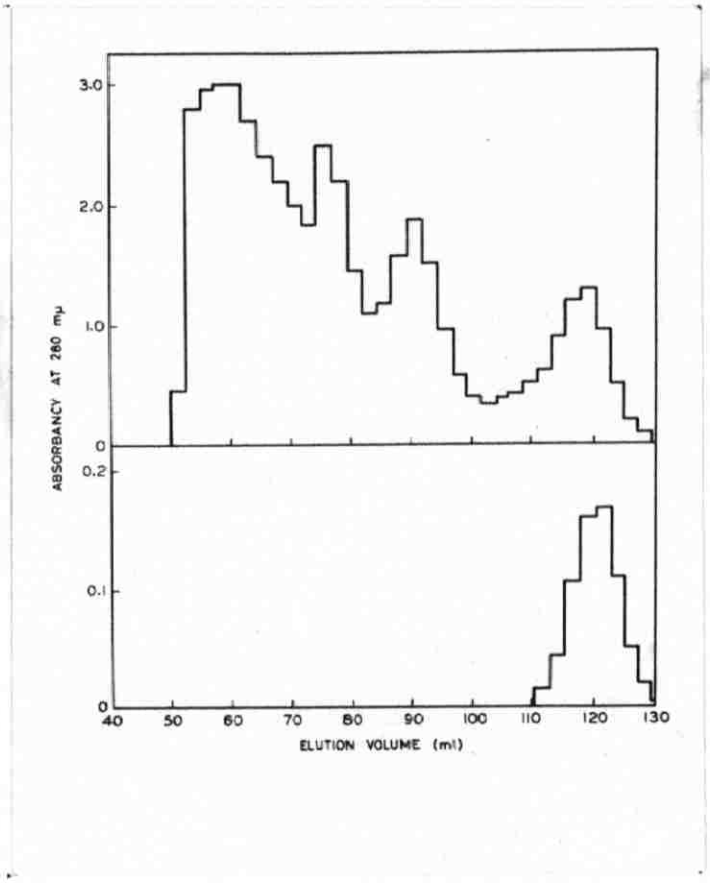

Figure 3 - Elution Profile of Equine Cytochrome c on Sephadex G-75
Column dimensions 41.5 x 2.5 cm
0.1M phosphate buffer pH 7.0
Flow rate = 5.0 ml/hr.
(a) Elution Profile of Polymer-monomer Mixture
(b) Rechromatography of Monomer Fraction

dialyzed hemoglobin was filtered and stored in the frozen state at $-20^{0}C$. The concentration of this stock solution was determined by its optical density at 275 mu and 414 mu using extinction coefficient of 2.95×10^{4} and 12.50×10^{4} respectively. The concentration was found to be 3.50×10^{-3}M.

Potassium Chromate. Analytical reagent potassium chromate (Mallinckrodt, New York) was used without further treatment. The spectrum showed no maximum in the region 300 - 600 mu (Figure 4). The wavelength 460 mu was chosen for measuring concentrations. Beer-Lambert's law was found to hold.

Solutions of 0.4M chromate in the desired buffer were applied to the column.

Buffer Solutions. Phosphate buffers with a total phosphate concentration of 0.1M were prepared. Buffers of higher ionic strength were prepared by adding solid NaCl. The pH was checked on a previously standardized pH meter (Radiometer, pH-meter-4, type RH-M4C) and the pH was adjusted with saturated sodium hydroxide solution to 7.00. In the cases when the buffer concentration required was less than 0.1M, the pH of the stock 0.1M phosphate buffer was first adjusted to 7.00 and then the desired dilution was made and the pH was measured. Ionic strengths were calculated assuming pK 7.0 for the acid dissociation of dihydrogen phosphate. The calculated ionic strength and the measured pH of the buffer solutions are given in Table I.

All other chemicals were of reagent grade and were used without further treatment.

All solutions were made using distilled deionized water whose

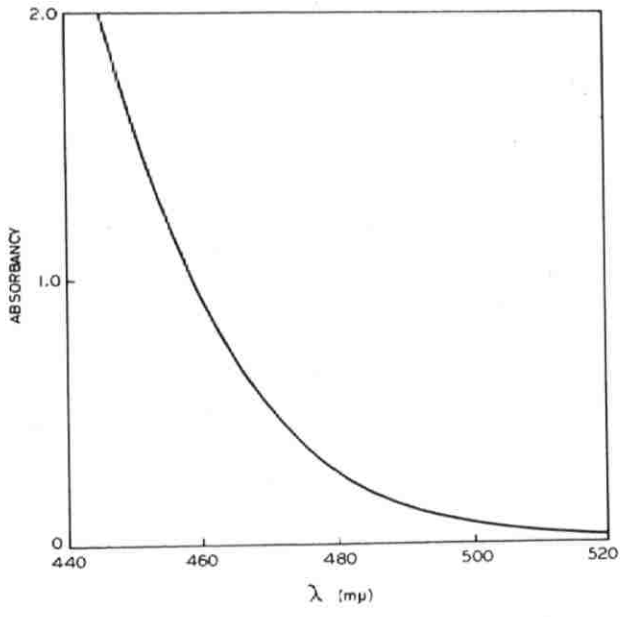

Figure 4 - Spectrum of Potassium Chromate

0.001M Potassium Chromate in 0.1M Phosphate buffer pH 7.0

specific conductance was better than 10^{-6} ohm^{-1} cm^{-1}.

