CHEMICAL MODIFICATION OF HORSE HEMOGLOBIN

by

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I hereby recommend that this dissertation prepared under my direction by John David Sakura

entitled Chemical Modification of Horse Hemoglobin

be accepted as fulfilling the dissertation requirement of the degree of Doctor of Philosophy

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ABSTRACT

The physical properties of chemically modified hemoglobin can give information concerning the nature of interactions between subunits. Complete conversion of all lysyl to homoarginyl residues gave a product similar to native at neutral pH. At pH 10, i.e., in the region of lysyl ionization, native hemoglobin showed a greater tendency to dissociate than guanidinated hemoglobin. Apparently lysyl groups at the subunit contacts can be involved either in specific interactions with sufficient freedom to allow replacement of the ϵ-amino by guanido groups or in general electrostatic interactions.

From concentration difference spectra, an increase in Soret extinction was observed upon subunit association. Difference spectra between isolated and recombined methemoglobin chains gave similar changes in extinction ($\Delta\epsilon/\epsilon$ = 10,000 - 20,000/150,000). The change in extinction arises from monomer-dimer interaction only. Since the ligand-binding properties of hemoglobin are closely associated with its spectrum, the dimer appears to be the fundamental structural unit.

Formation constants (K_{12}) for horse, human, and guanidinated methemoglobin dimers range from 1200-2500 $l \cdot gm^{-1}$. The concentration at which methemoglobin is half-dissociated into monomers is 1-10 μgm/ml. Based upon literature estimates, K_{24} is 100 times smaller; thus association occurs in two distinguishable steps.

Problems arose with handling dilute protein solutions and were attributed to surface effects. These problems appear as anomolous concentration difference spectra of monomeric heme proteins. Corrections (myoglobin blanks) for this effect were applied to all data.

INTRODUCTION

Structure of Hemoglobin

The physiological importance of hemoglobin is its ability to combine reversibly with oxygen. The oxygen affinity of hemoglobin depends on the concentrations of oxygen, carbon dioxide, phosphates, and pH. The cooperativity of oxygen binding, i.e., heme-heme interaction, seems to arise from conformational changes within the protein. The following introduction examines this question and the relation of interactions between subunits to the properties of hemoglobin.

Molecular Weight

Based upon an iron content of 0.35%, the minimum molecular weight of hemoglobin was established as 16,770 (Englehart 1825). Early osmotic pressure measurements (Huefner and Gansser 1907) gave a molecular weight of 16,700, but the results were not reproducible. Adair (1925) obtained reproducible values of 67,000 using collodion membranes. This work was done using salt and salt-free solutions, with protein concentration ranging from 40 to 120 mg/ml. Svedberg and Nichols (1927) measured the sedimentation coefficients of blood proteins from a number of vertebrate species. They determined for hemoglobin $s_{20,w} = 4.4 \times 10^{-13}$, corresponding to a molecular weight of 69,000. Studies on horse carbon-monoxide hemoglobin over the pH range 6.0 to 9.0 further confirmed Adair's conclusion of a molecular weight of 68,000 (Svedberg and Nichols 1927). Using sedimentation equilibrium Svedberg and Fahraeus (1926)

obtained a molecular weight of 66,000 for both horse carbon monoxide and methemoglobin. From a measurement of the diffusion coefficient of carboxyhemoglobin, Tiselius and Gross (1934) reported evidence suggesting dissociation at high dilution. However, Lamm and Polson (1936), using a refractometric procedure, found the diffusion coefficient constant between 3.8 to 0.8% protein. Below 0.8% they observed an increase in diffusion coefficient, i.e., dissociation. Recent light scattering measurements by Rossi-Fanelli, Antonini, and Caputo (1961) confirmed Adair's result that the molecular weight of human hemoglobin is 67,500.

Amino Acid Sequence

Mammalian hemoglobins contain two separate chains (Rhinesmith, Schroeder, and Pauling 1957) termed alpha and beta (Rhinesmith, Schroeder, and Martin 1958). Smith and co-workers (Smith, Haug, and Wilson 1957; Haug and Smith 1957) dissociated horse globin at low pH (pH 1.0 - 2.0) and demonstrated the presence of two components (alpha and beta chains) by Tiselius electrophoresis.

Wilson and Smith (1959), using an Amberlite IRC-50 column and an acidic urea gradient, obtained very pure (97%) horse alpha and beta chains. Alpha and beta chains of human adult globin (Ingram 1959) and human fetal globin (Hunt 1959) partially separated with this method. Hill et al. (1962) reported excellent separation of human globin chains by counter-current distribution. This method has successfully resolved alpha and beta chains of horse (slow component), pig, rabbit, llama, carp, and lamprey globin (Braunitzer et al. 1964).

Bucci and Fronticelli (1965) have developed a procedure for isolation of native human alpha and beta chains. Human oxy- or carboxyhemoglobin is treated with an excess of p-chloromercuribenzoate (PMB), at moderate salt concentration. The chains are separated on a carboxymethylcellulose column. PMB is removed from the modified hemoglobin chains by treatment with β-mercaptoethanol (Tyuma, Benesch, and Benesch 1966; Geraci and Li 1969; Geraci, Parkhurst, and Gibson 1969) or dodecanethiol (De Renzo et al. 1967).

Amino acid sequences of a large number of hemoglobins have been reported (Dayhoff 1969). Human hemoglobin has 574 amino acid residues and a molecular weight of 64,450 (Konigsberg, Guidotti, and Hill 1961). The alpha and beta chains have molecular weights of 15,126 and 15,866, respectively. Other data of particular interest are for sperm whale myoglobin (Edmunson and Hirs 1961, Edmunson 1965), horse alpha chain (Braunitzer and Matsuda 1963), and horse beta chain (Hunt 1959, Smith 1964).

X-ray Crystallography

The high resolution structure of myoglobin can be summarized as follows (Kendrew 1962):

1. The molecule is compact with no channels through the molecule.
2. Polar groups are on the surface, except those that perform a specific function, e.g., the heme-linked histidine.
3. The interior of the molecule is made up of non-polar residues, closely packed and in van der Waals contact with their neighbors. Some nonpolar groups are at the surface.

4. 75% of the amino acid residues are arranged in right-hand alpha helices.
5. There are eight helical segments, ranging in length from 7 to 24 residues; the corner regions between helices often contain proline.

These conclusions are general in that they carry over to hemoglobin.

A Fourier synthesis of horse oxyhemoglobin at 5.5Å resolution (Cullis et al. 1962) shows the four separate subunits. At high resolution (2.8Å), helical and nonhelical regions appear (Perutz et al. 1968). Table 1 compares the alpha and beta chains of horse oxyhemoglobin and myoglobin. The Kendrew nomenclature (Kendrew et al. 1961) identifies helical regions and corners between helices.

Oxygenation and the Nature of Subunit Interactions

The structure of human deoxyhemoglobin at 5.5Å resolution (Muirhead et al. 1967) clearly defines the polypeptide chains and the positions of the heme group. A low resolution comparison of the structures of horse oxyhemoglobin and human deoxyhemoglobin determines the general characteristics of the effect of oxygenation upon hemoglobin structure. Figure 1 shows the relative positions of the alpha and beta chains and locates the interactions between like ($\alpha_1\alpha_2$) and unlike ($\alpha_1\beta_2$ and $\alpha_2\beta_1$) subunits.

Deoxygenation produces significant changes in quarternary structure. At the α_1-β_1 contact, the beta subunit rotates 3.7 degrees about a screw axis and moves 0.3Å along it; the displacement, relative

TABLE 1

DIFFERENCES BETWEEN THE α- AND β-CHAINS OF HEMOGLOBIN AND MYOGLOBIN

	α	β	M
No. of residues:	141	146	153
Segment			
NA	=M	Contains 3 residues	Contains 2 residues
A	=M	=M	
AB	2 amides A-16-B1 differ	-2 residues	
B	=M	=M	
BC	=M	=M	
C	$3\cdot0_{10}$ helix	$3\cdot0_{10}$ helix	α-helix
CD	All different	2 amides CD5-7 differ	
D	Absent	=M	
E	Irregular	E18-20 irregular	1 kink
EF	3 amides EF2-5 differ, but $\alpha=\beta$		
F	=M	=M	
FG	=M	=M	
G		G1-3 in $3\cdot0_{10}$ helix differ	
GH	=M	Amide GH1-2	
H	Contains 21 residues		Contains 26 residues
HC	Contains 3 residues		Contains 4 residues

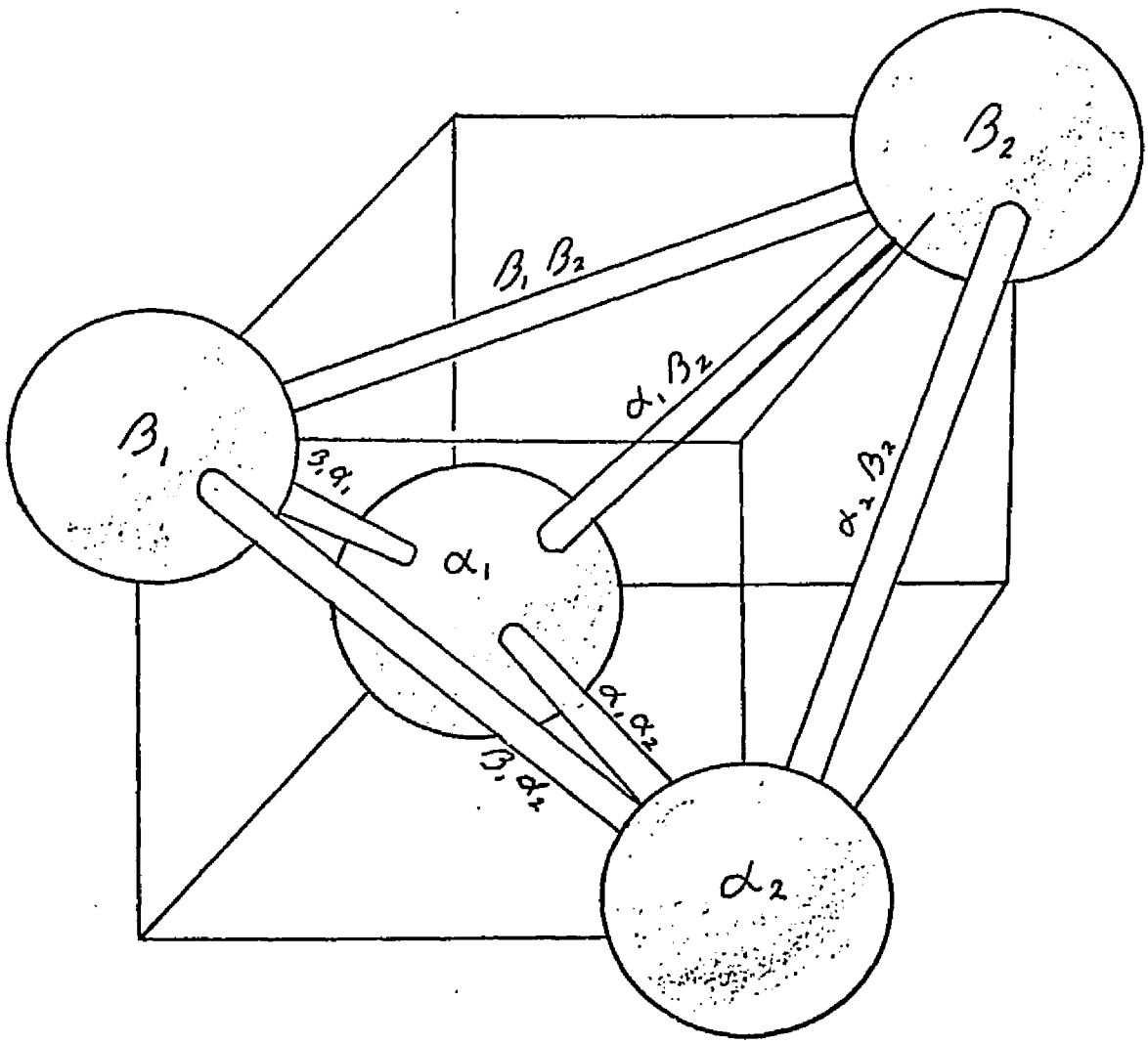

Figure 1. Spatial representation of alpha and beta chains.

to the alpha chain, is 1.0Å. At the α_1-β_2 contact, the beta subunit rotates 13.5 degrees and moves 1.9Å about a screw axis, with a 5.7Å relative displacement of atoms.

The β_1-β_2 contacts break upon deoxygenation, and the α_1-α_2 contacts undergo changes in both distance and angle of contact (Muirhead et al. 1967; Bolton, Cox, and Perutz 1968). The α_1-β_2 contact is closer to the heme groups; thus changes at this site more likely affect heme environment (Perutz et al. 1968, Perutz 1969).

Table 2 summarizes interactions between subunits of horse oxyhemoglobin.

There are 34 residues, or 110 atoms coming with 4Å distance at the α_1-β_1 contact. The majority of interactions are between nonpolar side chains. Five probable hydrogen bonds include:

1. Arginine G18(116)β[1] to the main chain carbonyl of proline GH2(114)α [it is also linked to glutamate B8(26)β].
2. Arginine B12(30)β to the carbonyl of phenylalanine GH5(117)α.
3. Serine G14(107)α to glutamine H5(127)β.
4. Aspartic H9(126)α to tyrosine C1(35)β[COO^- to OH^-].
5. Histidine G10(103)α to the main chain carbonyl of asx G10(108)β.

The $\alpha_1\beta_2$ contact comprises 19 residues, involving 80 atoms within 4Å of each other. The one clear hydrogen bond is between

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1. Arginine G18(116)β indicates that the arginine is in the beta chain, is the eighteenth residue of helical segment G, and is residue 116 counting from the amino end.

TABLE 2

SUMMARY OF POSSIBLE INTERACTIONS
BETWEEN UNLIKE CHAINS

Contact	No. Hydrogen Bonds	No. Hydrophobic Bonds	No. Residues	No. Atoms
$\alpha_1\beta_1$	5	extensive	34	110
$\alpha_1\beta_2$	1	less than $\alpha_1\beta_1$	19	80
$\alpha_1\alpha_2$	3 (possible)	none	6	
$\beta_1\beta_2$	2 (possible)	none	4	

aspartic acid G1(94)α and asparagine G4(102)β. Threonine C6(41)α possibly hydrogen bonds to histidine F64(97)β. The remainder of the contacts are nonpolar, with the exception of a possible π - π interaction between the phenolic groups of tyrosine C7(42)α and the guanidinium group of arginine C6(40)β.

No contacts between like chains are apparent, although the terminal residues of like chains may form salt bridges and/or hydrogen bonds. Possible interactions of this kind are given in Table 3.

Dissociation into Subunits

This section discusses conditions under which hemoglobin dissociates and summarizes the effect of dissociation on heme-heme interaction.

Urea and Guanidine Hydrochloride

Burk and Greenberg (1930) determined by osmotic pressure measurements the molecular weight of horse hemoglobin as 34,000 in 6.7 M urea. Steinhardt (1938) and Kurihara and Shibata (1960) observed a decrease in the sedimentation coefficient of horse hemoglobin in 4.0 M urea, to a value corresponding to a molecular weight of 39,000. Human carbonyl hemoglobin dissociates into dimers in 8 M urea and in 2.5 M guanidine hydrochloride (Kawahara, Kirshner, and Tanford 1965). In greater than 2.5 M guanidine hydrochloride, human carbonyl hemoglobin (Tanford, Kawahara, and Lapanje 1967) and bovine methemoglobin (Castellino and Barker 1968) unfold and dissociate to monomers.

TABLE 3

SUMMARY OF POSSIBLE INTERACTIONS
BETWEEN LIKE CHAINS

α-α	(arg H24(141)COO^-	--	NH_3^+lys H10(127)		
	(arg H24(140)gua^+	--	[internal anion]	--	gua^+arg H24(141)
	(lys G6(99)NH_3^+	--	[internal anion]	--	NH_3^+ lys G6(99)
β-β	(his H24(146)COO^-	--	NH_3^+lys H10(132)		
	(his H24(146)im	--	NH_3^+val NA1(1)		

Concentrated Salt Solutions

The molecular weight of hemoglobin decreases at sodium chloride concentrations greater than 0.5 M (Anderson 1940, Gutfreund 1949, Benhamou and Weil 1957). Increased salt concentration progressively dissociates hemoglobin until the molecular weight reaches 40,000 in 3 M NaCl (Rossi-Fanelli et al. 1961). Hemoglobin dissociates more effectively in $MgCl_2$ and $CaCl_2$ than in NaCl (Cecil 1962, Kirshner and Tanford 1964). Benesch, Benesch, and Williamson (1962) observed a significant difference in the degree of dissociation of human oxy- and deoxyhemoglobin in 2.0 M NaCl, suggesting different subunit interactions for the two forms. Several authors have studied the concentration dependence of the molecular weight at high salt. Rossi-Fanelli et al. (1961) and Benesch et al. (1962) observed no change in molecular weight down to 0.2 mg/ml protein in 2 M NaCl. However Benhamou et al. (1960) found concentration-dependent dissociation over approximately the same range and measured the dissociation constant (tetramer $\longrightarrow$ dimer) for human oxyhemoglobin in 1.0 M NaCl as 5×10^{-4} M/l. Kirshner and Tanford (1964) also found dissociation of human carboxyhemoglobin into dimer, with the constant ranging from 2.5×10^{-6} M/l in 0.02 M NaCl to 6.0×10^{-4} M/l in 3.0 M NaCl. From osmotic pressure data, Guidotti (1967) calculated the dissociation constant (tetramer $\longrightarrow$ dimer) for carboxyhemoglobin in 0.2 M NaCl as 5×10^{-6} M/l and 12×10^{-5} M/l in 2.0 M NaCl. The stability of tetramer was: deoxyhemoglobin $>$ carboxyhemoglobin $>$ oxyhemoglobin. In a study of the concentration-difference spectra of horse oxyhemoglobin in 1.0 M NaCl. Mizukami and Lumry (1967) fit the data to a two-step dissociation:

$$Hb_4 \rightleftharpoons 2\ Hb_2 \qquad K_1 = 1.3 \times 10^{-5}\ M \qquad (1)$$

$$Hb_2 \rightleftharpoons 2\ Hb_1 \qquad K_2 = 1.9 \times 10^{-5}\ M \qquad (2)$$

Alkaline and Acid pH

Human hemoglobin is the tetramer at pH 6 to 10 (Svedberg and Nichols 1927). Field and O'Brien (1955) observed the dimer for human carboxyhemoglobin at pH 4.5 acetate buffer. At this pH, hemoglobin displays normal oxygen capacity and spectrum; the protein denatures at pH below 3.5. In acetate-free buffer, however, dissociation does not occur at pH 5.2 (Chiancone and Gilbert 1965). Human carboxyhemoglobin dissociates reversibly into dimers at pH 11.0; at pH 11.5 denaturation sets in (Hasserodt and Vinograd 1959). Kurihara and Shibata (1960) concluded from measurements of sedimentation and diffusion coefficients that a conformation change occurs at pH 10.0 to 10.5, followed by dissociation to dimer at higher pH. Gottlieb, Robinson, and Itano (1967) found progressive dissociation of human carboxyhemoglobin between pH 9 and 11.6. They suggest that either tyrosine α 42 or β 35 was responsible for the integrity of hemoglobin tetramers.