Table I

Ionic Strength and pH of Buffers Used

Concentration	I	pH
2M NaCl, 0.1M PO_4	2.2	7.00
1M NaCl, 0.1M PO_4	1.2	7.00
0.1M PO_4	0.2	7.00
0.01M PO_4	0.02	7.02
0.001M PO_4	0.002	7.03

B. Methods

Packing the Column. A glass chromatographic column 46 cm long and 2.54 cm inner diameter (purchased from Pharmacia, Uppsala, Sweden) was used all through. The column was carefully mounted vertically and the outlet closed. It was filled to one third with buffer and the already swollen gel suspension was then added. When a layer of about 5 cm had formed, the outlet was opened and a slow stream of buffer allowed to flow out. A horizontal zone of packed material was then visible which allowed visual control of the process. If the zone became skew the packing was interrupted. After some gel had packed, the supernatant was removed and more sephadex suspension was added. This was repeated until the height of the gel column was about 40 cm. New suspension was always added before all gel previously present had packed.

After packing, the column was connected to a constant volume peristaltic pump (Harvard Apparatus Co., Dover, Mass.) set to deliver

at a flow rate of 5.0 ml/hr. The column was left overnight to equilibrate under constant flow of buffer. Before addition of the sample the bed surface was stabilized mechanically by using an applicator (a 4.5 cm long plastic cylinder with a fine-mesh nylon net) that fits snugly in the column above the packed gel. The outlet of the column was closed and the applicator was carefully fitted so that it protruded about 0.5 cm above the top of the open column. The applicator was then pressed down on the gel and the column was closed by screwing on the cap tightly. The column was left to equilibrate further for at least six hours before applying any samples. The settled column height was 41.5 cm.

Loading the Sample. The sample to be added was prepared in the buffer which was used as the eluant. The density of the solution was increased by adding enough solid sucrose to make the final solution 15% in sucrose. All solutions were filtered before being applied to the column. A thin plastic tube connected to an Agla micrometer syringe (Burroughs Wellcome and Co., London, England) was positioned in the supernatant buffer in the column a few millimeters above the nylon net of the applicator. The outlet of the column was closed and 0.50 ml of sample was delivered from the Agla syringe in about 30 seconds to form a narrow zone between the bed and the buffer above. The plastic tube was removed and the inlet in the cap of the column was tightly closed. The elution was started by opening the column outlet and turning on the pump.

Elution and Fraction Cutting. A time operated fraction collector (Gilson Medical Electronics, Middleton, Wisconsin) was used to cut the

column effluent into 0.88 ml fractions. The volume of the fractions was measured by weight difference taking into account the density of the buffer. The samples were applied consecutively at intervals of at least 5 hours, the highest molecular weight sample being applied first.

Analysis of Fractions. All samples, with the exception of chloride and glycine, were analyzed by reading absorbancy at the appropriate wavelength on a Zeiss PMQII spectrophotometer. Table II shows the wavelength and the corresponding molar extinction coefficients.

Table II

Molar Absorbancies

Sample	Mol. Weight	(mμ)	ϵ
BD	2×10^6	262	6.6×10^6
HSA	64,500	280	-
Cyt. c	13,000	414	-
Mb	18,000	280	3.96×10^4
		410	16.6×10^4
Hb	64,500	275	2.95×10^4 *
		414	12.5×10^4 *
K_2CrO_4	194	460	9.25×10^2

* per mole Fe

Chloride was analyzed by titrating the contents of each test tube with standard 0.1N $AgNO_3$ using two drops of 5% potassium chromate as an indicator.

Glycine was determined with ninhydrin by using the analytical section of a Technicon amino acid analyzer (Technicon International Ltd., Chauncey, New York).

All chromatographic runs were performed at room temperature (about 20°C.).

RESULTS

Dependence of Elution Volume on Molecular Weight: Column Calibration

Elution volumes of BD, the monomer fraction of HSA, Mb, the monomer fraction of cytochrom c, potassium chromate, sodium chloride and glycine were calculated from the elution profiles by taking the weight average elution volume which is given by:

$$V_e = \sum h_i v_i / \sum h_i$$

where h_i is the ordinate of fraction i on the elution profile, and

v_i is the total volume of fraction i.

The results are given in Tables III and V.

The V_e values obtained were plotted versus the logarithm of the molecular weights for each of these substances as shown in Figure 5. As seen from the plots, the elution volume was found to be linearly dependent on log M_w in the range of molecular weight of 10,000 - 70,000, in agreement with the work of Andrews[5] and of Whitaker.[40]

Apparent Elution Volumes of Hemoglobin Solutions: Determination of M_w

The V_e values of Hb solutions were obtained for the following ranges of initial concentration C_0, 3.5 - 0.35 mM and 1.75 - 0.35mM in phosphate buffers pH 7.0 and ionic strengths 0.002, 0.02, 0.2, 1.2 and 2.2. The V_e values are also given in Tables III and V. Number average molecular weights were calculated from the linear plots of

Figure 5, assuming the linearity to hold. The horizontal lines in the plots of Figure 5 represent the values of the hemoglobin solutions at the five ionic strengths mentioned above.

The ~~weight~~ number average molecular weight, M_w ~~was calculated from~~ is given by the equation:

$$M_w = \sum n_i M_i \; / \sum n_i \qquad (6)$$

where n_i is the number of molecules of species i, and

M_i is the molecular weight of species i.

Since we have an equilibrium involving the two species $\alpha_2\beta_2$ and given by: $\alpha_2\beta_2 \rightleftharpoons 2\alpha\beta$

Equation 6 reduces to:

$$M_w = (n_2M_2 + n_4M_4) \; / \; (n_2 + n_4)$$

$$= M_2(n_2 + 2n_4) \; / \; (n_2 + n_4) \qquad (7)$$

where n_2 and n_4 are number of molecules of species $\alpha\beta$ and $\alpha_2\beta_2$ respectively, and M_2 and M_4 are the molecular weights of species $\alpha\beta$ and $\alpha_2\beta_2$.

Variation of M_w with the Ionic Strength of the Buffer Medium

The M_w values of hemoglobin solutions at the five ionic strengths obtained above were plotted against the square root of the ionic strengths, as shown in Figure 6. The two dashed lines, labelled M_4 and M_2, correspond to the molecular weights of $\alpha_2\beta_2$ and $\alpha\beta$, thus giving a visual estimate of the degree of dissociation. The three symbols used for the points on the plot correspond to the three initial concentrations of hemoglobin: 1.75, 0.875, and 0.350 mM. As seen from the figure, M_w

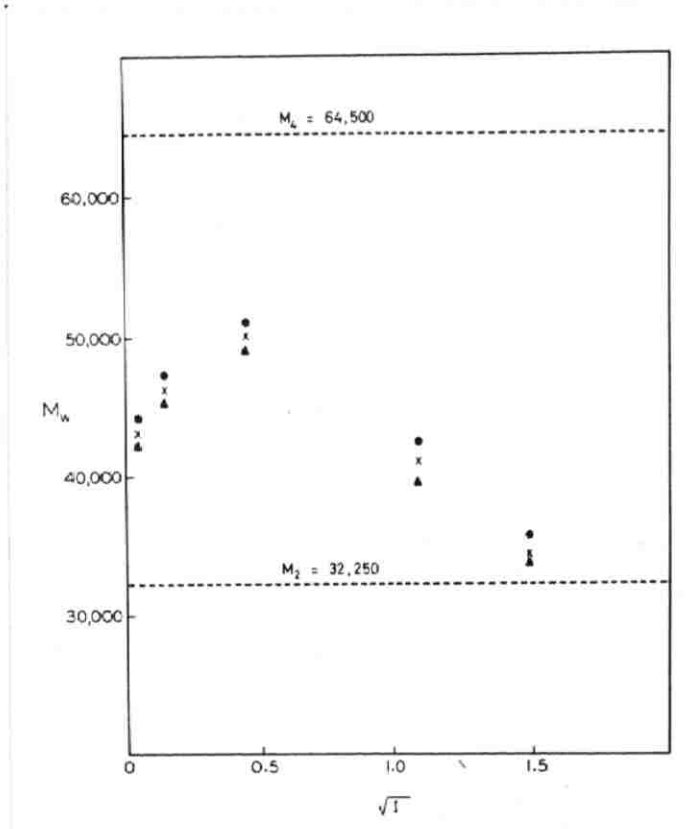

Figure 6 - Variation of M_w with $\sqrt{I}$

▲ - C_0 = 0.350×10^{-3} M

× - C_0 = 0.875×10^{-3} M

● - C_0 = 1.75×10^{-3} M

(C_0 = initial concentration of Hb sample)

is smaller at high and very low ionic strengths. Even at moderate ionic strength, ionic strength 0.2, the M_w obtained is less than M_4 implying dissociation of the hemoglobin at this ionic strength.

Variation of M_w with Initial Hemoglobin Concentration

The M_w values obtained above were plotted versus the logarithm of C_0, where C_0 is the initial concentration of the Hb solutions applied to the column, expressed in moles of iron per liter solution. The plots are shown in Figure 7. The five plots correspond to the five ionic strengths used. The five lines are seen to be parallel. This suggests that the effect of the column on the observed value of M_w in all five cases is of the same kind. It is also seen that M_w decreases, hence dissociation increases, with decreasing initial Hb concentration.

Calculation of Apparent Dissociation Constants, K_{diss}.

For the equilibrium

$$\alpha_2 \beta_2 \rightleftharpoons 2\,\alpha\beta$$

the dissociation constant is given by

$$K_{diss} = \left[\alpha\beta\right]^2 / \left[\alpha_2 \beta_2\right] \qquad (8)$$

The initial concentration C_0 expressed in terms of iron concentration is

$$C_0 = 4\left[\alpha_2 \beta_2\right] + 2\left[\alpha\beta\right].$$

Dividing by $\alpha_2 \beta_2$ we obtain:

$$C_0/\left[\alpha_2\beta_2\right] = 4 + 2\left[\alpha\beta\right] / \left[\alpha_2 \beta_2\right] \qquad (9)$$

If we define $r = \left[\alpha\beta\right] / \left[\alpha_2 \beta_2\right]$ then equation (9) becomes:

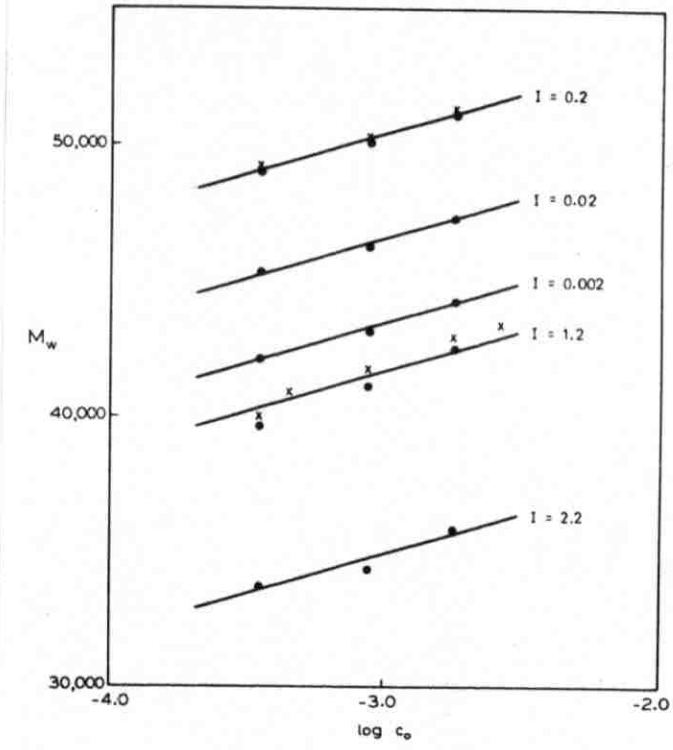

Figure 7 - Variation of M_w with log C_o at Different Ionic Strengths

- • - values derived from Table III
- x - values derived from Table V

$$C_0 / \left[\alpha_2\beta_2\right] = 4 + 2r$$

and $$\left[\alpha_2\beta_2\right] = C_0 / (4 + 2r).$$

Hence $$\left[\alpha\beta\right] = r.C_0 / (4 + 2r).$$

Substituting these values in the equilibrium expression of equation (8) we obtain:

$$K_{diss} = r^2 . C_0 / (4 + 2r). \qquad (10)$$

The ratio r can be obtained from experimental data through the following derivation:

Rearrangement of equation (7) gives

$$M_w = (n_2/n_4 + 2) . M_2 / (n_2/n_4 + 1)$$

But $n_2/n_4 = \left[\alpha\beta\right] / \left[\alpha_2\beta_2\right] = r$.

So $M_w = (r + 2) . M_2 / (r + 1)$.

Rearranging this equation and noting that $M_4 = 2M_2$, we obtain

$$r = (M_4 - M_w) / (M_w - M_2)$$

Tables IV and VI give the calculated K_{diss} values with the corresponding values of r and R, where $R = r^2 / (2r + 4)$ (see equation 10).

Variation of K_{diss} with Initial Concentration of Hemoglobin

The logarithm of K_{diss} obtained above was plotted against the initial hemoglobin concentration, C_0, as shown in Figure 8. The five plots in the figure correspond to the five ionic strengths 0.002, 0.02, 0.2, 1.2 and 2.2. As seen from the plots, K_{diss} increases with increasing initial concentration. It is also apparent that although dissociation occurs at both high and low ionic strengths, yet the change at high ionic strengths

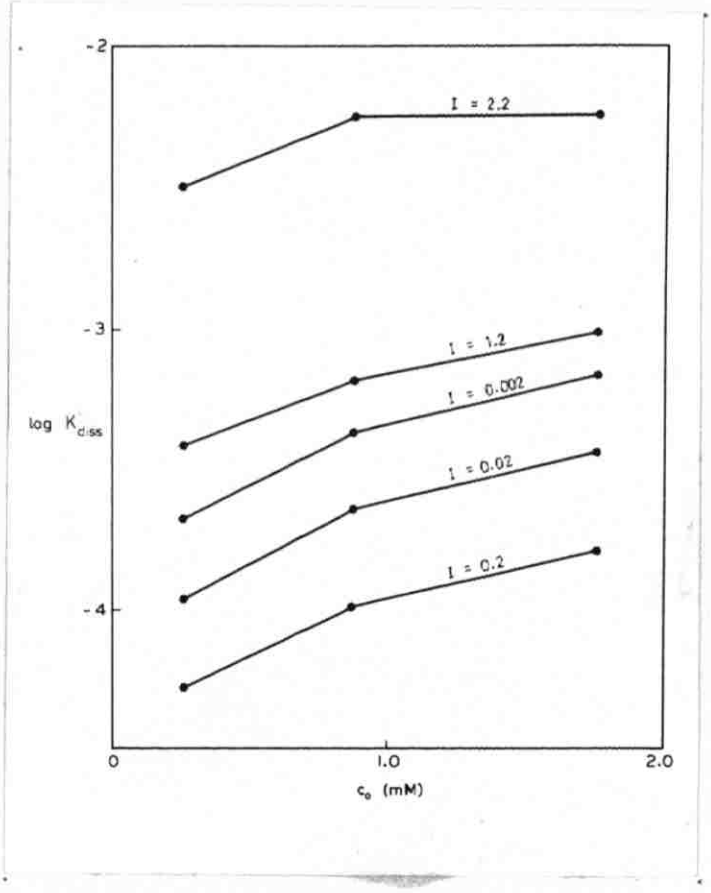

Figure 8 - Variation of log K_{diss} with C_o at Different Ionic Strengths

Table III

Chromatographic Characteristics on Sephadex G-75 (early data)

a. Column dimensions 41.5 x 2.5 cm.

0.1M phosphate buffer, pH 7.00

Flow rate = 5.0 ml/hr.

Sample	V_e (ml)	K_d	log M_w	M_w
BD	52.5	0.000	6.301	2×10^6
HSA	64.0	0.090	4.810	64,500
Mb	107.0	0.426	4.255	18,000
Cyt. c	116.0	0.504	4.114	13,000
NaCl	180.5	1.000	1.766	58
Hb 1.75 mM	70.0	0.137	4.710	51,300
0.875 mM	71.0	0.145	4.701	50,200
0.350 mM	71.5	0.148	4.692	49,200

b. Column dimensions 41.5 x 2.5 cm.

0.1M phosphate buffer and 1M NaCl, pH 7.00

Flow rate = 5.0 ml/hr.

BD	55.5	0.000	6.301	2×10^6
HSA	68.0	0.092	4.810	64,500
Mb	108.0	0.385	4.255	18,000
Cyt. c	117.0	0.451	4.114	13,000
Glycine	192.0	1.000	1.892	78
Hb	79.5	0.176	4.634	43,100

Table III cont'd.

c. Column dimensions 37.5 x 2.5 cm.
O.1M phosphate buffer + 2M NaCl, pH 7.00
Flow rate = 17 ml/hr.