Neutral pH

Observations by Tiselius and Gross (1934) indicate that a decrease in sedimentation velocity and an increase diffusion coefficient follow dilution of horse or human hemoglobin. However Kegeles and Gutter (1951), Gutfreund (1949), and Schumaker and Schachman (1957) reported no dissociation over the range 89 to 0.5 mg/ml. With improved photoelectric scanning systems, Gilbert (1965), Ansevin and Yphantis

(1964), Schachman and Edelstein (1966), and Edelstein (1967) have reported dissociation of human hemoglobin at concentration below 0.1 mg/ml, in solutions of moderate pH and ionic strength. Gel filtration experiments at pH 7.0 by Guidotti (1964), Ackers and Thompson (1965) and Chiancone et al. (1968) have shown dissociation of human hemoglobin to dimers. Although they made no estimate of the degree of dissociation beyond dimer, half dissociation occurred at a protein concentration of 0.1 mg/ml (Chiancone et al. 1968).

Reaction with p-chloromercuribenzoate of the β-93 sulfhydryl of human carboxyhemoglobin causes dissociation to dimers; further reaction of the β-112 and α-104 sulfhydryl groups dissociates to monomer (Rosemeyer and Huehns 1967). The authors suggest splitting of tetramer occurs at the $\alpha_1\beta_2$ contact site giving symmetric $\alpha_1\beta_1$ dimers; Vinograd and Hutchinson (1960) first suggested this mode of dissociation.

The molecular weight of hemoglobin decreases to 30,000 after acetylation of 60% of the amino groups with acetic anhydride (Bucci et al. 1963). Guanidination of 70% of lysine groups resulted in no change in molecular weight (Rossi-Fanelli, Antonini, and Caputo 1964). Similar results were obtained with human carboxyhemoglobin (82% lysine converted to homoarginine) between pH 6 to 10 (Itano, Robinson, and Gottlieb 1964). Above pH 10, guanidinated hemoglobin showed less tendency to dissociate than unmodified hemoglobin.

Heme-Heme Interaction

The oxygen affinity of hemoglobin increases as more oxygen is bound (Bohr 1903). This cooperative effect, which has been called

"heme-heme" interaction, enables hemoglobin to undergo transition from an oxygenated to a deoxygenated state with only a small change in oxygen partial pressure between arterial and venous sytems.

Models

Several models have been proposed to explain the sigmoidal character of hemoglobin oxygen equilibrium.

The Adair equation (Adair 1925) assumes the binding of ligand (X) in four steps:

$$Hb + X \underset{k_1}{\overset{k_1'}{\rightleftharpoons}} HbX \qquad K_1 = k_1'/k_1 \tag{3}$$

$$HbX + X \underset{k_2}{\overset{k_2'}{\rightleftharpoons}} HbX_2 \qquad K_2 = k_2'/k_2 \tag{4}$$

$$HbX_2 + X \underset{k_3}{\overset{k_3'}{\rightleftharpoons}} HbX_3 \qquad K_3 = k_3'/k_3 \tag{5}$$

$$HbX_3 + X \underset{k_4}{\overset{k_4'}{\rightleftharpoons}} HbX_4 \qquad K_4 = k_4'/k_4 \tag{6}$$

Hill (1910) gave the following empirical equation for the equilibrium of hemoglobin with ligands:

$$\frac{Y}{1 - Y} = KX^n \tag{7}$$

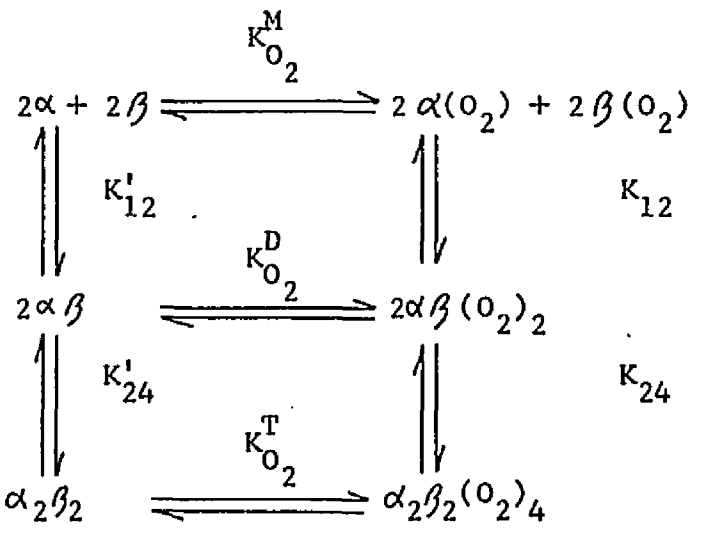
Y is the fractional saturation with ligand; X is the ligand activity; K and n are empirical constants. An overall measure of heme-heme

interaction is n which has a value close to 3.0 (Roughton, Otis, and Lyster 1955). The allosteric model proposed by Monod, Wyman, and Changeux (1965) assumes equilibrium between two conformation states ($S \rightleftharpoons T$). Binding of ligand shifts the equilibrium to conformation state (T). Wyman (1967), starting from basic thermodynamic principles, introduced an allosteric binding potential to express the linkage between pre-existing equilibrium conformations. He concluded that the major part of the allosteric interaction in hemoglobin is within the $\alpha\beta$ subunits. The total free energy of interaction is 3.0 kilocalories/site, approximately ten percent of the total free energy of oxygenation. Koshland, Nemethy, and Filmer (1966) presented models which resemble the Monod-Wyman-Changeux proposal in that binding of ligand changes the subunit conformation, but which differ in that the change can cause no, partial, or equal changes in the neighboring subunits, depending on the strength of subunit interactions.

Dissociation

Several authors have stressed the importance of understanding subunit dissociation for explaining the cooperativity of oxygen binding: Briehl (1963); Schejter, Adler, and Glauser (1963); Benesch, Benesch, and MacDuff (1965); Nichol, Jackson, and Winzor (1967); and Noble (1969).

The following cycle shows why this is true:



(8)

K_{O_2} are averaged constants for the addition of one mole of oxygen per mole heme.

$$K^T_{O_2} / K^M_{O_2} = K_{12} K_{24} / K'_{12} K'_{24} \qquad (9)$$

The weaker oxygen binding observed for the hemoglobin tetramer reflects the stronger subunit interactions of deoxyhemoglobin. The sigmoid character of the binding process is equivalent to nonlinear dependence of the observed constant $K^{obs}_{O_2}$ on degree of saturation. $K^{obs}_{O_2}$ is a complicated function of the constants of the above scheme and of similar constants characterizing equilibria involving other (partially oxygenated) polymeric species. Thus, although only a first step, definition of the association properties of fully oxygenated and deoxygenated hemoglobin is necessary for understanding the allosteric properties of the molecule. Presumably a combination of crystallographic and chemical approaches can define the strength of the individual side-chain interactions between subunits, and the way in which ligand binding changes them.

Conformation Changes

Ligand-induced conformational changes in hemoglobin have been associated with heme-heme interaction. Possibly the simplest experiment demonstrating conformational differences between oxy and deoxy forms was cited by Haurowitz (1953). When oxygen diffuses into crystals of deoxyhemoglobin, the crystals break, indicating that the oxy and deoxy derivatives are not isomorphous. Flash photolysis of sheep carbon monoxide hemoglobin generates a rapidly reacting form (Gibson 1959), which may have lost its ligand but not undergone a structural alteration to the deoxy form. Oxy and deoxy human hemoglobin show different reactivity toward alkylating reagents (Benesch and Benesch 1962a, Guidotti 1967) and carboxypeptidase A or B (Zito, Antonini, and Wyman 1964). Mutant hemoglobins have provided indirect evidence implicating the contact sites between subunits and heme-heme interaction (Perutz and Lehmann 1968). Five mutant hemoglobins, J. Captetown, Chesapeake, Yakima, Kempsey, and Kansas, have amino acid replacements at the $\alpha_1\beta_2$ contact site. All five show diminished heme-heme interaction. The oxygen equilibrium curve of one of several hemoglobins with mutations at the $\alpha_1\beta_1$ site, hemoglobin E, has been measured and shows normal heme-heme interaction (Bellingham and Huehns 1968).

Ogawa and McConnell (1967) have attached a spin label consisting of a synthetic organic free radical to the reactive -SH group (β-93) of horse and human hemoglobin. The authors concluded the following from paramagnetic resonance spectra of oxy, deoxy, and mixed chain derivatives, e.g., α(oxy) β(CN met) and α(CN met) β(oxy):

1. During oxygenation a conformation change occurs about -SH (β-93) that is related to the heme-heme interaction.
2. The conformation change depends on the state of oxygenation of the β-heme, and to a lesser extent on the state of the α-heme.
3. The resonance spectrum reflects conformational changes at the $\alpha_1\beta_2$ contact, rather than in the region about the heme groups.

Shulman et al. (1969) in nuclear magnetic resonance (NMR) and electron paramagnetic resonance (EPR) experiments, detect no change in the heme group when neighboring hemes are liganded, i.e., no heme-heme interactions. Hence, the authors conclude that the subunit conformation changes responsible for cooperativity of ligand binding are propagated only as far as the $\alpha_1\beta_2$ contact site.

These experiments suggest that cooperativity is to be explained by changes at or near the subunit contacts, rather than changes about one heme due to liganding of another. Determination of the groups and types of forces involved should provide a better understanding of hemoglobin allostery.

Physiological Unit

Hemoglobin when dissociated into dimer subunits shows heme-heme interaction. Although hemoglobin is certainly tetrameric at its concentration in the red blood cell, it is appropriate to ask whether interactions with half of the molecule (i.e., within the dimer) are entirely responsible for cooperativity. Table 4 gives the Hill

TABLE 4

SUMMARY OF HEME-HEME INTERACTION OF MODIFIED HEMOGLOBINS

Type of Modification	Heme-Heme Interaction (n)	Oxygen Affinity	Comments	References
None				
1. Native hemoglobin	2.9	Normal	1.0 M phosphate buffer, pH 7.0, 20°	Roughton et al. (1955)
2. Single chain α-SH	1.0	High	Monomer	Tyuma et al. (1966)
3. Single chain β-SH	1.0	High	Tetramer	Tyuma et al. (1966)
Dissociation				
1. Concentrated salts (2 M NaCl)	3.0	Normal	Dissociation to dimer and reversible. Molecular weight determinations performed in a three component system.	Rossi-Fanelli et al. (1961)

TABLE 4--Continued

Type of Modification	Heme-Heme Interaction (n)	Oxygen Affinity	Comments	References
2. High pH (9.5-10.5)	3.0	Normal	Dissociation to dimer and reversible. Conflicting molecular weight data; conformation changes at pH 10.0.	Antonini, Rossi-Fanelli and Caputo (1962)
3. Low pH (4.8-6.0)	3.0	Normal	Dissociation to dimer and reversible. Specific effect of acetate.	Antonini et al. (1962)
4. Urea	2.0	High	Dissociation to dimer, reversible, little conformational change.	Kawahara et al. (1965)
5. Organic phosphate free	2.9	High	No dissociation upon removal of phosphate.	Benesch and Benesch (1969)
Chemical Modification				
1. N-ethyl malemide	2.0	High	-SH groups differ in reactivity to -SH reagents. Effect on heme-heme interaction and molecular weight unclear.	Riggs (1961), Benesch and Benesch (1962a)

TABLE 4--Continued

Type of Modification	Heme-Heme Interaction (n)	Oxygen Affinity	Comments	References
2. Acetylation	2.0	High	n decreases with extent of reaction. At 80% reaction, mainly dimer.	Bucci et al. (1963)
3. Guanidination	2.0	Normal	No decrease in molecular weight at 70% reaction.	Zito and Antonini (1962)
4. Digestion with carboxy-peptidase A	1.0	Normal	No major change in conformation, molecular weight.	Zito, Antonini, and Wyman (1964)
5. Digestion with carboxy-peptidase B	2.8	High	Same as above.	Zito et al. (1964)
6. Digestion with carboxy-peptidase A + B	1.0	High	Same as above.	Zito et al. (1964)
Mutation at $\alpha_1\beta_2$ contact				
1. Kansas	2.0	Low	Dissociates into dimers.	Bonaventura and Riggs (1968)
2. J. Capetown	2.0	High		Lines and McIntosh (1967)

TABLE 4--Continued

Type of Modification	Heme-Heme Interaction (n)	Oxygen Affinity	Comments	References
3. Chespeake	1.3	High	Normal Bohr effect; conformation change during oxygenation differs from normal Hb.	Nagel, Gibson, and Charache (1967)
4. Yakima	1.1	High	Normal Bohr effect.	Jones, Brimhall, and Lisker (1968)
5. Kempsey	2.0	High	Normal Bohr effect.	Reed et al. (1968)
Mutations at $\alpha_1\beta_1$ contact				
1. Hemoglobin E	2.8	Low	Possible dissociation to dimer	Bellingham and Huehns (1968)
2. Tacoma	N.A.	High	Increased instability of protein	Cited by Perutz and Lehmann (1968)

coefficient, n, and the oxygen affinity for a variety of modified hemoglobins. Normal values of n (near 3) were observed for hemoglobin dissociated in concentrated salt solutions and at high and low pH. Chemical modification of hemoglobin generally decreases heme-heme interaction and increases oxygen affinity. Limited reports of the oxygen-binding properties of hemoglobins with mutations at the contact sites indicate a decrease in heme-heme interaction and variable oxygen affinity. Thus the relation between state of association of hemoglobin and its functional properties remains unclear. Based upon crystallographic results, Perutz (1969) suggests that the tetramer is the functional unit. A similar conclusion is reached by Gibson and Parkhurst (1968) on the basis of kinetic evidence. Since the dimer (in 2.0 M salt) possesses normal cooperativity, Antonini, Chiancone, and Brunori (1967) proposes the dimer as the important physiological unit. Brunori et al. (1969) and Guidotti (1967) present results consistent with Antonini's conclusion. It is not clear how these authors account for values of n greater than two if only two subunits interact.

Properties of Isolated α-β Chains

Table 5 gives the physical properties of isolated alpha and beta chains. Both show, compared with native hemoglobin, increased oxygen affinity and no heme-heme interactions, and considerably greater rates of combination with oxygen. The spectral properties of most forms of the isolated chains differ from those of the corresponding hemoglobin derivative. Most notable is the spectrum differences

TABLE 5

PROPERTIES OF ISOLATED α, β CHAINS

			References
1. State of Aggregation[a]			
α chain	MW = 23,000 to 30,000		Tyuma et al. (1966)
β chain	MW = Tetramer		Tyuma et al. (1966), Bucci et al. (1965)
2. Oxygen Affinity[b]			
Species	n	log $P_{\frac{1}{2}}$ (mm Hg)	
α chain	1.0	-0.0	Tyuma et al. (1966)
β chain	1.0	-3.4	Antonini et al. (1965)
$\alpha\beta$ chain	2.7	+1.22	Tyuma et al. (1966)
3. Absorption[c]			
Soret	λ_{max}	ϵ^{mM}	
α (deoxy)	429	111	
β (deoxy)	428	110	Antonini et al. (1966)
$\alpha\beta$ (deoxy)	430	127	
α (met)	404	150	
β (met)	409	125	Geraci and Li (1969)
$\alpha\beta$ (met)	407	135	

TABLE 5--Continued

	λ_{max}	ϵ^{mM}	References
Ultraviolet			
α(oxy)	272	N.A.	Tyuma et al. (1966)
β(oxy)	275	N.A.	
$\alpha\beta$(oxy)	275	N.A.	

4. Circular Dichroism[c]

	λ_{max}	$\theta \times 10^{-3}$	Geraci and Li (1969)
Visible			
α(oxy)	576,542	13.0	
β(oxy)	576,550	5.0	
$\alpha\beta$(oxy)	576,542	12.0	
Soret			
α(oxy)	414	96	
β(oxy)	421	44	
$\alpha\beta$(oxy)	420	98	
α(deoxy)	434	100	
β(deoxy)	435	72	
$\alpha\beta$(deoxy)	433	168	
α(met)	406	80	
β(met)	414	64	
$\alpha\beta$(met)	410	90	

TABLE 5--Continued

	λ_{max}	$\theta \times 10^{-3}$	References
Ultraviolet			
α(oxy)	260 (broad)	100	Goodall and Shooter (1969), Beychok et al. (1967)
β(oxy)	260 (broad	40	
$\alpha\beta$(oxy)	260 (broad)	60	

5. Kinetic Properties

	k_{on}[d] (M^{-1} sec^{-1})	k_{off}[e] (sec^{-1})
α-SH	5.0×10^{-7}	28
β-SH	7.1×10^{-7}	16
Sheep hemoglobin[f]		22

a. Over a range of 1.0-6.0 mg/ml.

b. Measurements at pH 7.02, 0.1 M phosphate buffer, 3 mg/ml.

c. Samples generally in 0.2 or 0.05 M phosphate buffer, pH 7.0.

d. Noble et al. (1969) used flash photolysis and stopped flow methods.

e. Brunori et al. (1966) used stopped flow methods.

f. Brunori and Schuster (1969) using temperature-jump methods observed two relation times: τ_f and τ_s. The results were obtained using τ_s which exhibits apparent bimolecular kinetics under certain conditions ($\bar{Y} \geq .4$). Both flow, flash photolysis, and temperature jump relaxation methods give similar kinetic parameters for isolated human hemoglobin chains.

($\Delta\epsilon_{430nm}$ = 15,000) between the separated deoxyhemoglobin chains and their equimolar mixture (Antonini, Brunori, and Anderson 1968). A similar difference holds for the circular dichroism spectrum. Nagai, Sugita, and Yoneyama (1969) and Geraci and Li (1969) reported that the arithmetic mean of the isolated band in the Soret region is not equal to the band intensity of reconstituted hemoglobin. Similar effects of recombination on circular dichroism and absorption spectra have been observed for oxy, carboxy, and met chains (Geraci and Li 1969) and have been interpreted as indicating that the heme environment changes upon association.

Statement of the Research Problem

The mechanism of ligand binding to hemoglobin remains unclear. Since the properties of the alpha and beta chains are unlike those of the tetramer, subunit contacts must affect ligand binding and be central to cooperativity. As expected, the crystallographic structures of oxy- and deoxyhemoglobin reveal significant differences in subunit interaction. Kinetic, equilibrium, and crystallographic data (Antonini et al. 1968; Gibson, personal communication) have shown that dissociation into subunits is linked to oxygenation. Conformational changes associated with oxygenation are reflected in changes at the subunit contacts and in the environment about the liganded heme (Shulman et al. 1969, Ogawa and McConnell 1967).