Sample	V_e (ml)	K_d	log M_w	M_w
BGG	50.2	0.000	5.193	156,000
HSA	60.5	0.105	4.810	64,500
Mb	102.0	0.530	4.255	18,000
Cyt. c	113.0	0.622	4.114	13,000
Glycine	158.0	1.000	1.875	75
Hb	80.0	0.305	4.540	34,700

d. Column dimensions 40.5 x 2.5 cm.
O.1M phosphate buffer + 1M NaCl, pH 7.00
Flow rate = 11.5

Sample	V_e (ml)	log M_w	M_w
HSA	68.0	4.810	64,500
Mb	116.0	4.255	18,000
Cyt. c	127.5	4.114	13,000
Hb 2.63 mM	83.0	4.638	43,400
1.75 mM	83.5	4.633	42,900
0.875 mM	84.5	4.621	41,800
0.438 mM	85.0	4.613	41,000
0.350 mM	86.0	4.602	40,000

Table IV

M_w Values and Calculated K_{diss} Values (early data)

M_w data of Table III b.

Ionic strength of buffer = 1.2, pH 7.00

M_w	C_0 (mM)	r	R	$K_{diss} \times 10^4$ (m/l)
43,400	2.63	1.89	0.459	12.0
42,900	1.75	2.03	0.511	8.94
41,800	0.875	2.38	0.646	5.65
41,000	0.438	2.69	0.776	3.40
40,000	0.350	3.16	0.968	3.38

Table V

Chromatographic Characteristics on Sephadex G-75

Column dimensions: 41.5 x 2.5 cm.

Flow rates = 5.0 ml/hr.

a. Ionic strength of buffer = 2.2

Sample	V_e (ml)	K_d	log M_w	M_w
BD	56.5	0.000	6.301	2×10^6
HSA	68.1	0.078	4.810	64,500
Mb	118.4	0.418	4.255	18,000
Cyt. c	131.3	0.505	4.114	13,000
K_2CrO_4	204.7	1.000	2.288	194
Hb 1.75 mM	91.4	0.235	4.554	35,800
0.875 mM	93.0	0.246	4.535	34,300
0.350 mM	93.5	0.250	4.527	33,700

b. Ionic strength of buffer = 1.2

BD	61.4	0.000	6.301	2×10^6
HSA	72.1	0.073	4.810	64,500
Mb	119.2	0.397	4.255	18,000
Cyt. c	131.4	0.480	4.114	13,000
K_2CrO_4	207.1	1.000	2.288	194
Hb 1.75 mM	87.8	0.181	4.628	42,500
0.875 mM	89.0	0.189	4.614	41,100
0.350 mM	90.7	0.208	4,585	39,500

Table V cont'd.

c. Ionic strength of buffer = 0.2

Sample	V_e (ml)	K_d	log M_w	M_w
BD	60.7	0.000	6.301	2×10^6
HSA	72.3	0.072	4.810	64,500
Mb	121.4	0.413	4.255	18,000
Cyt. c	133.4	0.499	4.114	13,000
K_2CrO_4	207.5	1.000	2.288	194
Hb 1.75 mM	81.8	0.144	4.705	51,100
0.875 mM	82.4	0.148	4.700	50,100
0.350 mM	83.5	0.145	4.698	49,000

d. Ionic strength of buffer = 0.02

BD	59.2	0.000	6.301	2×10^6
HSA	69.6	0.071	4.810	64,500
Mb	123.5	0.440	4.255	18,000
Cyt. c	137.5	0.536	4.114	13,000
K_2CrO_4	205.3	1.000	2.288	194
Hb 1.75 mM	82.5	0.159	4.676	47,300
0.875 mM	83.5	0.166	4.665	46,200
0.350 mM	84.5	0.173	4.655	45,300

Table V cont'd.

e. Ionic strength of buffer = 0.002

Sample	V_e (ml)	K_d	log M_w	M_w
BD	57.6	0.000	6.301	2×10^6
HSA	67.2	0.080	4.810	64,500
Mb	122.1	0.444	4.255	18,000
Cyt. c	136.3	0.542	4.114	13,000
K_2CrO_4	203.0	1.000	2.288	194
Hb 1.75 mM	83.3	0.177	4.645	44,200
0.875 mM	84.3	0.184	4.635	43,100
0.350 mM	85.4	0.191	4.624	42,100

Table VI

M_w Values and Calculated K_{diss} Values

I	$\sqrt{I}$	C_o(mM)	M_w	r	R	$K_{diss} \times 10^4$(m/l)
2.2	1.483	1.75	35,800	8.08	3.24	56.7
		0.875	34,300	14.73	6.48	56.7
		0.350	33,700	21.24	9.71	34.0
1.2	1.095	1.75	42,500	2.15	0.557	9.75
		0.875	41,100	2.64	0.751	6.57
		0.350	39,500	3.45	1.09	3.82
0.2	0.447	1.75	51,100	0.71	0.092	1.61
		0.875	50,100	0.81	0.117	1.02
		0.350	49,000	0.93	0.147	0.52
0.02	0.141	1.75	47,300	1.14	0.207	3.62
		0.875	46,200	1.31	0.260	2.28
		0.350	45,300	1.47	0.311	1.09
0.002	0.045	1.75	44,200	1.70	0.391	6.84
		0.875	43,100	1.97	0.489	4.28
		0.350	42,100	2.27	0.609	2.13

is more pronounced. Whereas, an increase in the ionic strength from 0.2 to 1.2 corresponds to a ten fold increase in the K_{diss}, and a change from 0.2 to 2.2 corresponds to a hundred fold increase in K_{diss}; a decrease from 0.2 to 0.02 corresponds to a two fold increase in K_{diss} and a change from 0.2 to 0.002 corresponds to a four fold change in K_{diss}.

Variation of K_{diss} with Ionic Strength of the Buffer Medium

The logarithms of K_{diss} were plotted versus the square root of the ionic strengths, as shown in Figure 9. The three plots correspond to the three initial concentrations: 1.75, 0.875, and 0.350 mM. The K_{diss} values are found to have a minimum at the ionic strength 0.2. The rise at the higher ionic strengths is seen to be steeper than that at the lower ionic strengths. The three sets of K_{diss} plots are seen to fall in the order of decreasing initial concentrations.

Analysis of the Shape of Elution Profiles

Martin and Synge[32] suggested the possibility of comparing chromatographic elution profiles with a Gaussian normal error function. Figure 10a gives a normal error plot and normalized elution profiles of Hb and of Mb.

The Gaussian curve is the plot of the function $y = \exp(-x^2)$, the values used for the plot were taken from Jost.[27] The area under the Gaussian curve is given by

$$\int_{-\infty}^{+\infty} e^{-x^2} dx = \sqrt{\pi} = 1.78$$

and the width of the cruve at half the height is 1.68.

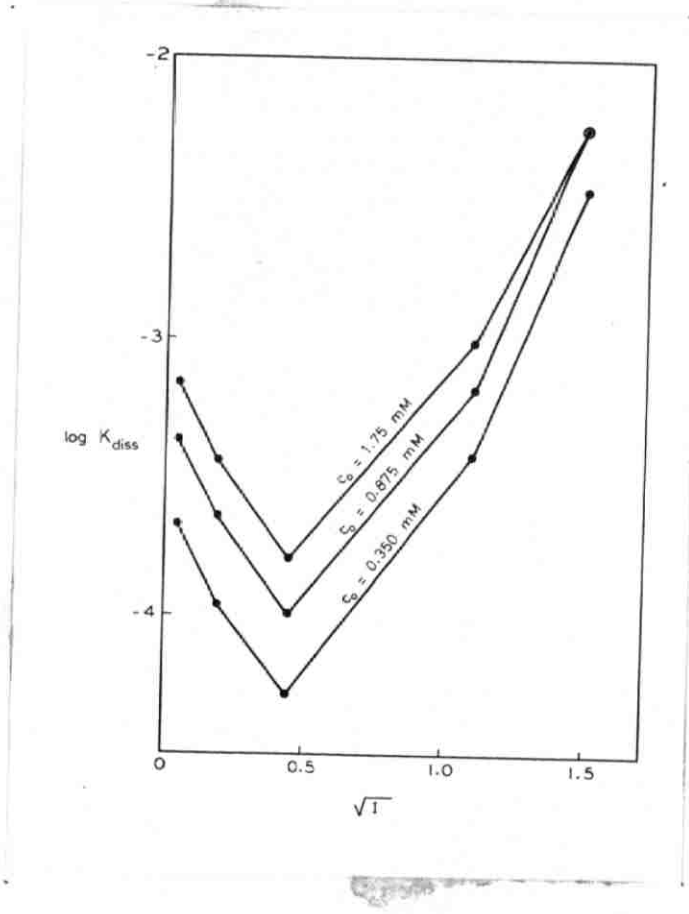

Figure 9 - Variation of log K_{diss} with $\sqrt{I}$ for the Three Hemoglobin Concentrations

The Mb elution profile at I = 2.2 was chosen for analysis. The height was normalized to unity by dividing all the absorbancy readings by the maximum reading. The width at half the height was found to be 9.52 ml which was normalized with the width at half the height of the Gaussian curve. This normalization can in fact be considered equivalent to a normalization of the area under the curve, since the area under the Gaussian curve is very close to the area of the triangle formed by joining the maximum through the points at half the height.

The Hb elution profiles at I = 2.2 and C_0 = 0.875 mM was chosen, because at this ionic strength dissociation is very pronounced. The height and width were normalized. The position of the weight average elution volume falls after the position of the maximum because of the dissymmetry of the hemoglobin elution profile.

Qualitative inspection of the three curves leads to the following two observations:

1. The correlation between the myoglobin elution profile and the normal error curve is very close. This, of course, implies the symmetry of the myoglobin elution profile, since the Gaussian curve is perfectly symmetrical.
2. The dissymmetry in the hemoglobin elution profile is very striking. This is a characteristic of the elution profile of a system in a rapid dissociation equilibrium, which in fact turns out to be

indicative of a single equilibrium system represented by

$$\alpha_2\beta_2 \rightleftharpoons 2\,\alpha\beta$$

The first, derivative of the Gaussian error curve and of the normalized elution profile of the hemoglobin band is shown in Figure 10b. It is evident that the first derivative of the trailing edge has no minimum. On the basis of the theory of Ackers and Thompson[2], discussed earlier, it can be concluded that there exists a dimerization equilibrium only. This conclusion is valid, even though the treatment of Ackers and Thompson applies strictly to a continous feed profile. Awad and Cameron[7] have found that in the chromatography of NaCl on sephadex G-75 the leading and trailing edges of a narrow band are approximately superimposable on those of the continous feed elution profile. The difficulty in making quantitative calculations based on the leading or trailing edge of a narrow band profile lies in the uncertainty of determining the prevailing total concentration of monomer and dimer.