Several questions can be asked about how heme-heme interaction and changes in ligand binding depend upon changes in subunit structure and interaction.

1. What are the subunit interactions? Although the crystallographic structure indicates which side-chain contacts might be important in the association, chemical techniques such as selective modification can define those that in fact contribute significantly.

2. Does liganding of one heme affect the structure about neighboring hemes? Spectrum differences between the hemoglobin tetramer and isolated chains should bear upon this matter. It is by no means necessary that one heme be perturbed by liganding of another. The chemistry of the former might be affected by changes in protein structure about the pocket which the ligand fills, rather than about the heme itself (i.e., the ligand contacts groups that the heme does not contact).

3. What best accounts for the cooperativity of hemoglobin? The Adair model (Adair 1925) explains binding of oxygen in four successive steps, each having a particular equilibrium constant. Allosteric mechanisms involving just two (Monod et al. 1965) or more than two conformations (Koshland et al. 1966) have been proposed. Both dimer and tetramer have been postulated as the functional unit. Information concerning the heme chemistry and the strength of subunit interactions could help decide between various schemes.

The data to be described relate to the above points. Guanidination of lysine residues examines the importance of these in the self-association of hemoglobin. If lysine residues are critical, their modification to homoarginine should dissociate the molecule. Literature data conflict on this point. I find that modification

affects self-association at alkaline but not neutral pH. Apparently either lysyl interactions involve side chains that have considerable freedom, or the net charge determines association behavior.

Dissociation into monomer subunits occurs at concentrations below the range of most experimental techniques. Reports of dissociation beyond dimer are of uncertain validity. One needs a simple method to detect changes in molecular weight in the microgram/milliliter region in order to evaluate monomer-dimer and dimer-tetramer association constants. We shall report concentration difference spectra for methemoglobin and other derivatives in the 1-100 microgram/milliliter range. These spectrum changes in the Soret region depend only upon the monomer-dimer equilibrium and isolate it from the dimer-tetramer equilibrium. Difference spectra between isolated α(met) and β(met) chains and their equimolar mixtures substantiate the results obtained by dilution. Since the spectrum changes presumably reflect changes in conformation about the heme group, the $\alpha\beta$ dimer appear to be the fundamental structural unit of hemoglobin.

EXPERIMENTAL

Materials

Horse hemoglobin was prepared according to the method of Rupley (1964). Freshly drawn citrated horse blood (Tucson Dog Food) was centrifuged, and packed cells were washed at least five times with 0.9% saline. The cells were lysed by addition of a large volume of deionized water, followed by centrifugation one hour at 32,000 g. Horse hemoglobin was twice crystallized by adjusting to 2.0 M ammonium sulfate. Conversion to the met form was by addition of potassium ferricyanide (1.5 molar excess, heme basis). Excess ferricyanide was removed by exhaustive dialysis against water (three changes), 0.1 M ammonium acetate (one change) and water (three changes). Human hemoglobin was prepared, using the method of Geraci et al. (1969), from freshly drawn human blood (J.D.S.) that has been heparinized or oxalated. Thirty milliliters of blood was spun at 3,500 g for 15 minutes, followed by three successive washes with 0.9% saline. The cells were lysed by the addition of 1.5 volumes deionized water. After standing 30 minutes, the cell debris was removed by centrifuging one hour at 32,000 g. Additional precipitate, obtained after adding 1.0 ml 1.0 M sodium phosphate, pH 7.0, was removed by brief centrifugation. The oxyhemoglobin solution was dialyzed four hours against two changes of deionized water.

Human methemoglobin was prepared as follows: 2 ml oxyhemoglobin solution (ca. 50 mg/ml) was adjusted to pH 7.0 by passing it

through a short (2 x 6 cm) Sephadex G-25 column, equilibrated with 0.05 M phosphate buffer, pH 7.0. A fivefold molar excess (heme basis) of potassium ferricyanide was added and the excess ferricyanide was removed using the short G-25 column. Human cyanmethemoglobin was prepared by adding KCN to a methemoglobin solution (final concentration 0.01 M CN^-).

We used the single column method of Geraci and Li (1969) to prepare alpha and beta chains. To 10 ml of human oxyhemoglobin (50-70 mg/ml) was added 0.1 ml of 1.0 M K_2HPO_4 and 0.4 ml of 2.0 M NaCl; 50 mg p-chloromercuribenzoate (PCMB; Sigma Chemical Co., Lot 97B-5200) was dissolved in 2 ml of 0.1 N NaOH and adjusted with 1 N acid to first turbidity. After adding the PCMB solution, the pH of the hemoglobin mixture was adjusted with 1.0 N acetic acid to 6.0. The mixture was left overnight at 4°. Following centrifugation, the mixture was adjusted to pH 8.0 with 1.0 N NaOH and passed over a G-25 column equilibrated with 0.01 M phosphate buffer, pH 8.0. The volume of the hemoglobin mixture was reduced to 10 ml by ultrafiltration (Amicon Diaflo, Model 50) and applied to a diethylaminoethyl (DEAE) - cellulose column (Whatman Grade 22), 2 x 9 cm, equilibrated with 0.01 M phosphate buffer, pH 8.0). A band corresponding to α-PMB eluted in about 20 ml. Following this, the column was washed over four hours with 240 ml of 30 mM β-mercaptoethanol (2$\lambda\beta$-mercaptoethanol/milliliter 0.01 M K_2HPO_4). The column was freed of mercaptoethanol by washing with 150-ml buffer, pH 8.0. The β-SH band was eluted with 0.1 M phosphate buffer, pH 7.0.

The α-PMB solution was adjusted to pH 7.0 with 1.0 M KH_2PO_4 and passed over a short G-25 Sephadex column, 0.01 M phosphate, pH 6.6. The solution was incubated for 10 minutes at 0° with 15 mM mercaptoethanol, then applied to 2 x 2-cm carboxymethyl-cellulose (CMC) column (Biorad, Cellex-CM) equilibrated with the same buffer. The column was washed with 30 ml of 15 mM mercaptoethanol in pH 6.6 buffer, and the α-SH chains were eluted with 0.2 M Tris-HCl buffer, pH 8.0. Thiol content of the chains was determined by the method of Boyer (1954) adapted to hemoglobin by Benesch and Benesch (1962b). Chain purity and function was examined by cellulose acetate strip electrophoresis (Gelman Instruments, Model 51170) of the alpha and beta chains and alpha-beta mixtures, using .15 M Tris-borate buffer, pH 8.5.

Sperm whale metmyoglobin (Seravac) was passed over a Sephadex G-100 column (2.5 x 50 cm) equilibrated with 0.05 M phosphate buffer, pH 7.0. The leading edge was asymmetric, and only fractions following the peak maximum were pooled for use.

Guanidinated horse methemoglobin (Hb-G) was prepared according to the method of Hughes, Saroff, and Carney (1949). A 300-fold molar excess (heme basis) of O-methylisourea hydrogen sulfate (OMIU) was dissolved in a minimum of water (approximately 5 ml/gm). The sulfate was removed by addition of saturated $Ba(OH)_2$ solution at 70°. At this temperature, 1 ml of $Ba(OH)_2$ contains approximately 1 mM Ba^{++}. After cooling the mixture, the copious white precipitate was removed by centrifugation. The supernatant was checked for sulfate by adding a drop of $Ba(OH)_2$ solution. The pH of the OMIU solution was adjusted to 10.2

with concentrated HCl. The methemoglobin solution was carefully adjusted to pH 10.1 with 1.0 N NaOH, and the OMIU solution was slowly added. The final pH was adjusted to 10.2.

Methods

Sedimentation Velocity

Sedimentation velocity measurements were with a Spinco Model E ultracentrifuge equipped with Corning Filter (red, No. 2418) and using red-sensitive spectroscopic plates (Kodak I-N). All runs were at room temperature and 59,780 rpm. Values of the sedimentation coefficient were corrected to $s_{20,w}$ (Schachman 1957).

Spectra

Spectra and absorbancies were determined with either Zeiss PMQII or Cary Model 15 instruments.

Protein Concentration

These were determined from absorbance, using the following extinction coefficients: $\epsilon^{M}_{540nm} = 13.8 \times 10^3$ (Antonini 1965) for the oxyhemoglobin; $\epsilon^{M}_{406nm} = 155 \times 10^3$ (Antonini 1965) and $\epsilon^{M}_{500nm} = 9.5 \times 10^3$ (Lemberg and Legge 1949) for human horse and guanidinated horse methemoglobin; $\epsilon^{M}_{540nm} = 11.5 \times 10^3$ (Antonini et al. 1965, Tyuma et al. 1966) for the cyanmethemoglobin. The concentration of α(met) and β(met) chains was determined by conversion to the cyanmet derivative, $\epsilon^{M}_{540nm} = 11.5 \times 10^3$, a procedure used by Geraci and Li (1969), Tyuma et al. (1966), Banerjee et al. (1969), and Antonini et al. (1965).

Analysis of Lysine Containing Peptides

Lyophilized guanidinated methemoglobin (238 mg) was dissolved in 15 ml of 0.1 N KCl and adjusted to pH 8.0 with 0.1 N NaOH. Alpha-chymotrypsin (Worthington DDl 6110-1, 3x crystallized), 2.5 mg, was added and reacted for 16 hours at 38°. Constant pH was maintained with a Radiometer Model TTTlc SBR2 instrument and 0.1 N NaOH. Peptides were separated on a 0.9 x 15 cm Dowex 1-X2 column equilibrated with 0.05 M ammonium acetate buffer, pH 8.0. Two successive discontinuous linear gradients were established: (1) 100 ml of 2 M acetic acid into 100 ml of 2 M acetic acid; (2) 50 ml of glacial acetic acid into 50 ml of 2 N acetic acid. Following this, the column was washed with 50 ml of 50% acetic acid, then 50 ml of glacial acetic acid. Fractions (3.5 ml) were collected and analyzed for ninhydrin color after alkaline hydrolysis. Pooled fractions were lyophilized and samples hydrolyzed 20 hours under N_2 in 1:1 HCl-H_2O at 108°. Lysine content was determined with a 0.9 x 15 cm column of Chromobeads Type A (Technicon Corp.) equilibrated with sodium citrate buffer, pH 5.28. Lysine standards eluted at 1 hour 39 minutes.

Concentration Difference Spectra

Concentration difference spectra were measured at room temperature (25 $\pm$ 2°) using a Cary 14 spectrophotometer equipped with 0-0.1 slide wire. In a typical experiment the reference hemoglobin solution was placed in a 0.5-cm cell and dilutions of 1:1, 1:10, and 1:20 were used to fill the 1-cm, 5-cm, and 10-cm cells (obtained from Pyrocell

and Opticell). The product of hemoglobin concentration (c) times pathlength (l) was constant. The cells were read from 510 to 390 nm with the 0.5-cm cell as reference; reference and sample cells were then reversed and the spectra read again. The slit control was set at ca. 20, dynode voltage at 2, and scan speed at 2-5 Å/sec. The concentrations were such that the optical density never exceeded 2.5. The minimum optical density was about 0.25. The maximum slit width was 0.3 mm. Blanks with buffer in all cells, run after each day's determinations, established the instrument baseline. All cells, pipettes, and volumetrics were cleaned in 1:1 HNO_3-H_2SO_4, rinsed with dilute NH_4OH, thoroughly rinsed with deionized water, and air dried. Absorbance differences (ΔA) were read from the Cary chart as the difference between response at the difference spectrum maximum and 500 nm; ΔA ranged from 0.1 to 0.005 absorbance, with an uncertainty of $\pm$ 0.001. Values of $\Delta\epsilon$ were obtained from ΔA through division by c·l.

Errors in $\Delta\epsilon$ come from cell pathlength, solution preparation, and instrument noise and operation. Spectrophotometer cells (Opticell and Pyrocell) were calibrated to $\pm$ 0.001 cm using a Unitron microcomparator (Table 6). The measurements were checked by concentration difference measurements on standard basic chromate solutions (Haupt 1952) at 375 nm. These results were within expectation (i.e., volumetric error) of the values calculated from comparator data. The contribution of cell pair mismatch to measured $\Delta\epsilon$ was ca. 0-0.003 or less and was corrected for as follows:

where
$$\Delta\epsilon = \Delta\epsilon_{obs} - \epsilon\left[\frac{\Delta l_s}{l_s} - \frac{\Delta l_r}{l_r}\right], \qquad (10)$$

TABLE 6

ERRORS IN CELL PATHLENGTH

	Nominal Cell Pathlength	Measured Cell Pathlength[a]
A.	0.50 cm	0.4984 cm
	1.00 cm	1.009 cm
	5.00 cm	4.9907 cm
	10.00 cm	9.9913 cm
	Cell Pair	**% Mismatch**
B.	1.0 - 0.5 cm	+0.32
	5.0 - 0.5 cm	+0.13
	10.0 - 0.5 cm	+0.23

a. Measured with Unitron Microcomparator. The results average five or more readings taken along various planes through the cell. All readings were to $\pm$ 0.001 cm.

$\Delta\epsilon_{obs}$ is the measured change, $\Delta\epsilon$ the corrected change, and ϵ the extinction coefficient of the hemoglobin at the wavelength of interest. The measured sample cell path less the nominal value, l_s, is Δl_s; Δl_r and l_r are the corresponding values for the reference cell. With this correction, the estimated error in c·l comes essentially from solution preparation. Volumetric error is estimated as ca. 0.002ϵ. Instrument noise was 0.001 absorbance and contributed an error to $\Delta\epsilon$ of ca. 0.005 at the highest concentration and ca. 0.004 at the lowest. Other errors in instrument operation were looked for but not found (see Results). A systematic error in solution preparation is discussed in the Results chapter. Neglecting this, the expected error in $\Delta\epsilon$ is 0.003-0.006, i.e., 450 to 900 extinction units.

Extraction of Association Parameters

We assume that four hemoglobin species dominate association equilibria: α, β, $\alpha\beta$, $\alpha_2\beta_2$. The precision of the present data and the strength of the $\alpha\beta$ interactions justifies neglecting other dimer and trimer forms. In this regard, the $\alpha\beta$ dimer is formed about 100 times more strongly than it forms the tetramer. We write for a concentration difference spectrum measurement:

$$\Delta\epsilon_r^i = \frac{A^i}{c^i \cdot l^i} - \frac{A^r}{c^r \cdot l^r} = \frac{\Delta A_r^i}{c^r \cdot l^r} \qquad (11)$$

where $\Delta\epsilon_r^i$ is the extinction coefficient (basis: moles heme/liter) at total protein concentration c^i less that at c^r, the reference cell concentration (moles heme/liter); A^i and A^r are corresponding absorbances

measured in cells of pathlength l^i and l^r (cm); ΔA_r^i is the observed absorbance difference. In a given set of measurements:

$$c^i \cdot l^i = c^r \cdot l^r \tag{12}$$

Introducing extinction coefficients and concentrations of the several species,

$$A^i = (\epsilon_\alpha \cdot \alpha^i + \epsilon_\beta \cdot \beta^i + \epsilon_{\alpha\beta} \cdot \alpha\beta^i) l^i + (\epsilon_{\alpha_2\beta_2} \cdot \alpha_2\beta_2{}^i) l^i \tag{13}$$

$$\Delta A_o^i = (\Delta\epsilon_{12} \cdot \alpha\beta^i + \Delta\epsilon_{14} \cdot \alpha_2\beta_2{}^i) l^i \tag{14}$$

$$\Delta\epsilon_o^i = \frac{\Delta A_o^i}{c^i l_1{}^i} \tag{15}$$

$$\Delta\epsilon_{jk} = \epsilon_{jk} - \frac{\epsilon_\alpha + \epsilon_\beta}{2} \tag{16}$$

$$\Delta\epsilon_r^i = \Delta\epsilon_o^i - \Delta\epsilon_o^r \tag{17}$$

$$\Delta\epsilon_r^i = \Delta\epsilon_{12} \left(\frac{\alpha\beta^i}{c^i} - \frac{\alpha\beta^r}{c^r}\right) + \Delta\epsilon_{14} \left(\frac{\alpha_2\beta_2{}^i}{c^i} - \frac{\alpha_2\beta_2{}^r}{c^r}\right) \tag{18}$$

The transformation of $\Delta\epsilon_{jk}$ sets the concentration of zero change in absorbance at infinite dilution; i.e., ΔA_o^i expresses the experimental absorbance A^i as the difference between it and that of solution of equal total protein concentration, with all protein present as separated chains. The quantities $\Delta\epsilon_{jk}$, because they define the effect of association, are ultimately those of interest, but these must be extracted from the data, $\Delta\epsilon_r^i$.

Assuming simple equilibria and neglecting activity coefficients,

$$K_{12} = \frac{\alpha\beta^i}{\alpha^i \cdot \beta^i} = \frac{\alpha\beta^i}{(\alpha^i)^2} \tag{19}$$

$$K_{24} = \frac{\alpha_2\beta_2{}^i}{(\alpha\beta^i)^2} = \frac{\alpha_2\beta_2{}^i}{(K_{12})^2(\alpha^i)^4} \tag{20}$$

$$c^i = 2\alpha^i + \alpha\beta^i + \alpha_2\beta_2{}^i \tag{21}$$

$$= 2\alpha^i + K_{12}(\alpha^i)^2 + K_{24}(K_{12})^2(\alpha^i)^4$$

Equations (18) to (21) show that:

$$\Delta\epsilon_r^i = f(\Delta\epsilon_{12}, \Delta\epsilon_{14}, K_{12}, K_{24}, c^i, c^r) \tag{22}$$

It is convenient to change concentration units (c^i, c^r) to gm/liter (the experimental units) but to leave the basis of $\Delta\epsilon_{jk}$ and $\Delta\epsilon_r^i$ as moles heme/liter. This offers no problem, because equation (18) contains only ratios of concentration terms. K_{12} and K_{24} therefore have dimension $(gm/l)^{-1}$.

Four variable parameters $\Delta\epsilon_{jk}$ and K_{jk} were fit to the data (dependent variable $\Delta\epsilon_r^i$; independent variables c^i, c^r), using the nonlinear least squares program described by Banerjee and Rupley (to be published, 1970) and modified as follows. Equation (21) is fourth order, and thus the expression (22) cannot be written as an explicit function of the independent variables, which the least squares program requires. In order to avoid this problem, once during each iteration

(i.e., for each current value of K_{12} and K_{24}) the program solved equation (21) by Newton-Raphson approximation to give values of α^i and, using equations (19) and (20), values of $\alpha\beta^i$ and $\alpha_2\beta_2{}^i$. The least squares program then could operate with the analytically simple equation (18). Output $\Delta\epsilon_r^i$ (calculated) is difficult to plot and to visualize in comparison with input values $\Delta\epsilon_r^i$. Thus we used equations (14), (15), (16), (19), and (20) to compute theoretical curves $\Delta\epsilon_o^i$ vs. c^i. Location in the plot of experimental points $\Delta\epsilon_o^i$ assumed $\Delta\epsilon_o^{r'}$ fell on the theoretical curve. An error in the reference solution would bias an entire group of data points. To correct for this, within each group of data with the same reference solution, the program corrected $\Delta\epsilon_o^r$ by the average values, for that group, of $\overline{DY} = \overline{(\Delta\epsilon_o^i \text{ (calc)} - \Delta\epsilon_o^i)}$. The program provided for refitting of the data (called the second approximation) after this correction had been made. Correcting the fit generally reduced the standard deviation ca. twofold. The second approximation did not significantly reduce deviations.