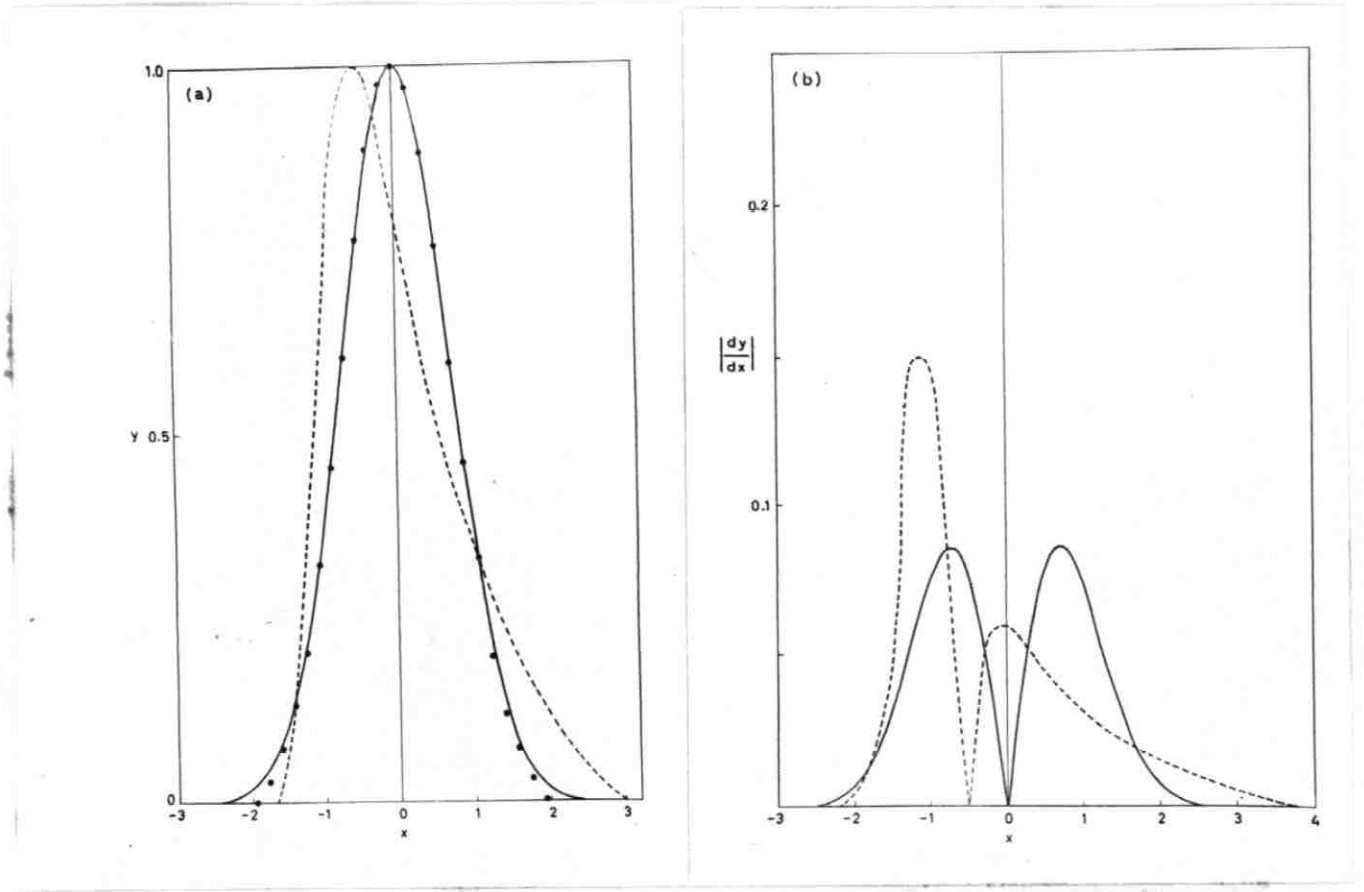

Figure 10 - (a) Gaussian Error Function and Elution Profiles. (b) Profile of the first Derivatives

——— the function $y = \exp(-x^2)$

• the normalized Mb values

---- the normalized Hb curve

——— first derivative of Gaussian function

---- first derivative of Hb curve

DISCUSSION

1. There is no doubt that human hemoglobin dissociates in high salt concentration. This dissociation has been studied and confirmed by different techniques in different laboratories: Gutfreund[23] confirmed it by osmotic pressure measurements, Rossi-Fanelli et al[34,35,36] by light scattering measurements, Benesch et al[8,9] using equilibrium sedimentation measurements by the Archibald method, Kirshner and Tanford[28] using sedminentation velocity, and recently Ackers and Thompson[2] and the present work using molecular sieve chromatography.

The situation regarding the dissociation of hemoglobin at low ionic strength has not been clearly reported previously. Antonini et al[6] say: "there are also indications that a similar dissociation may occur as the ionic strength is reduced below the physiological level". Rossi-Fanelli et al[35] say: "experiments now in progress indicate that a similar phenomenon (i.e., dissociation) may exist at low salt concentration". Wyman[44], says: "there is some indication that oxyhemoglobin also dissociates at low ionic strength". The present work shows that hemoglobin does dissociate in the ionic strength region below 0.1 and that the molecular weight of hemoglobin reaches a maximum at around I = 0.2, (see Figure 6). The maximum M_w value obtained, 51,000, is less than that of the undissociated $\alpha_2 \beta_2$ molecule calculated from amino acid sequence data, but this low value is due to the low total concentration

of hemoglobin present.

Obviously, there are two effects operative in the mechanism of dissociation. One of which is dominant at low ionic strength and the other dominant at high ionic strength. The mechanism of splitting in high ionic strength may be sought in the electrostatic effects which cause other types of protein-protein dissociation; it may be supposed that in concentrated salt solutions the electrostatic forces holding the molecule together are weakened by a shielding effect of the salts on the charges of the protein molecules. Rupture of hydrophobic bonds owing to the high ionic strength of the medium may also be involved in the splitting. The mechanism of dissociation at low ionic strength is more difficult to explain. It could be that the two subunits are held together by electrostatic forces involving foreign ions. Upon lowering of ionic strength the number of these foreign ions is decreased and hence dissociation takes place. There is an optimum ionic strength for which the two effects involved are minimum. This ionic strength corresponds to physiological conditions.