Spectrum Measurements of Isolated Chains

Spectra for α(met), β(met), and $\alpha_2\beta_2$(met) in the Soret region were measured at 10° with a Cary 15 instrument equipped with thermostated cell holders in both reference and sample compartments. In a typical experiment, freshly prepared α(oxy) and β(oxy) solutions were adjusted to pH 7.0 using a 2 x 2 cm Sephadex G-25 column equilibrated 0.05 M phosphate buffer, pH 7.0. At time 0, a fivefold excess of $Fe(CN)_6^{-3}$ was added to each solution, followed by removal of the excess

ferricyanide by passage over the short Sephadex G-25 column. Protein concentrations were determined spectrophotometrically, after conversion to cyanmet form by dilution into 0.2 M KCN ($\epsilon^{M}_{540nm} = 11.5 \times 10^3$; Antonini et al. 1965). Cyanmethemoglobin is stable and its color at 540nm is independent of subunit association (i.e., $\Delta\epsilon$ between isolated and recombined cyanmet chains is small, ca. 2% at 427nm, and is negligible elsewhere). Equimolar solutions of α(met) and β(met) were prepared, and the spectra of the pure species and a 1:1 mixture read from 450-390nm at times to 500 minutes.

Similar experiments were done using the Cary 14 at room temperature with α(cyanmet), β(cyanmet), and $\alpha_2\beta_2$(cyanmet) in 0.02 M KCN, 0.05 M phosphate buffer, pH 7.0. Equimolar amounts of α(cyanmet) and β(cyanmet) were read in tandem 1-cm cells against a 2-cm cell containing a 1:1 mixture of the two chains. Owing to instability of the met derivatives, difference spectra of this kind could not be measured for them and had to be constructed by subtraction of spectrum measurements extrapolated to a reference time.

RESULTS

Guanidination of Horse Methemoglobin

Amino groups react with O-methyisourea (OMIU) to give the guanido derivative:

$$RNH_2 + CH_3\text{-}O\text{-}C(NH)NH_2 \longrightarrow RNHC(NH)NH_2 + CH_3OH \qquad (23)$$

The reagent preferentially converts ϵ-amino groups of lysine to homoarginine in model dipeptides (Greenstein 1938) and proteins (Krimmel 1968) with slower reaction of the α-amino. Horse methemoglobin was guanidinated for 6 to 7 days at pH 10. Figure 2 gives percent unreacted amino groups as a function of time of reaction. Free amino groups were determined by formol titration (French and Edsall 1945). Amino acid analysis of guanidinated methemoglobin confirmed that nearly complete reaction had occurred. After 6 days, 1.5 $\pm$ 0.2 moles lysine/mole hemoglobin tetramer (an average of amino acid analyses on two different preparations) remained. In one experiment, longer reaction did not further reduce the amino content. Figure 3 compares the spectra of guanidinated and native horse oxyhemoglobin. Addition of sodium dithionite followed by equilibration with air at pH 7.0 converted Hb-G(met) to the oxy form. The spectrum of guanidinated horse methemoglobin also resembles that of the native. Guanidinated methemoglobin moved as a single peak in sedimentation velocity experiments. Table 7 gives $S_{20,w}$ values for guanidinated and native horse methemoglobin.

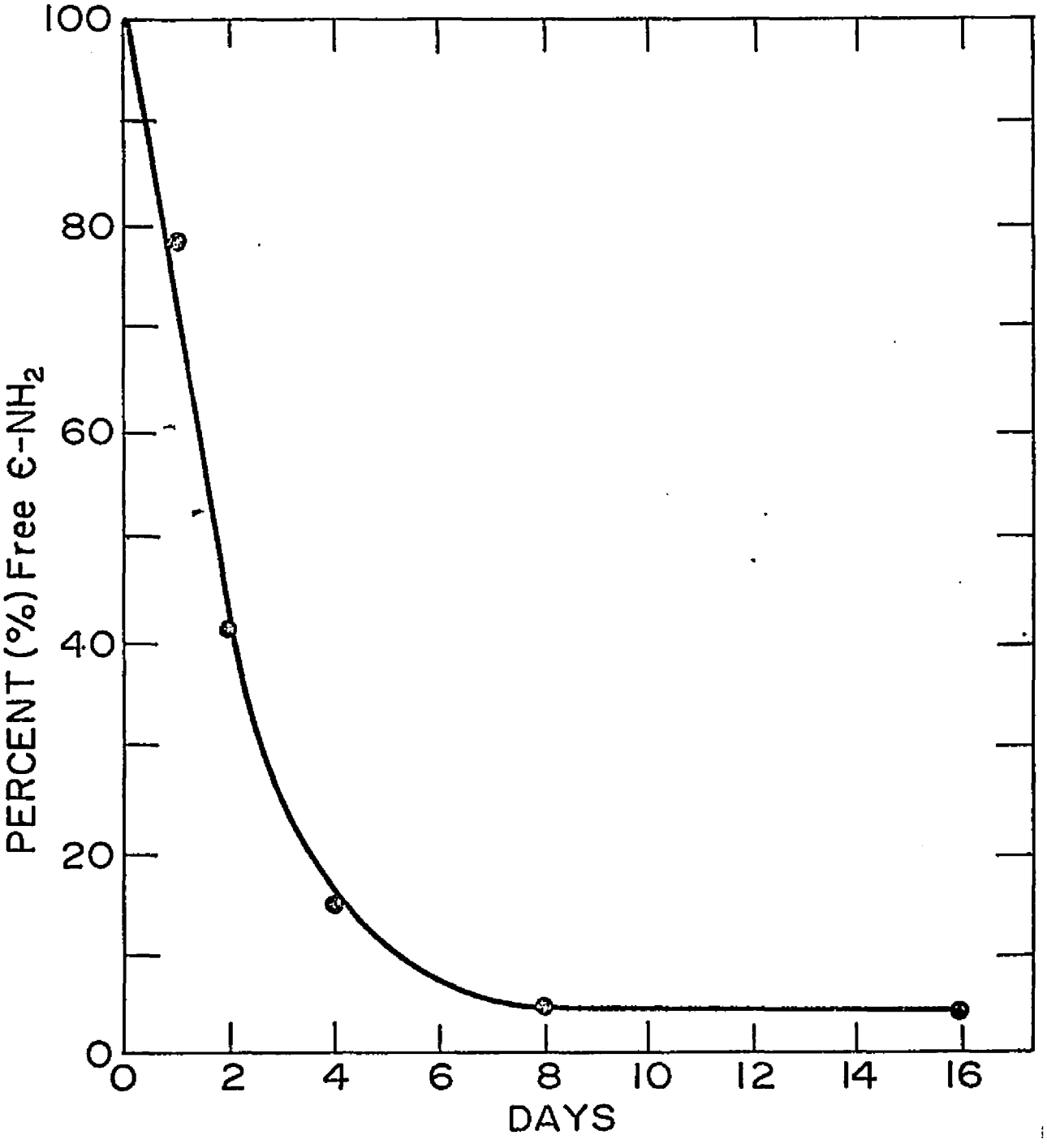


Figure 2. Guanidination of horse methemoglobin.

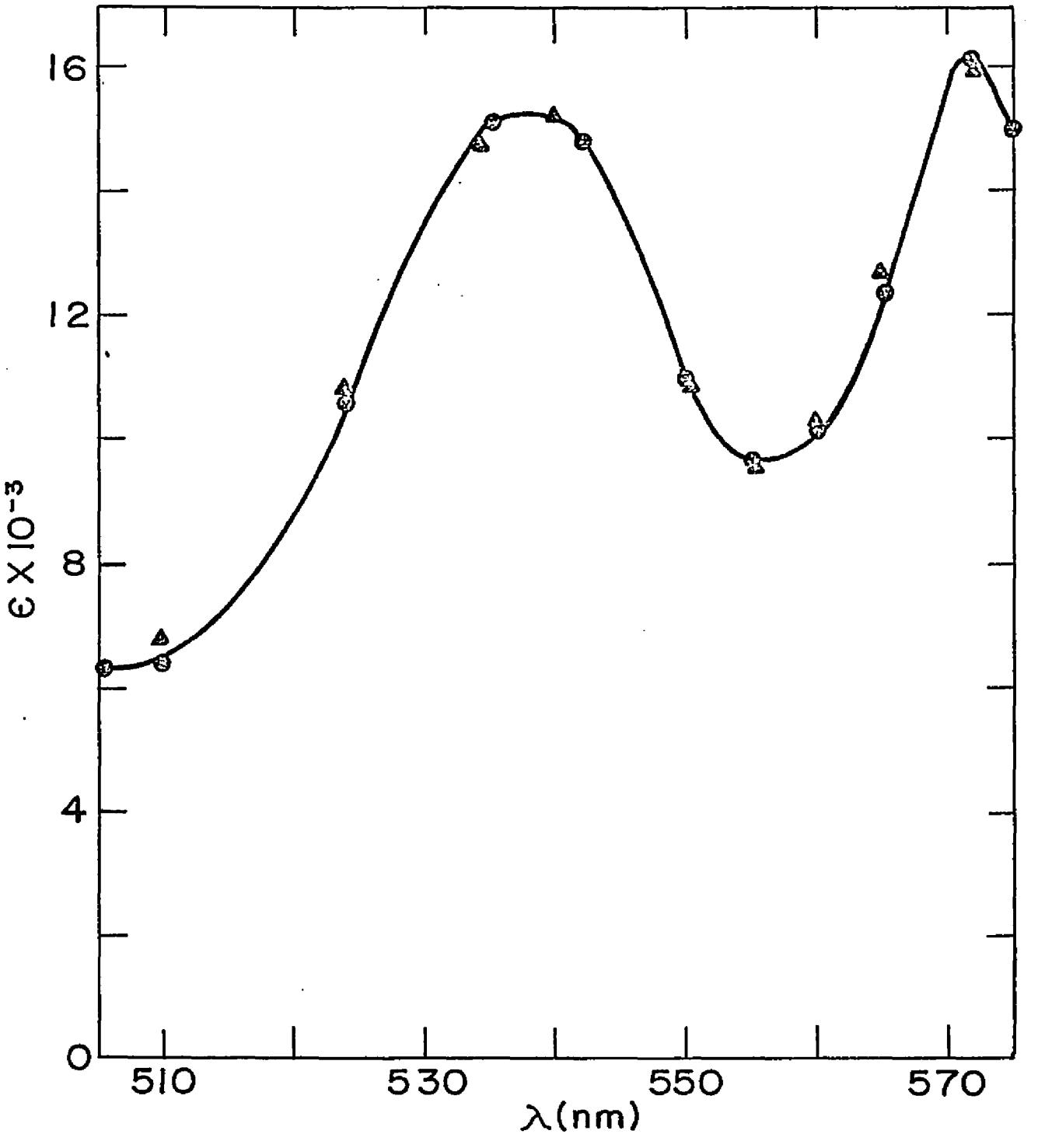


Figure 3. Visible spectrum of native horse oxyhemoglobin (closed circles) and guanidinated horse oxyhemoglobin (triangles), 0.1 M phosphate buffer, pH 7.0.

TABLE 7

SEDIMENTATION COEFFICIENTS FOR HORSE METHEMOGLOBIN AND DERIVATIVES

		Conc. mg/ml	$S_{20,w}$
pH 7.0[a]	Hb(met)	2.1	4.26
		7.3	3.97
	Hb-G(met)	2.2	4.09
pH 10.2[b]	Hb(met)	1.9	3.63
		8.8	3.93
	Hb-G(met)	2.2	4.17

a. 0.1 M phosphate buffer.

b: Universal buffer (Frugoni 1957), $\mu = 0.1$. Itano et al. (1964) reported a similar difference (ca. 10%) between native human carboxyhemoglobin and its guanidinated derivative (82% lysyl conversion).

The sedimentation constant of Hb-G(met) is the same at pH 7 and 10.2 and is equal to that of the native protein measured at pH 7; $S_{20,w}$ for native hemoglobin is significantly lower at pH 10.2 than pH 7. This agrees with literature reports (Antonini 1965, Chiancone et al. 1968).

The extent of guanidination has been used to determine whether ϵ-NH_2 groups are unreactive owing to folding of the protein. Klee and Richards(1957) presented evidence that one lysine out of ten in ribonuclease reacted slowly. Banaszak, Eylar, and Gurd (1963) found essentially complete conversion of the lysyl groups of sperm whale metmyoglobin. The failure to react 1.5 moles of lysine indicates that one mole of lysine per $\alpha\beta$ dimer (the symmetry unit of hemoglobin) may be buried. To examine this, Hb-G was digested with chymotrypsin. Ten to 20 peptide bonds per heme were hydrolyzed, estimated from the extrapolated end value of base uptake. Some insoluble material remained after digestion. Amino acid analysis showed that it contained less than 5% of total lysine recovered. Figure 4 gives the separation of chymotryptic peptides on Dowex 1-X2. Five major u.v. absorbing components eluted with the gradient. A significant amount of material (50% of the A_{280nm}) recovered remained tightly bound to the column and eluted only with glacial acetic acid. Table 8 gives the lysine content of five groups of pooled fractions. Lysine was distributed approximately equally among the first three. Seventy-six percent of the lysine applied was recovered. Because of the essentially uniform distribution of the 1.5 moles of unreacted lysine in three groups of peptides, no one lysine can be unreactive. Specifically, no lysine

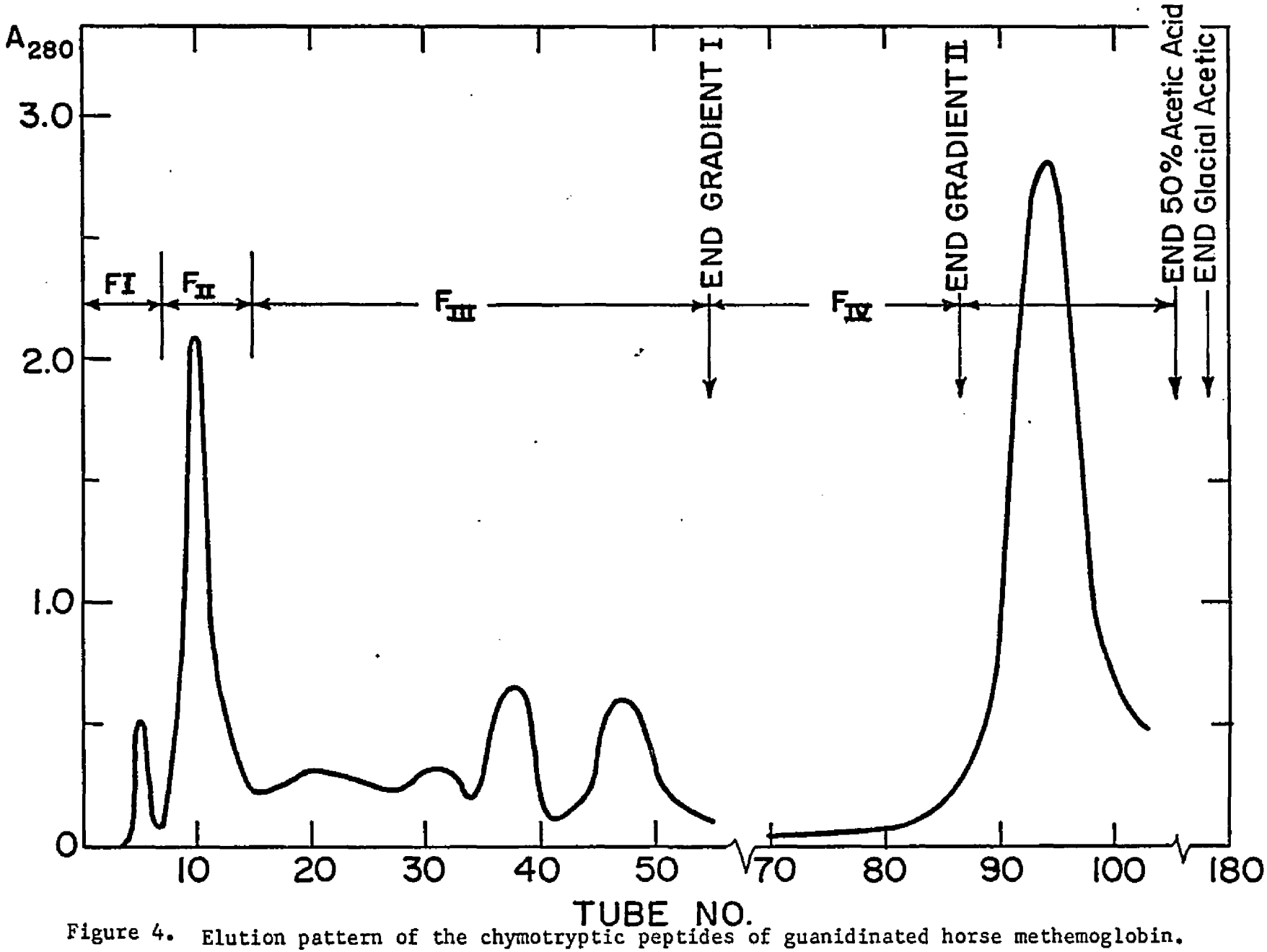


Figure 4. Elution pattern of the chymotryptic peptides of guanidinated horse methemoglobin.

TABLE 8

DISTRIBUTION OF LYSINE AMONG CHYMOTRYPTIC PEPTIDES OF GUANIDINATED HORSE HEMOGLOBIN[a]

Fraction No.	μM Lysine Found	% Total Recovered
I	0.827	34
II	0.895	37
III	0.619	26
IV	0.075	3
V	0.000	-

a. Total μM lysine applied to column = 3.15; total μM lysine recovered from column = 2.42.

can be less than 75% reacted, and this rises to ca. 90% if the free lysine in groups I and II is distributed among two or more side chains, as is likely.

Concentration Difference Spectra

Figure 5 shows concentration difference spectra of human methemoglobin at pH 7. In accord with their presumed origin in dissociation of the tetramer into subunits the spectrum differences increase with greater difference between sample and reference concentrations. The change is principally in band intensity, which decreases with increased dilution. The difference spectrum maximum (λ_{max} = 407nm) is only 1-2nm to the red of the absorption maximum. The spectra of methemoglobin and cyanmethemoglobin did not vary with time. Both horse and human oxyhemoglobin had time-dependent concentration-difference spectra; no further studies were made on these species.

Figure 6 shows concentration-difference spectra for various hemoglobin derivatives, under several solvent conditions. Those for native (B) and guanidinated (G) horse methemoglobin resemble that of human. Curves E and F give typical spectra for horse native and guanidinated horse methemoglobin at pH 10.0. At this pH the negative maxima are at 410nm for the guanidinated species and 415nm for the native. Increased salt concentration (curves C and D) gives a larger difference spectrum for the same concentration difference with no change in maximum. Literature data (e.g., Rossi-Fanelli et al. 1961) suggest that high salt favors hemoglobin dissociation. The concentration difference spectrum of human cyanmethemoglobin at pH 7.0 (curve A) is small, but

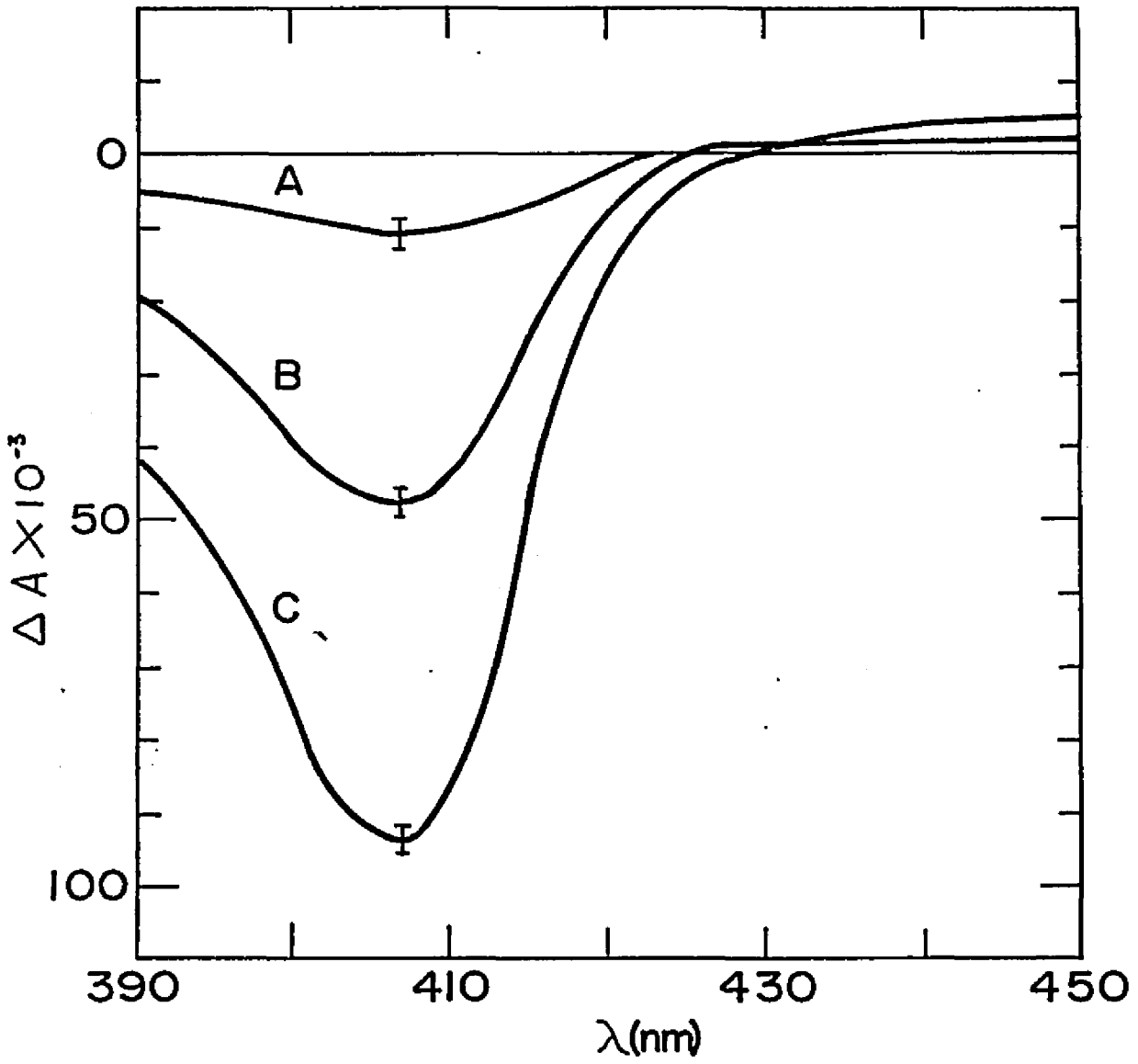


Figure 5. Concentration difference spectra of human methemoglobin.

A. 0.267 mg/ml (1.0-cm cell) vs. 0.534 mg/ml (0.5-cm cell).
B. 0.0534 mg/ml (5.0-cm cell) vs. 0.534 mg/ml (0.5-cm cell).
C. 0.0267 mg/ml (10.0-cm cell) vs. 0.534 mg/ml (0.5-cm cell).

Conditions: 0.05 M phosphate buffer, pH 7.0.

Method of Fisher and Cross, 1965.

Figure 6. Concentration difference spectra for hemoglobin derivatives.

A. Human cyanmethemoglobin, 0.05 M phosphate buffer, pH 7.0, 0.01 M KCN, 0.0249 mg/ml vs. 0.499 mg/ml.

B. Horse methemoglobin, 0.1 M phosphate buffer, pH 7.0, 0.0422 mg/ml (5.0-cm cell) vs. 0.422 mg/ml (0.5-cm cell).

C. Horse methemoglobin in 1.0 M NaCl, pH 7.0, 0.0329 mg/ml (5.0-cm cell) vs. 0.329 mg/ml (0.5-cm cell).

D. Horse methemoglobin in 2.0 M NaCl, pH 7.0, 0.0324 mg/ml (5.0-cm cell) vs. 0.324 mg/ml (0.5-cm cell).

E. Horse methemoglobin, universal buffer, pH 10.0, 0.0108 mg/ml (10-cm cell) vs. 0.216 mg/ml (0.5-cm cell).

F. Guanidinated horse methemoglobin in universal buffer, pH 10.0, 0.0407 mg/ml (10-cm cell) vs. 0.814 mg/ml (0.5-cm cell).

G. Guanidated horse methemoglobin, 0.1 M phosphate buffer, pH 7.0, 0.0153 mg/ml (10-cm cell) vs. 0.306 mg/ml (0.5-cm cell).

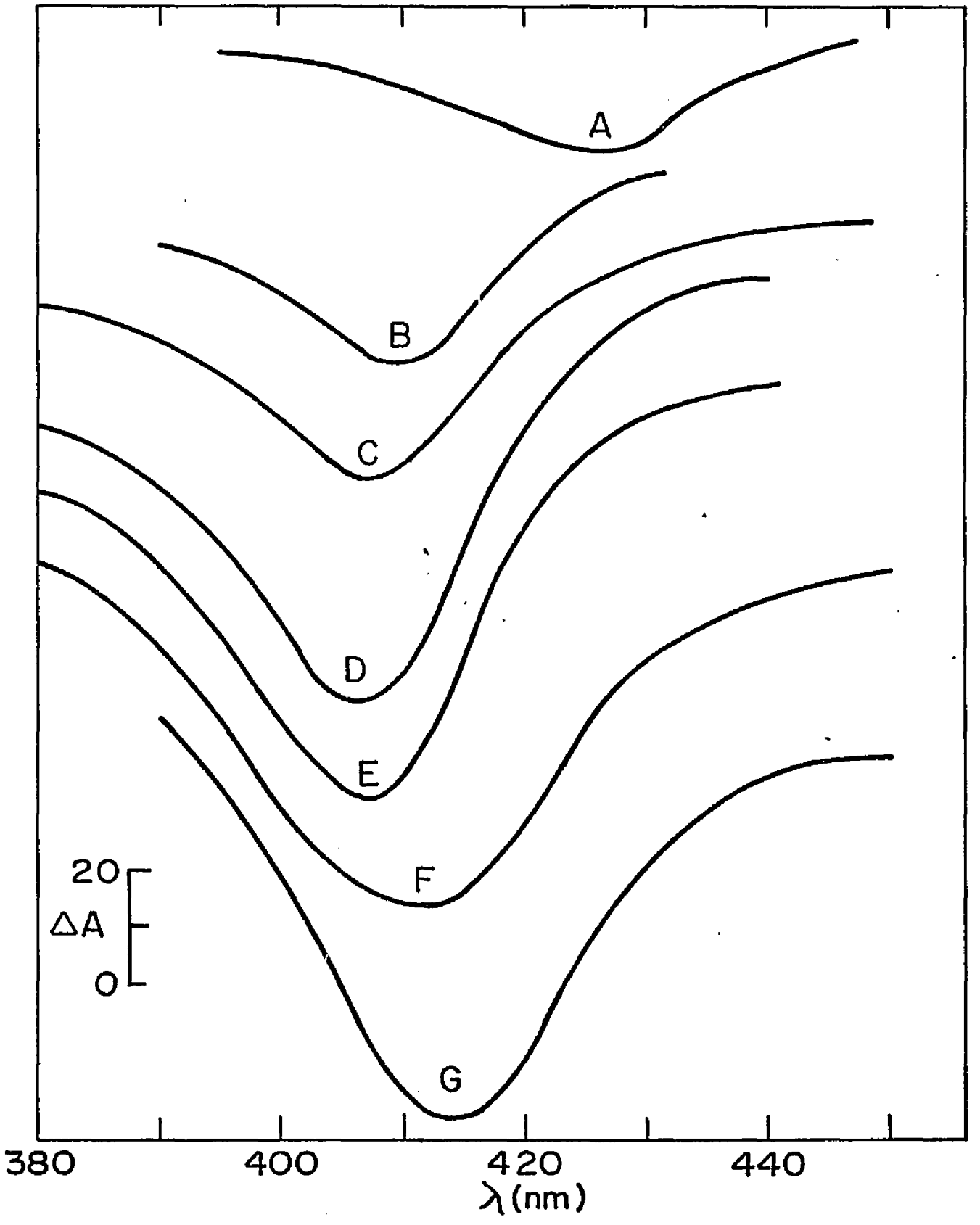


Figure 6. Concentration difference spectra for hemoglobin derivatives.

the maximum is at 425-427.5nm, indicating a considerable red shift due to dilution from the absorption maximum at 420nm.

It was particularly important to examine possible sources of error since the concentration difference spectra were small (e.g., $\Delta A/A = -0.049/1.57$) for human methemoglobin at 407nm and their shape and maxima resembled those of the parent absorption spectra. Experimental artifacts can arise from handling of the protein and instrument limitations.

Concentration difference spectra, centered at the absorption maximum and negative with dilution, were observed with monomeric heme and nonheme proteins: sperm whale metmyoglobin, ferricytochrome c and hen egg-white lysozyme. For a given total optical density and cell pair (concentration difference), the spectrum differences are less than those observed for methemoglobin. Figure 7 shows for metmyoglobin -- presumably the protein behaves similarly to hemoglobin -- the change in extinction at 407nm as a function of total optical density and cell combination. $\Delta\epsilon$ increased with dilution, i.e., larger changes at low total optical density and with the 10.0 - 0.5 cm cell combination.

The myoglobin data depend on the method of solution preparation. Curve A, Figure 8, shows the concentration difference spectra of myoglobin solutions prepared by <u>sequential</u> dilution of the 0.5-cm cell solution by 1:1, 1:5, and 1:1 for the 1-, 5-, and 10-cm cells. Comparable difference spectra for myoglobin solutions prepared by <u>direct</u> dilution (single-step) of concentrated stock myoglobin solutions, either by adding 500λ stock myoglobin to appropriate volumetric flasks filled with

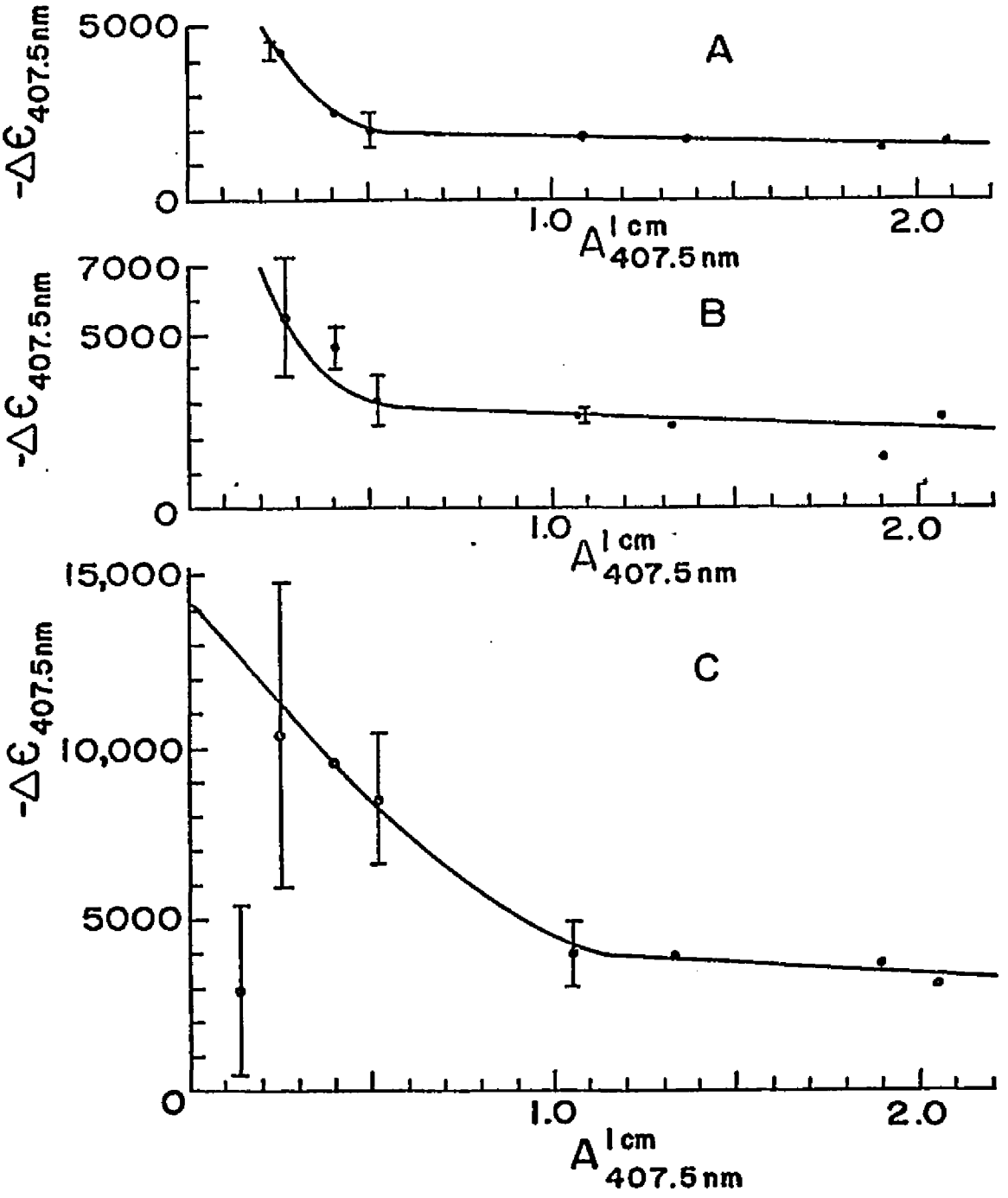


Figure 7. Metmyoglobin blanks.

1.0 cm vs. 0.5 cm (A), 5.0 cm vs. 0.5 cm (B), and 10.0 cm vs. 0.5 cm (C) cell combinations. Conditions: 0.05 M phosphate buffer, pH 7.0; samples prepared by sequential dilution.

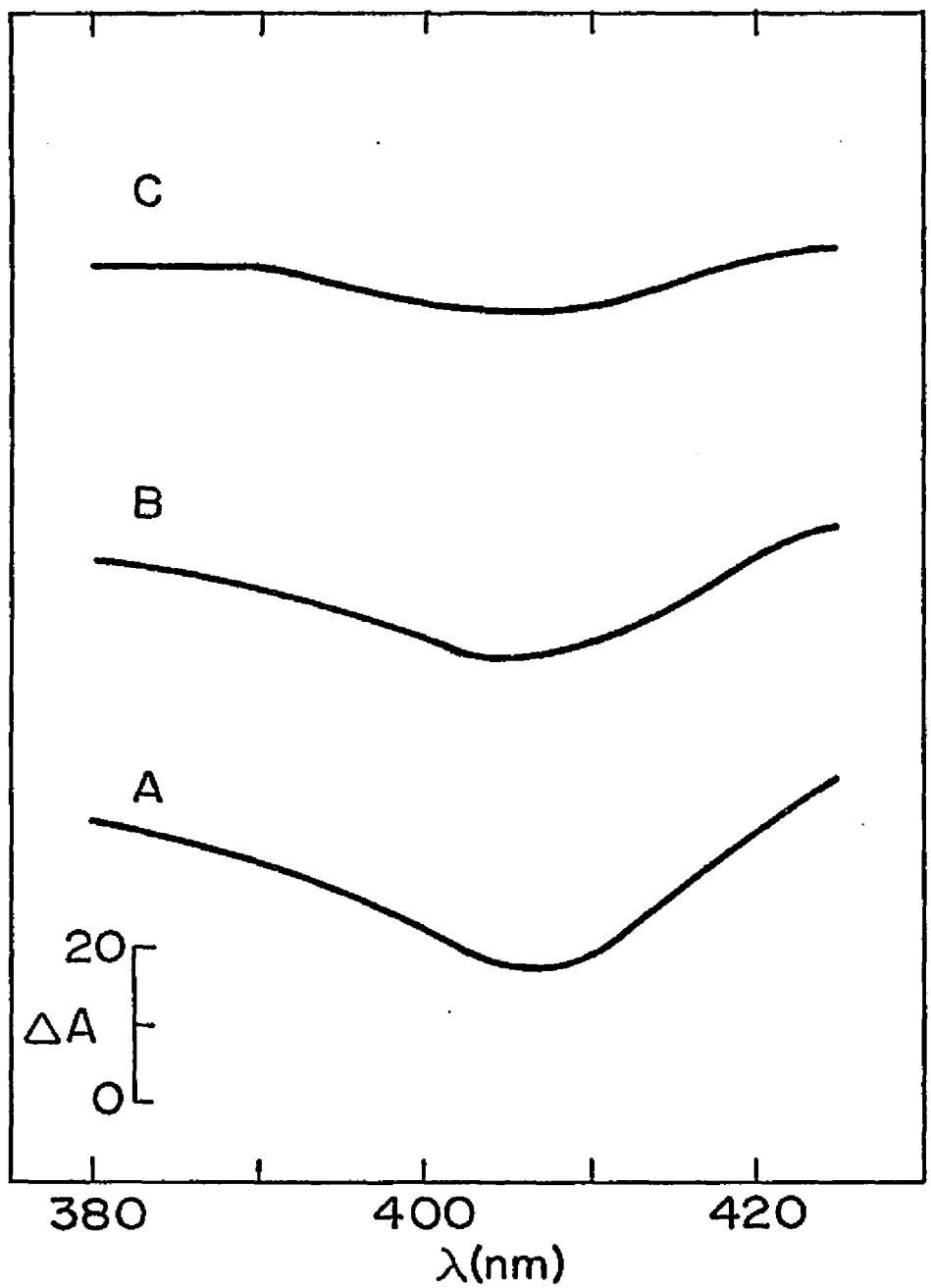


Figure 8. Concentration difference spectra of metmyoglobin.