2. Theoretically, the dissociation of the $\alpha_2\beta_2$ tetramer of hemoglobin can be symmetrical ($\alpha_2\beta_2 \rightleftharpoons 2\alpha\beta$), asymmetrical ($\alpha_2\beta_2 \rightleftharpoons \alpha_2 + \beta_2$), or non-specific ($2\alpha_2\beta_2 \rightleftharpoons 2\alpha\beta + \alpha_2 + \beta_2$). Furthermore, each of the dimer units $\alpha\beta$, α_2 and β_2 can undergo dissociation to the monomer species α and β. It has been assumed in the present work and by other workers[9,28,36] that the dissociation is symmetrical. Guidotti et al[22] offer experimental evidence in favor of the symmetrical dissociation of hemoglobin at high and low pH, as well as at high ionic strength. Other evidence cited by Guidotti et al. comes from the model

proposed for hemoglobin, which shows greater and tighter contacts between the unlike peptide chains than between the like chains. It has also been shown that hemoglobin containing only α chains exists as the monomer, while hemoglobin H, a hemoglobin consisting of only β chains, exists as tetramer. If therefore the $\alpha\beta$ unit were not the most stable configuration, appreciable quantities of the individual chain hemoglobins should be found in the red cell hemolysate, which is not the case.[22]

It has also been assumed that, under the conditions relevant to the present work, the dissociation involves one single equilibrium:

$$\alpha_2\beta_2 \rightleftharpoons 2\,\alpha\beta$$

and that the $\alpha\beta$ subunits do not dissociate further into monomer units. This assumption is justified by the mathematical deductions of Ackers and Thompson[2], which require that in a rapidly associating system there would be no minimum in the profile of the first derivative, if a single dimerization equilibrium is involved. For a more detailed discussion of this point see pages 14-17 and 44-48.

3. The K_{diss} values calculated here are by no means true thermodynamic values. The difficulty with narrow band chromatography lies in the problem of determining the effective total concentration of $\alpha\beta$ and $\alpha_2\beta_2$ species. As the sample moves down the column it gets diluted, the fraction of $\alpha\beta$ units increases, and the center of gravity of the band moves slower. This movement changes the M_w value. The estimated ratio, $r = [\alpha\beta] / [\alpha_2\beta_2]$ is obtained from the observed M_w value. Since

$$K_{diss} = RC_0 = r^2 \cdot C_0 / (2r + 4)$$

it follows that K_{diss} is very highly dependent on the movement down the column. This is true for all moving boundary experiments. The K_{diss} values calculated are thus not more informative than M_w, but have been calculated for rough comparison with reported literature values.

Ackers and Thompson[2] have avoided this problem, by their use of a continuous feed mechanism. In their approach there is no problem in determining the concentration, since it would be that of the plateau region. They assumed that at high concentrations of hemoglobin (approx. 10^{-3}M) the hemoglobin molecule exists as $\alpha_2\beta_2$ while at low concentrations (approx. 10^{-8}M) hemoglobin exists as $\alpha\beta$ subunits. In the light of the present work the validity of this assumption can be questioned.

Rossi-Fanelli et al[34] obtained M_w values for human oxyhemoglobin of 47,200 in 1M NaCl and 39,200 in 2M NaCl, where the hemoglobin concentration range was 5 to 15 x 10^{-5}M. Benesch et al[9] obtained an M_w value of 38,300 for the dissociation of human oxyhemoglobin in 2.0M NaCl at 20°C. The values obtained in the present work for an initial concentration range of 0.35 to 1.75 x 10^{-3}M were 42,500 and 35,800 in 1M and 2M NaCl respectively.

Kirshner and Tanford[28] calculated the K_{diss} of human CO-hemoglobin of concentration range 2 to 9 x 10^{-5}M in 1M and 2M NaCl. Their values are K_{diss} = 3 x 10^{-5} moles/liter and 1.8 x 10^{-4} moles/liter respectively. Our corresponding values are 9.7 x 10^{-4} moles/liter and 5.7 x 10^{-3} moles/liter. Ackers and Thompson[2] obtained a K_{diss} value of 4 x 10^{-6} moles/liter for the equilibrium constant of isoelectric human CO-hemoglobin

at the concentration range 0.3 to 14 x 10^{-6} moles/liter in 0.2M sodium phosphate buffer.

4. If we assume that the linearity of the plots of Figure 7 holds approximately for higher hemoglobin concentrations, we can obtain a rough estimate of the M_w value for hemoglobin under physiological conditions, that is to say a concentration of hemoglobin of about 30 g/100 ml at ionic strength of about 0.2. The M_w value would still be below the 64,500 value expected for $\alpha_2 \beta_2$. This ~~fact~~ may alter ~~reverse~~ the present picture of hemoglobin. One wonders if instead of considering hemoglobin as $\alpha_2\beta_2$ which under certain conditions dissociates into $\alpha\beta$ subunits, it may not be more correct to think of $\alpha\beta$ units which associate under physiological conditions to form $(\alpha\beta)_2$ aggregates. This view is shared by Guidotti et al.[22], who suggest a dynamic picture of the hemoglobin molecule, in terms of an equilibrium of the hemoglobin tetramer with its subunits under all solvent conditions.

5. During the past fifty years two types of hypotheses have been advanced to explain the classical Bohr effect, namely, the heme-heme interaction theories and the algebraic association - dissociation formulations. So far, there is no conclusive evidence to establish one in favor of the other. Recently, the pendulum seems to be swinging away from the heme-heme interaction hypotheses towards the concept of aggregation - disaggregation. There are a number of observations in favor of the latter. First, it is possible to formulate multiple equilibria, which when treated thermodynamically, can explain the mechanism of the Bohr effect, for example as developed by Schejter et al.[37] Second, the fact that hemoglobin H is resistant to dissociation and at the same

time shows no Bohr effect argues against heme-heme interaction. Third, the observation cited by Schejter et al.[37], that the hemoglobin of lamprey in excessively high concentrations shows both the Bohr effect and simultaneously an increase in the observed molecular weight, although under normal physiological conditions this hemoglobin is a monomer and does not exhibit a Bohr effect.

It would seem that it is perhaps not so much the "extent of dissociation" but rather the "tendency to dissociate" which is the important factor giving rise to the Bohr phenomenon.

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