A. Solutions prepared by sequential dilution.
B. Solutions prepared by direct dilution of concentrated metmyoglobin solutions.
C. Solutions prepared by direct dilution with weighed amounts of buffer.

Conditions: 0.05 M phosphate buffer, pH 7.0; $A^{1\ cm}_{407nm} = 1.5$.

buffer or by dilution with weighed amounts of buffer, are shown in curves B and C, respectively. The differences are notably smaller. Proteins at high dilution are more susceptible to denaturation and surface effects (Cohn and Edsall 1943, Putnam 1953). The additional volumetric transfers in the sequential method apparently produce the anomalous difference spectra. Concentration difference spectra for cyanmetmyoglobin, diluted sequentially, were small and suggest that cyanmet species are more stable than met.

We assume that horse and human methemoglobin react similarly to handling. Thus methemoglobin data used in estimating K_{assoc} were corrected by the myoglobin "blanks" of Figure 7, since most of the hemoglobin solutions were prepared by sequential dilution. For measurements at low total absorbance using the 10-cm cells, the metmyoglobin blank amounted to ca. 50% of ΔA observed. Thus 10-cm data points that deviated by more than five standard deviations were omitted from calculations.

Table 9 summarizes errors of instrument operation that could give anomalous concentration-difference spectra. These were looked for and not found with the Cary 14.

We found the Cary 15 spectrophotometer did not give accurate difference spectra. The following experiments used sperm whale metmyoglobin in 0.1 M sodium phosphate buffer, pH 7.0, and matched 1-cm silica cells (Pyrocell, S22-240). With the same solution in both cells, there was a small difference spectrum ($\Delta A/A$ = 0.005-0.007/1.54 at 408nm) with the shape of the Soret band. Reversal of sample and

TABLE 9

CARY 14 OPERATING CHARACTERISTICS

Possible Source of Error	Test and Conclusion
1. Slit artifacts.	1. Some spectrum obtained at different maximum slit widths (0.1 to 0.3 mm).
2. Temperature differential between cell compartments.	2. Cell reversal gave mirror-image difference spectra; also warming a 10-cm cell solution to 40° gave a reversible change in spectrum, the size of which showed a few degrees difference would be unimportant.
3. Positioning of cell in cell compartment.	3. No change in spectrum when the reference cell was placed in different positions along the light path.
4. Light scattering from solutions with long pathlength.	4. No change in spectrum after centrifuging solutions 1 hour at 45,000 rpm; calculation showed light-scattering by hemoglobin tetramer would not contribute.
5. Preferential adsorption of protein on cell walls.	5. Successive rinsing of the 10-cm cell with 500-ml met-myoglobin solution did not change the difference spectrum.
6. Stray light.	6. Level below quoted machine tolerance; calculation shows it would not contribute at even the highest absorbances (ca. 2).

reference cells did not change the spectrum. At lower total absorbance, the differences were similar but smaller ($\Delta A/A$ = 0.003-0.004/0.50 at 408nm). Measurements with the Cary 14 showed no difference spectra. Addition of a small amount of buffer to one cell solution generated a spectrum difference ($\Delta A/A$ = 0.032/1.56 at 408nm). Reversal of the cells gave mirror-image spectra with Cary 14 and Zeiss PMQII instruments. The Cary 15 gave ΔA = -0.034 and 0.030 for the normal and reversed cell readings. This accords with the readings with identical sample and reference solutions. Summarizing, difference spectra taken with a Cary 15 should be interpreted with caution. Cell blanks should be obtained using material with approximately the same optical density and spectrum as the solutions to be measured. Although the value for ΔA can be determined by averaging natural and reversed cell spectra, use of the Cary 15 compounds the difficulties inherent in difference spectrum measurements.

A check of the operating characteristics of the Cary 15 was made according to the manufacturer's suggestions. The following were observed to be within quoted tolerances: slide wire linearity, focus of the light beams, phototube response, and stray light. The positioning of the cells within the cell compartment and/or masking of the cell did not change the response. Introduction of a second empty cell into the light path had no significant effect, suggesting that cell imperfections and internal reflections did not contribute. Differences between the Cary 14 or Zeiss and the Cary 15 likely came from differences in phototube response. The Cary 14 and Zeiss PMQII have a single

phototube reading the light passed by both sample and reference solutions. The Cary 15 has two phototubes, which apparently respond unequally to produce difference spectra when both sample and reference compartments have solutions of high absorbance that varies with wavelength. Mismatch should be unimportant if one solution is of low absorbance or of absorbance that does not change with wavelength.

Extinction Values of Isolated Chains

Alpha(oxy) and beta(oxy) chains, prepared according to the method of Geraci and Li (1969), contained 1.0 $\pm$ 0.1 and 1.7 $\pm$ 0.2 moles free -SH/mole heme, respectively. In all preparations the -SH content of the β(oxy) chain was lower than values (2.0 $\pm$ 0.1) reported by Geraci et al. (1969) and Tyuma et al. (1966). Alpha(oxy) chains exhibited one major and two minor bands (less than 5% of the total protein) in cellulose acetate electrophoresis; beta(oxy) chains appeared homogenous. Chain recombination experiments showed that α(oxy) chains converted completely to hemoglobin, but β(oxy) chains were partially unreactive (20-30%), perhaps due to the incomplete removal of PMB.

Alpha and beta cyanmet chains were stable (judged by time independence of their spectra). Solutions of α(met) and β(met) chains became turbid at room temperature within 10 minutes after oxidations with five-molar excess ferricyanide. Geraci and Li (1969) reported similar observations. At 10°, the met derivatives were more stable in that their solutions remained clear, but their spectra were time dependent. In a typical experiment, α(oxy) and β(oxy) chains were oxidized and spectra determined at times to 500 minutes. An equimolar

mixture was prepared at 70 minutes, and its spectrum recorded at 120 and 535 minutes. The absorbance of α(met) and β(met) chains shows an initial rapid decrease during the first 100 minutes after oxidation, followed by a slow linear change (Figure 9). The slow change was also obtained for the chain mixture. The slopes of the slow changes are similar for all three species.

Comparison of extinction values at 407.5nm of the isolated alpha and beta chains with that of the equimolar mixture provides an estimate of the change in extinction for complete dissociation of the hemoglobin tetramer. This is useful for interpreting concentration-difference spectrum data. Table 10 gives extinction values at 10° for α(met), β(met), and the equimolar mixture 120 minutes after addition of ferricyanide. Table 11 gives the change in extinction calculated from absorbance values obtained as follows (Figure 9): (1) by extrapolation to 0 minutes (i.e., the start of the experiment); (2) by extrapolation to 70 minutes (the time of mixing chains); (3) values obtained at 500 minutes (end of experiment). The calculated $\Delta\epsilon$ agreed in sign (negative) and location of maximum (407.5nm) with concentration-difference results. If one assumes 30% unreacted beta chain, the change in extinction per mole heme between high concentration (tetramer) and infinite dilution (monomer) is ca. -14,000.

Table 12 gives the extinction values for isolated α(cyanmet) and β(cyanmet) chains, and an equimolar mixture. The stability of cyanmet chains permitted a difference measurement at room temperature of $\Delta\epsilon$ for recombination. Figure 10 shows chain combination difference

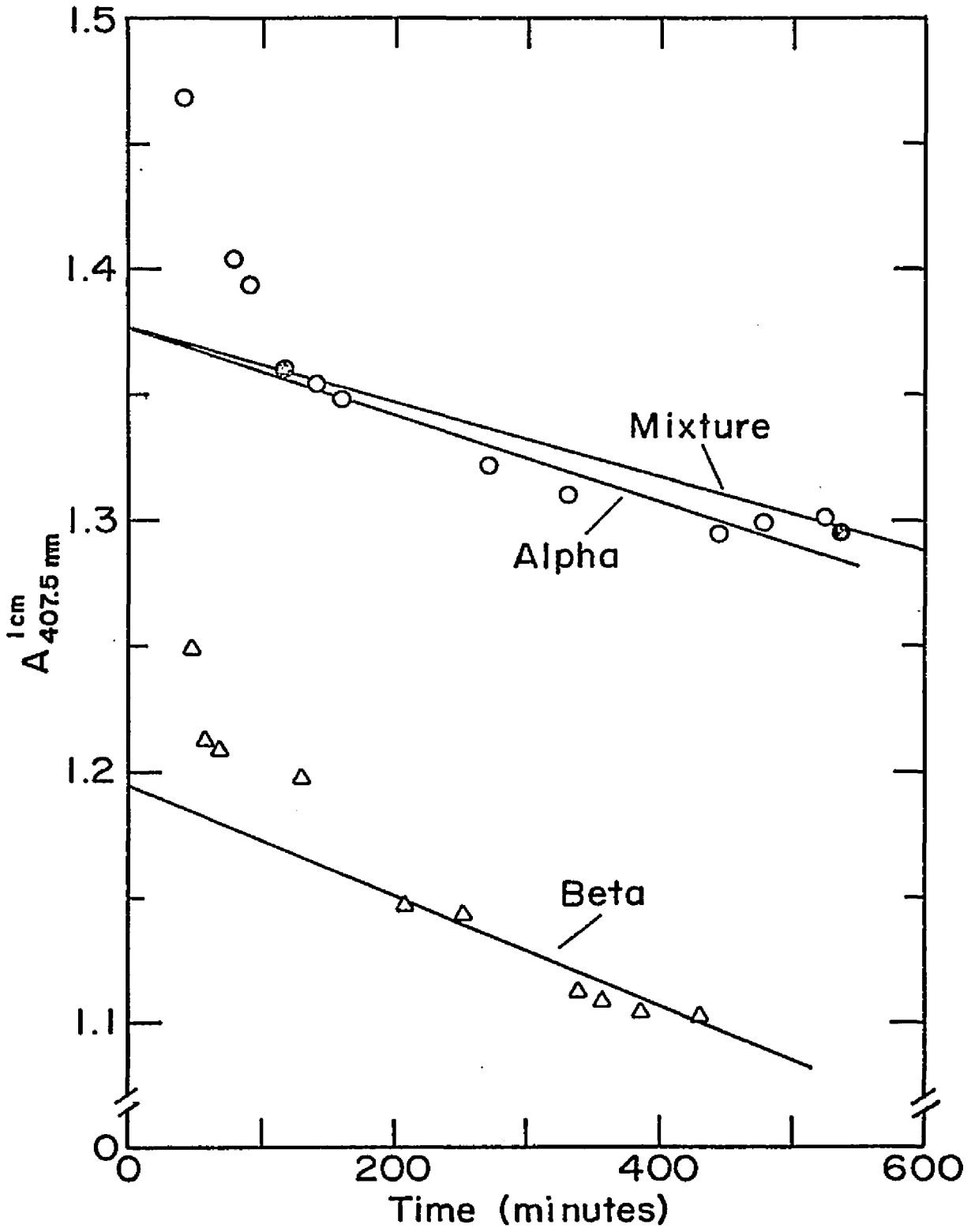


Figure 9. Time change of $A^{1\,cm}_{407.5nm}$ of isolated and recombined human methemoglobin chains after oxidation with ferricyanide.

Conditions: 0.05 M phosphate, pH 7.0, 10°.

TABLE 10

EXTINCTION VALUES OF α(MET), β(MET) AND $\alpha_2\beta_2$(MET) AT 10°

	λ_{max}(nm)	$\epsilon \times 10^3$ [a]
α(met)[b]	405	146
β(met)	407	123
$\alpha_2\beta_2$(met)	406	138

a. Similar values were obtained from estimates taken from Figure 1 of Geraci and Li (1969).

b. Interpolated between values read at 93 and 142 minutes.

TABLE 11

CALCULATED $\Delta\epsilon$ FROM CHAIN RECOMBINATION EXPERIMENTS[b]

Time (min.)	$\Delta\epsilon_{407.5nm}$[a]
0	-9,800
70	-9,600
500	-10,000
Average:	-9,800

a. Extinction values read to $\pm$ 500 ($\pm$ 0.010A).

b. alpha(met) + beta(met) vs. alpha-beta(met) chains.

TABLE 12

EXTINCTION VALUES FOR CYANMET DERIVATIVES

	λ_{max}(nm)	$\epsilon \times 10^3$
α(cyanmet)	419	119
β(cyanmet)	421	111
$\alpha_2\beta_2$(cyanmet)[a]	420	116
$\alpha_2\beta_2$(cyanmet)[b]	419	116

a. Value for a 1:1 mixture of α(cyanmet), β(cyanmet).

b. Human cyanmethemoglobin, based on $\epsilon_{540nm} = 11.5 \times 10^3$.

Figure 10. Difference spectra between isolated and recombined human cyanmethemoglobin chains.

A. Difference spectrum calculated from spectrum of each species.

B. Observed difference spectrum with isolated species in tandem cells vs. recombined chains.

Conditions: 0.05 M phosphate buffer, pH 7.0, 0.01 M KCN, 22°.

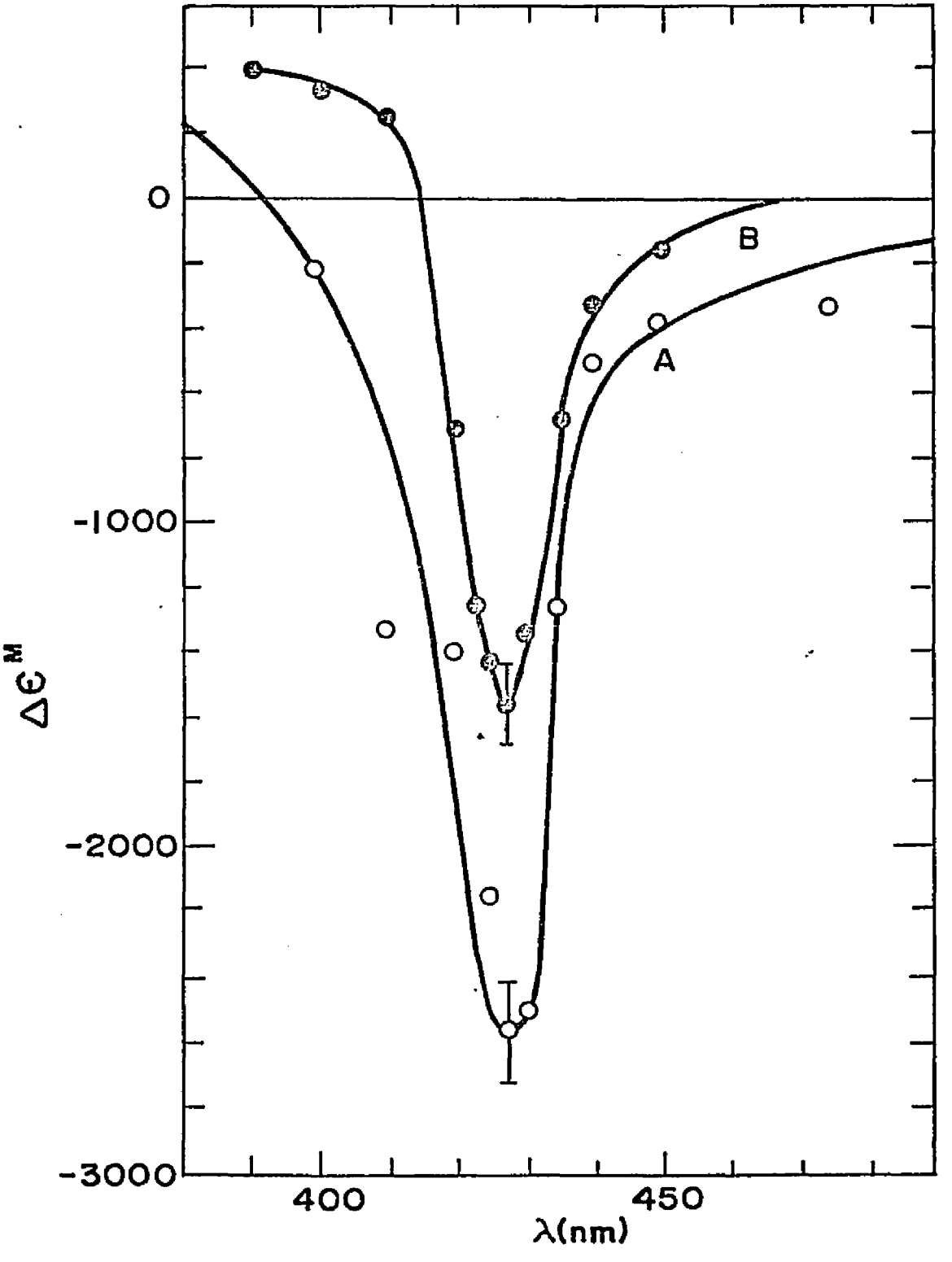


Figure 10. Difference spectra between isolated and recombined human cyanmethemoglobin chains.

spectrum measured by two methods: (1) that used for the met chains, i.e., subtraction of the direct spectra of the three species (curve A); and (2) the difference spectrum recorded between isolated chains (tandem 1-cm cells) and the equimolar mixture (2-cm cell) (curve B). Both methods give curves similar in size, shape, and maximum (427-428nm) to those observed in concentration difference experiments. Method 1 and method 2 give, respectively, $\Delta\epsilon_{max}$ = -2500 and -1500. Considering the small size of the perturbation (2% of the total absorbance), the agreement is adequate.

Calculation of Association Constants

Sets of concentration difference spectra were analyzed using the least squares procedure described in Methods. Literature values of K_{24} (Table 13) are so small that the dimer-tetramer equilibrium cannot be associated with significant changes in spectrum (see below). Unless noted, $\Delta\epsilon_{14}$ was set equal to $\Delta\epsilon_{12}$ (i.e., no spectrum change in association of dimer), and fitting concentrated on estimating K_{12} and $\Delta\epsilon_{12}$. The program generally returned physically unreasonable values of $\Delta\epsilon_{12}$ ($\frac{1}{4}$ to $\frac{1}{2}\epsilon$) if this parameter was free to vary. The trials reported have $\Delta\epsilon_{12}$ fixed at 10,000 or 20,000 for methemoglobin, and 5,000 or 10,000 for cyanmethemoglobin, values consistent with results of chain-combination experiments. Uncertainty in $\Delta\epsilon_{12}$ produces uncertainty in K_{12}. However, since we have chosen limits on $\Delta\epsilon_{12}$ that are wide and almost certainly include the true value, concentration-difference spectrum data define allowable ranges of K_{12}. Most studies of hemoglobin association have concerned the physiologically important oxy and deoxy

TABLE 13

SUMMARY OF REPORTED ASSOCIATION CONSTANTS

Author/Method	Hemoglobin Species	Conditions	Reported Constant	Association Constant ($l \cdot gm^{-1}$)
Chiancone et al. (1968)[a]	Human oxy	0.1 M Na^+, pH 7.0, 3°	$K_{24}=1.90\times10^{2}$ dl/gm	$K_{24}=19$
Gel filtration			$K_{12}=4.80\times10^{4}$ dl/gm	$K_{12}=19\times10^{3}$
			or	or
			$K_{24}=2.9\times10^{2}$ dl/gm	$K_{24}=29$
			$K_{12}=0.7\times10^{4}$ dl/gm	$K_{12}=2.8\times10^{3}$
Edelstein (1967)[b]	Human oxy	0.1 M PO_4^{-3} pH 6.8, 20°	$K_{42}=0.5\times10^{-5}$M	$K_{24}=12$
Sedimentation equilibrium			$K_{21}=0.9\times10^{-7}$M	$K_{12}=2.7\times10^{3}$
	Human deoxy	"	$K_{42}=0.7\times10^{-6}$M	$K_{24}=86$
			$K_{21}=0.2\times10^{-7}$M	$K_{12}=12\times10^{3}$
	Human oxy	2.0 M NaCl, pH 7.0	Dimer only	

TABLE 13--Continued

Author/Method	Hemoglobin Species	Conditions	Reported Constant	Association Constant ($l \cdot gm^{-1}$)
Kirshner and Tanford (1964)	Human CO	2.0 M NaCl 25°, pH 7.0	$K_{42}=18\times10^{-5}M$	$K_{24}=0.3$
Sedimentation velocity		1.0 M NaCl 25°, pH 7.0	$K_{42}=3.0\times10^{-5}M$	$K_{24}=2.0$
		0.02 M NaCl 25°, pH 7.0	$K_{42}=2.5\times10^{-6}M$ (est)	$K_{24}=24$ (est)
Kawahara et al. (1965)	Human CO	= 0.1	$K_{42}=1\times10^{-6}M$ (est)	$K_{24}=60$ (est)
Sedimentation velocity				
Guidotti (1967)	Human oxy	1.0 M NaCl pH 7.0	$K_{42}=2\times10^{-5}M$	$K_{24}=3$
Osmotic pressure, 20°	"	2.0 M NaCl pH 7.0	$K_{42}=12\times10^{-5}M$	$K_{24}=0.5$
	Human deoxy	2.0 M NaCl pH 7.0	$K_{42}=5\times10^{-6}M$	$K_{24}=12$
	Human CO	0.2 M NaCl pH 7.0	$K_{42}=5\times10^{-6}M$	$K_{24}=12$

TABLE 13--Continued

Author/Method	Hemoglobin Species	Conditions	Reported Constant	Association Constant ($l \cdot gm^{-1}$)
Ackers and Thompson (1965)	Human CO	0.2 M $PO_4^{=}$ pH 6.8	$K_{42}=4x10^{-6}M$	$K_{24}=15$
Gel filtration				
Benhamou et al. (1960)	Human oxy	1.0 M NaCl pH 7.0, 20°	$K_{42}5x10^{-4}M$	$K_{24}=0.1$
Light scattering				
Mizukami and Lumry (1967)	Horse oxy	1.0 M NaCl pH 7.0, 22°	$K_{42}=1.3x10^{-5}M$	$K_{24}=4.7$
Concentration difference spectra			$K_{21}=1.9x10^{-5}M$	$K_{12}=12$

a. Conversion of gm/dl (Gilbert constants) to gm/l basis: $K_{12} = 0.4L_{12}$

$K_{24} = 0.1L_{24}$

b. Conversion of moles/liter to gm/l basis: $K_{12} = 2.43x10^{-4}/K_{21}$

$K_{24} = 0.61x10^{-4}/K_{42}$

forms. Because of spectrum instability, we did not study oxyhemoglobin. Oxy and methemoglobin crystals are isomorphous, and we assume that the association properties of these proteins are essentially the same. Some fitting trials set K_{12} and K_{24} at values estimated for oxyhemoglobin by Chiancone et al. (1968), which accord well with the reports from other laboratories (Table 13).

Tables 14 and 15 summarize estimates of K_{12} and the quality of fit for horse and human methemoglobin and derivatives under various conditions. Figures 11 to 19 show difference spectrum data and theoretical curves calculated from the parameters of the tables. The tables give standard deviations of K_{12} and describe the fit by listing standard deviations and a qualitative comment (good or poor) on how well the data seem to fit the calculated curve.

Comparison of columns A and C of Table 14 and Figures 11 and 13 shows that a substantially better fit comes with $\Delta\epsilon_{12}$ set at 20,000. Freeing $\Delta\epsilon_{12}$ did not further reduce the standard deviation (960), although this parameter reached the unreasonable value of 48,000. Introduction of the Gilbert (Chiancone et al. 1968) value of K_{24} did not significantly change K_{12} or the quality of the fit (compare columns A and C with B and D and Figures 11 and 12), supporting the statement that Δ_{24} is zero (i.e., that $\Delta\epsilon_{12} = \Delta\epsilon_{14}$). In accord, the fit with $\Delta\epsilon_{12} = 0$ and $\Delta\epsilon_{24} = 20{,}000$ (column H and Figure 14) is particularly poor. Chiancone et al. (1968) proposed two sets of association parameters that differ principally in one (column G) having a 7-times larger value of K_{12} than the other (columns E and F). Calculations with both

TABLE 14

EQUILIBRIUM CONSTANTS FOR ASSOCIATION OF HUMAN AND HORSE METHEMOGLOBIN SUBUNITS

	A	B	C	D	E	F	G	H
K_{12} (Fixed)					2800	2800	19,000	2800
K_{24} (Fixed)	0.0	29	0.0	29	29	29	19	29
$\Delta\epsilon_{12}$ (Fixed)	20,000	20,000	10,000	10,000	20,000	10,000	20,000	$\Delta\epsilon_{12}$= 0.0, $\Delta\epsilon_{24}$=20,000
Human methemoglobin pH 7.0 (32)[a]								
K_{12}	1200	1350	550	400	-	-	-	-
S.D.	250	200	100	150	-	-	-	-
Fit	1.0[b] good	1.0 good	2.0 poor	2.0 poor	1.5 good	2.5 poor	3.0 poor	3.0 poor

TABLE 14--Continued

	A	B	C	D	E	F	G	H
Horse methemoglobin pH 7.0 (28)								
K_{12}	2500	2900	400	450	-	-		-
S.D.	500	550	200	150	-	-		-
Fit	1.0 good	1.0 good	1.5 poor	1.5 poor	1.0 good	2.0 poor		4.0 poor

a. Number of data points.

b. Standard deviation x 10^{-3}.

TABLE 15

EQUILIBRIUM CONSTANTS FOR ASSOCIATION OF SEVERAL HEMOGLOBINS UNDER VARIOUS CONDITIONS

	I	II	III
K_{12} (Fixed)		2500	
K_{24} (Fixed)	0.0	0.0	0.0
$\Delta\epsilon_{12}$ (Fixed)	20,000	20,000	10,000
Methemoglobin, pH 7.0 (28)[a]			
K_{12}	2500		400
S.D.	500		200
Fit	1.5[b], good		1.5, poor
Guanidinated methemoglobin, pH 7.0 (24)			
K_{12}	1300	-	250
S.D.	250	-	150
Fit	1.0, good	1.0, good	2.5, poor

TABLE 15--Continued

	I	II	III
Methemoglobin, pH 10.0 (16)			
K_{12}	250	-	34
S.D.	100	-	200
Fit	1.0, good	3.0, poor	3.0, poor
Guanidinated methemoglobin, pH 10.0 (12)			
K_{12}	2400	-	450
S.D.	350	-	50
Fit	0.5, good	-	0.5 (?)
Methemoglobin, 1.0 M NaCl, pH 7.0 (20)			
K_{12}	1050	-	200
S.D.	350	-	500
Fit	1.5, good	1.5, good	2.5, poor

TABLE 15--Continued

	I	II	III
Methemoblogin, 2.0 M NaCl, pH 7.0 (16)			
K_{12}	600	-	650
S.D.	200	-	700
Fit	1.0, good	1.0, poor	5.0, poor
Human cyanmethemoglobin[c] pH 7.0 (20)			
K_{12}	1850	-	250
S.D.	600	-	200
Fit	0.7, good	-	0.8, good

a. Number of data points.

b. Standard deviation x 10^{-3}.

c. Estimate of $\Delta\epsilon_{12} = \frac{1}{2}\Delta\epsilon_{12}$ (columns I and III).

Figure 11. Concentration difference spectra data for human methemoglobin; theoretical curves given for K_{12} = 1200, K_{24} = 0.0, $\Delta\varepsilon_{12}$ = 20,000.

Conditions: 0.05 M phosphate buffer, pH 7.0.
Upper curve: 0 - 0.6 mg/ml.
Lower curve: 0 - 0.12 mg/ml.

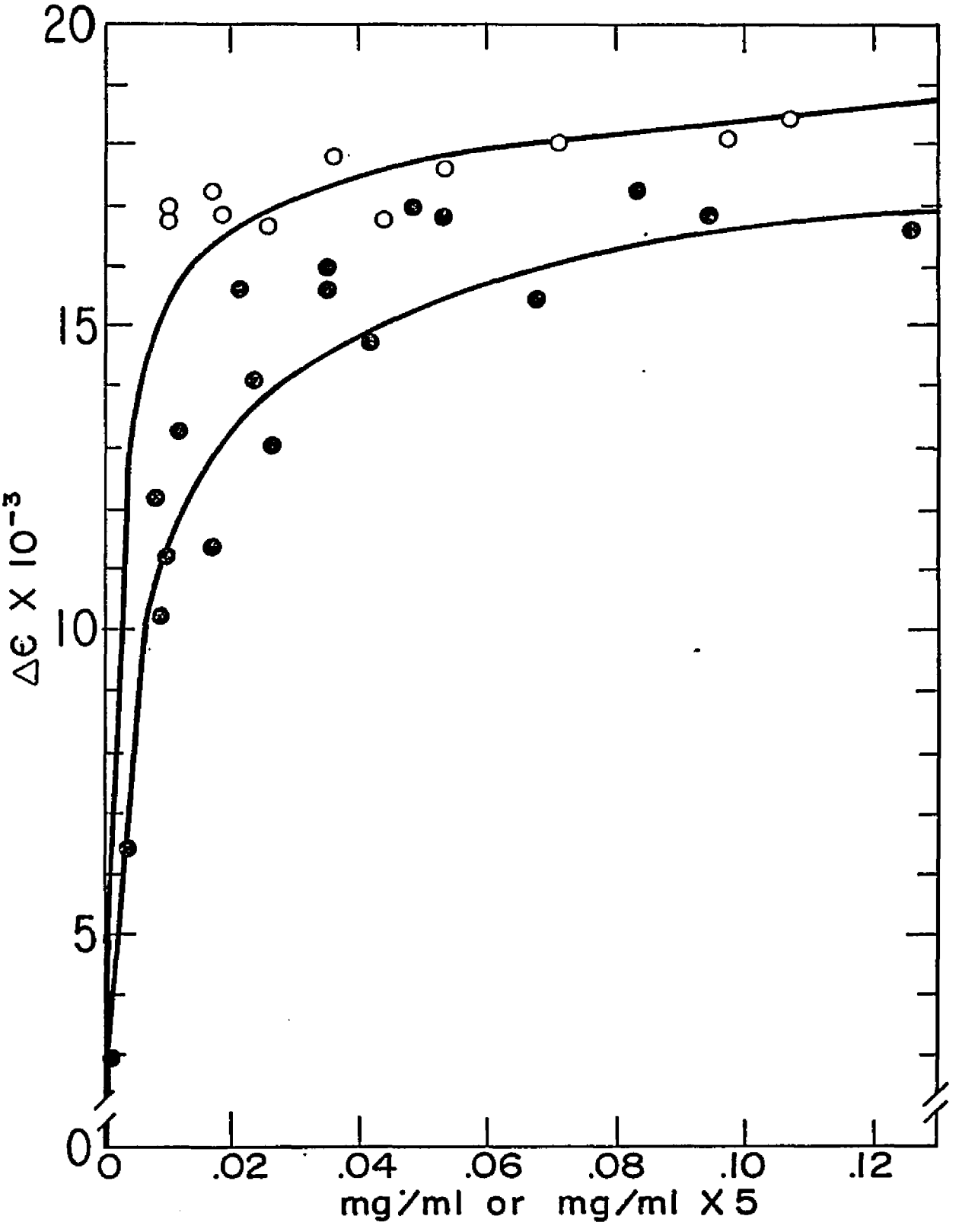


Figure 11. Concentration difference spectra data.

Figure 12. Concentration difference spectra data for human methemoglobin; theoretical curves given for $K_{12} = 1350$, $K_{24} = 29$, $\Delta\epsilon_{12} = 20{,}000$.

Conditions and concentration range: Same as Figure 11.

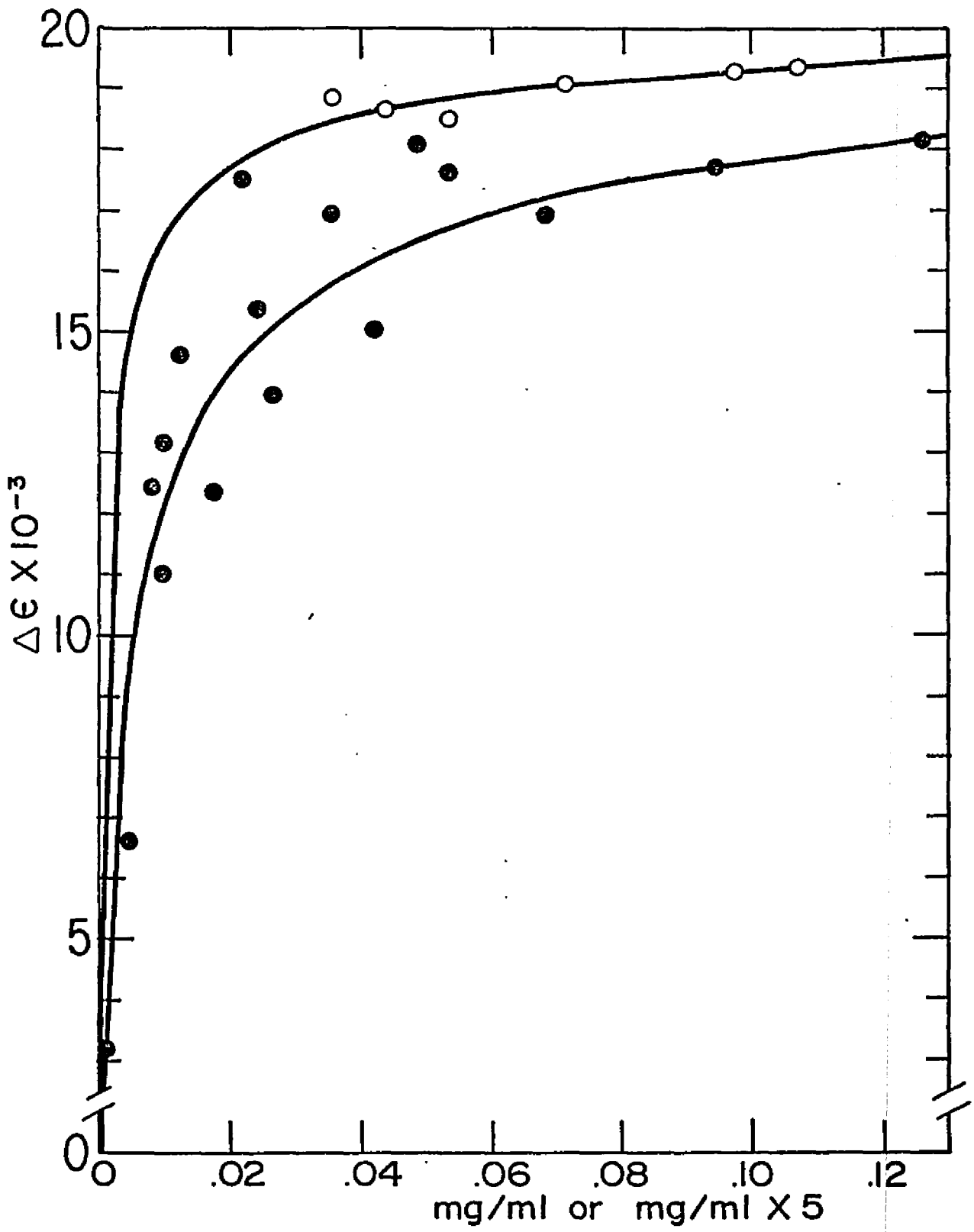


Figure 12. Concentration difference spectra data.

Figure 13. Concentration difference spectra data for human methemoglobin; theoretical curves given for $K_{12} = 550$, $K_{24} = 0.0$, $\Delta\epsilon_{12} = 10{,}000$.

Conditions and concentration range: Same as Figure 11.

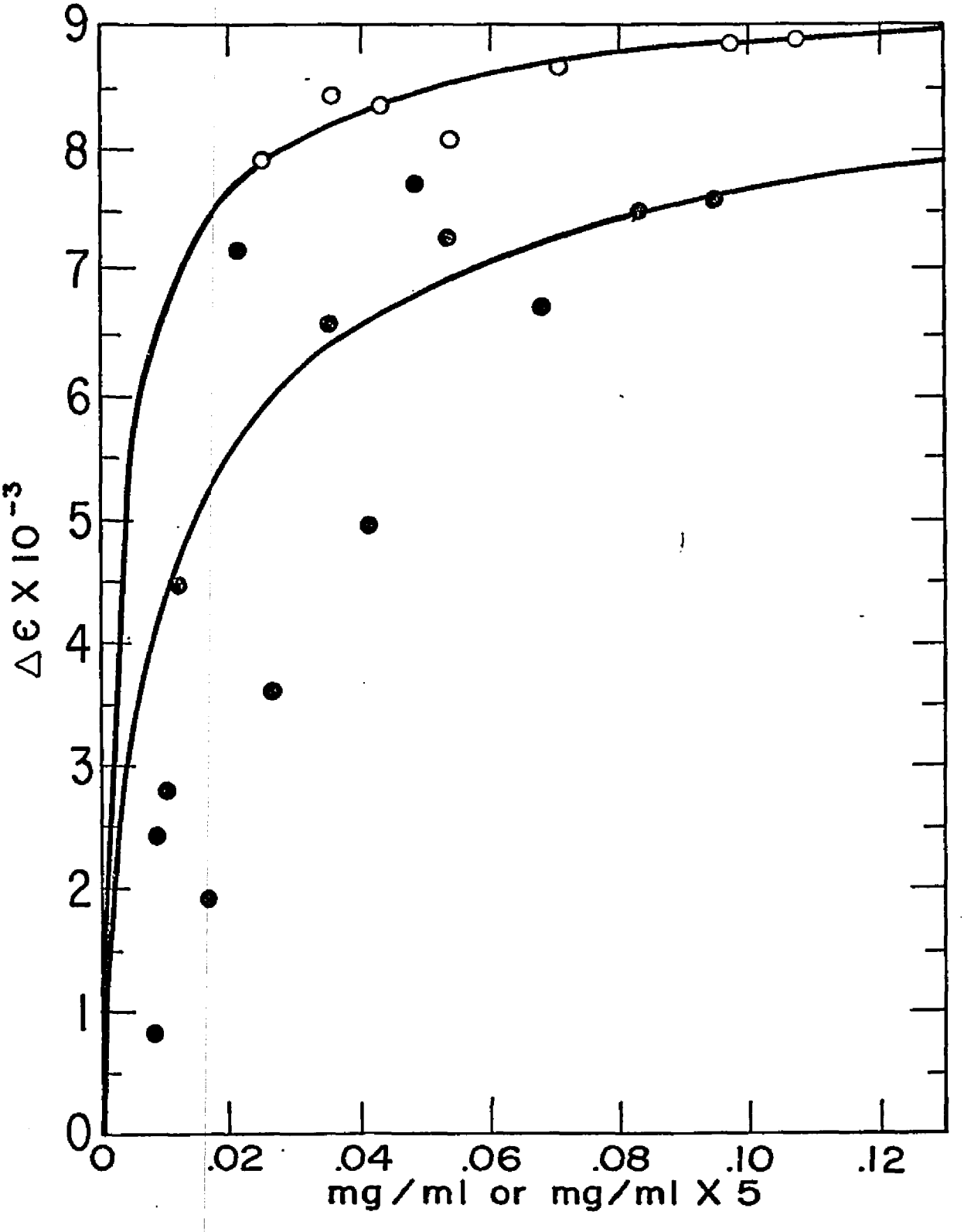


Figure 13. Concentration difference spectra data.

Figure 14. Concentration difference spectra data for human methemoglobin; theoretical curve given for $K_{12} = 2800$, $K_{24} = 29$, $\Delta\epsilon_{12} \approx 0.0, \Delta\epsilon_{24} = 20{,}000$.

Conditions: Same as Figure 11.

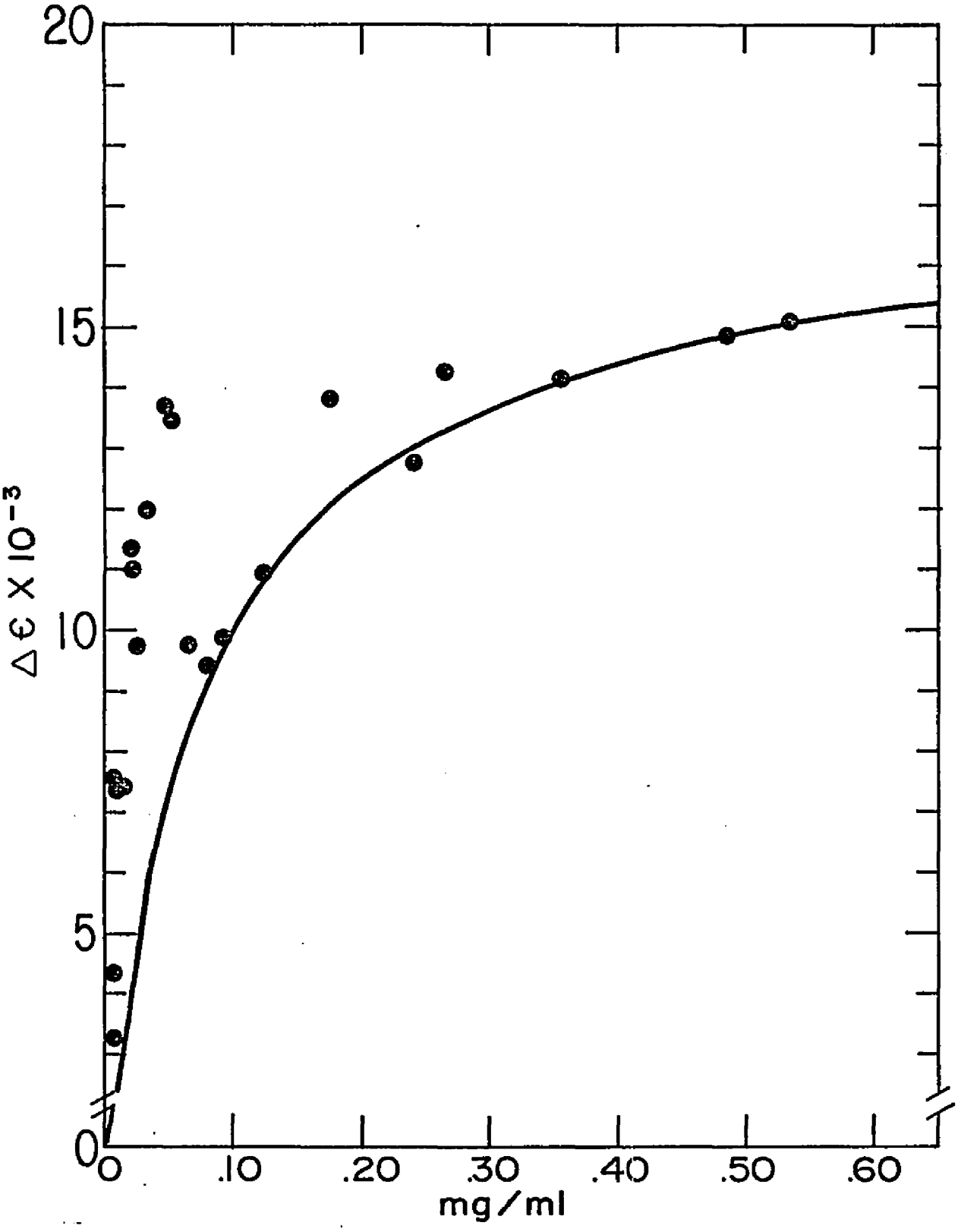


Figure 14. Concentration difference spectra data.

Figure 15. Concentration difference spectra data for human methemoglobin; theoretical curves given for K_{12} = 2800 and K_{24} = 29, i.e., Gilbert constants, $\Delta\epsilon_{12}$ = 20,000.

Condition and concentration range: Same as Figure 11.

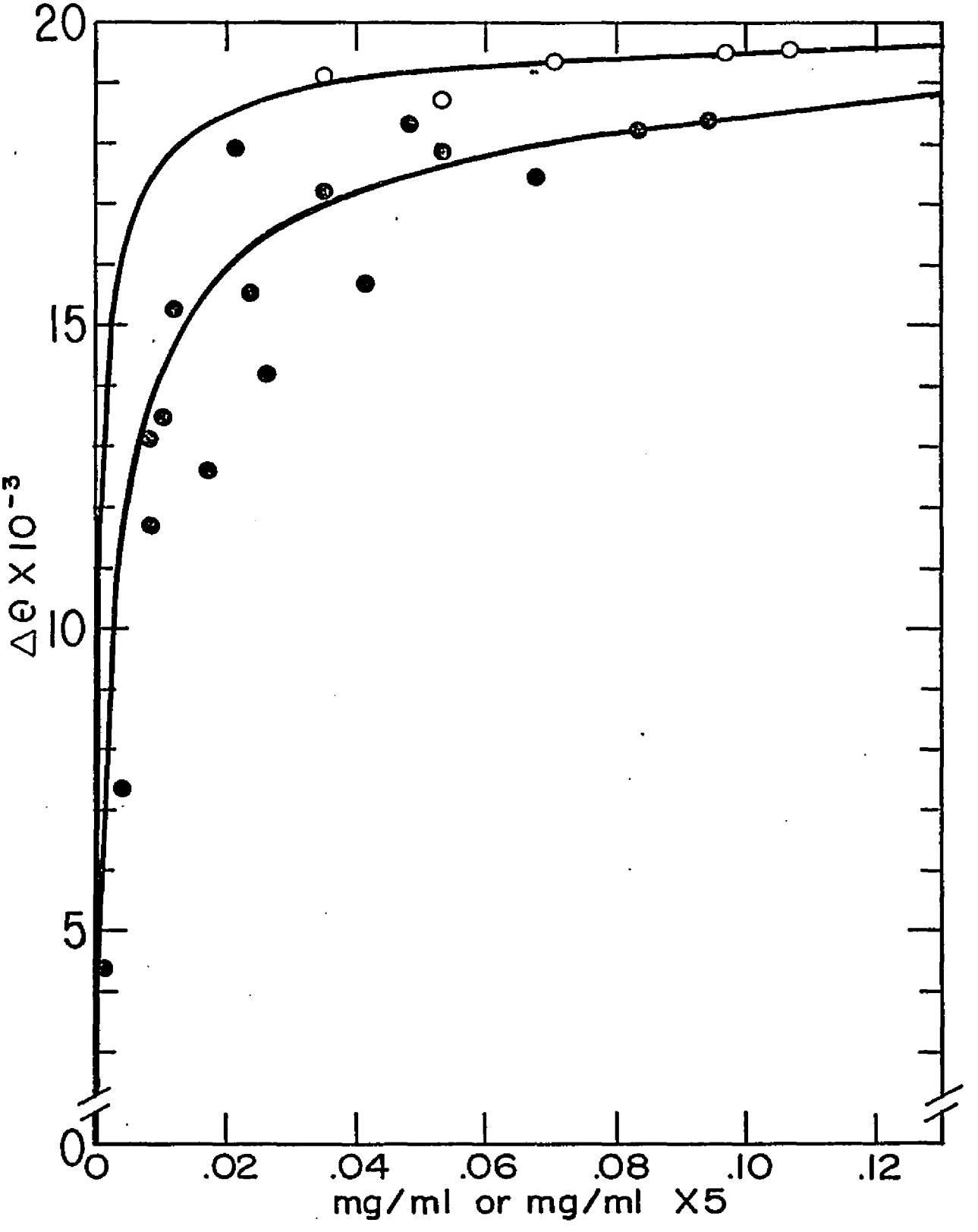


Figure 15. Concentration difference spectra data.

Figure 16. Concentration difference spectra data for horse methemoglobin, 0.1 M phosphate buffer, pH 7.0.

Theoretical curves given for $K_{12} = 2500$, $K_{24} = 0.0$, $\Delta\epsilon_{12} = 20{,}000$.
Upper curve: 0 - 0.6 mg/ml.
Lower curve: 0 - 0.12 mg/ml.

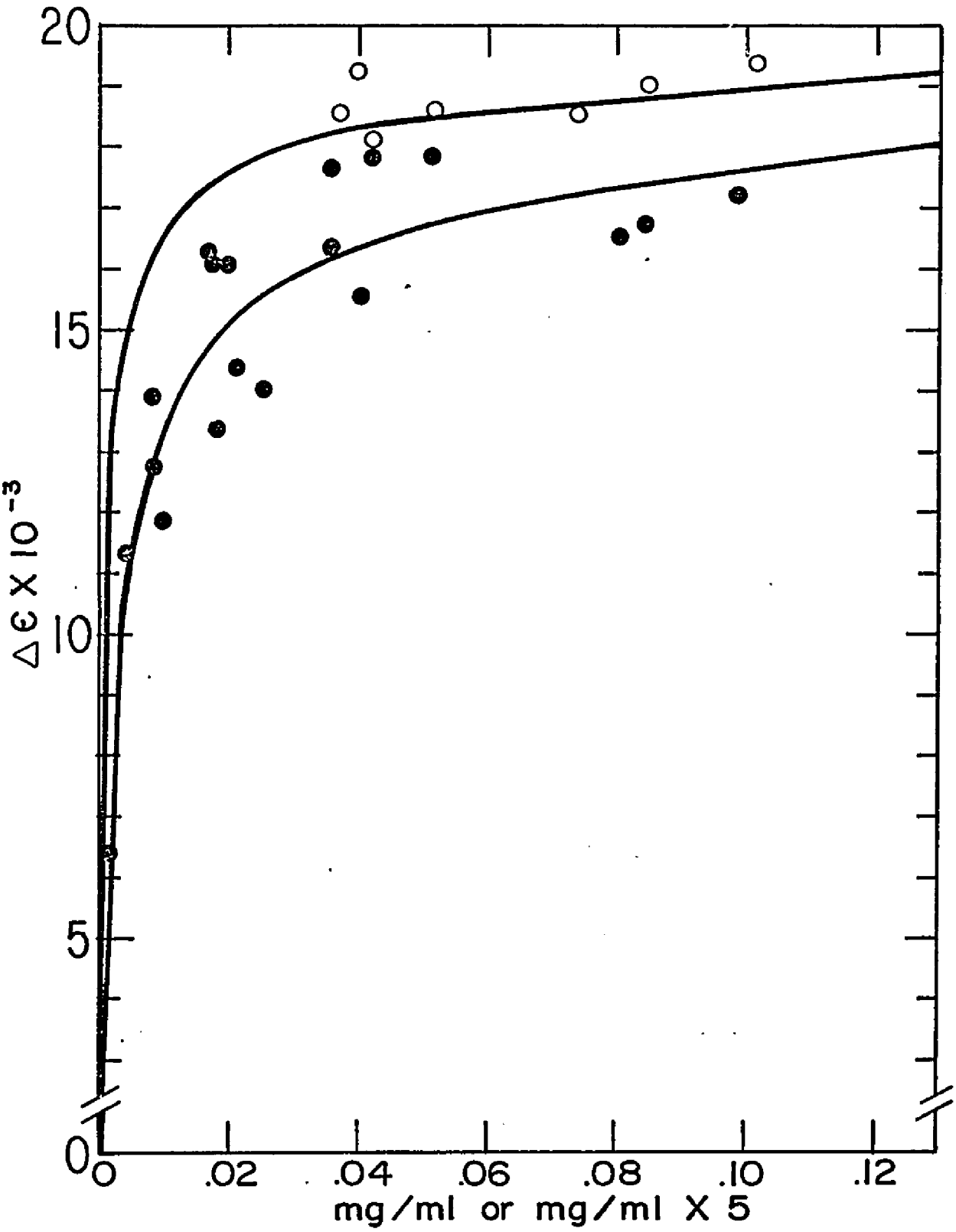


Figure 16. Concentration difference spectra data.

Figure 17. Concentration difference spectra data for guanidinated horse methemoglobin, 0.1 M phosphate buffer, pH 7.0.

Concentration range: Same as Figure 16.

Theoretical curves given for $K_{12} = 2500$, $K_{24} = 0.0$, $\Delta\epsilon_{12} = 20{,}000$, i.e., the parameters estimated for native horse methemoglobin.

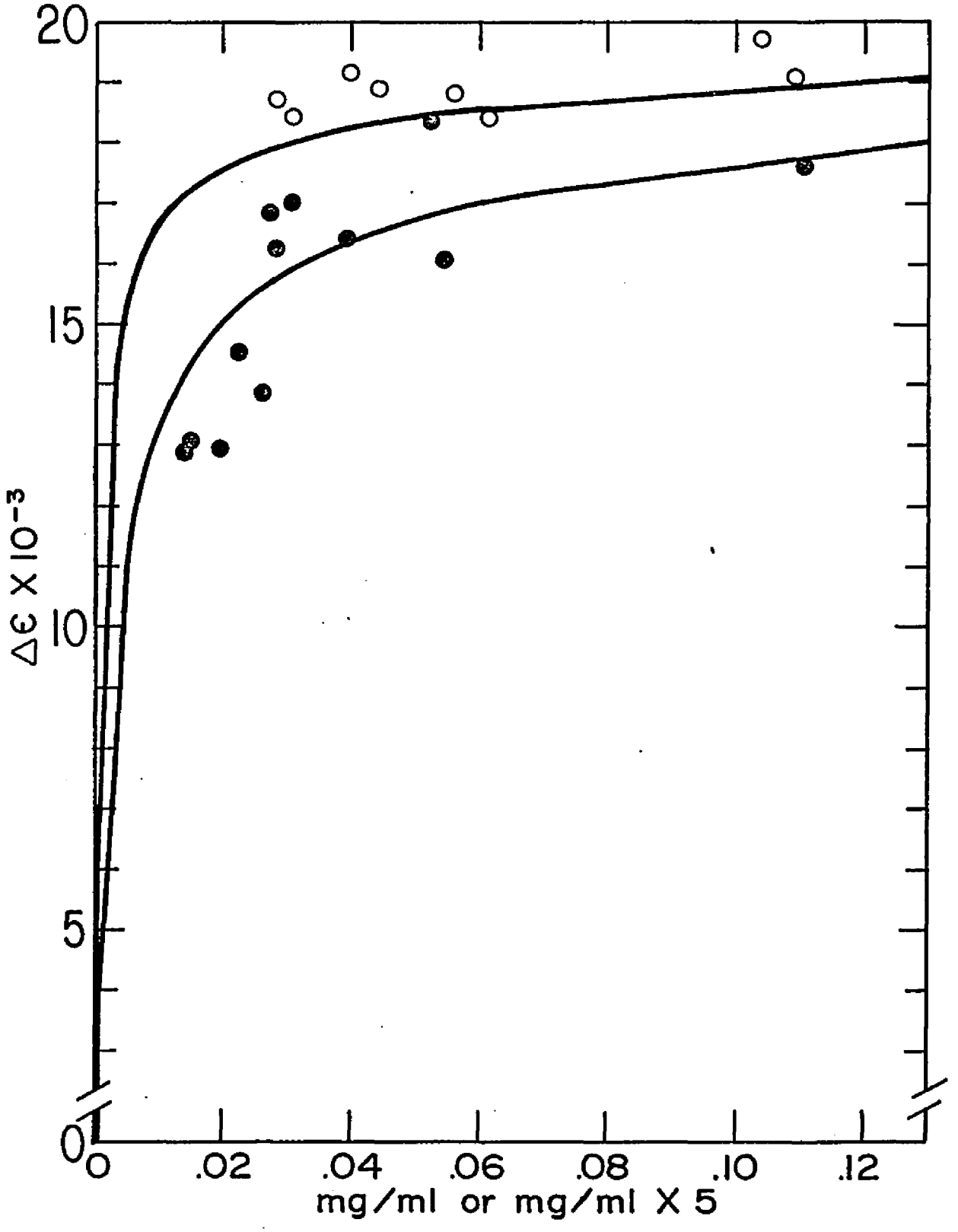


Figure 17. Concentration difference spectra data.

Figure 18. Concentration difference spectra data for guanidinated horse methemoglobin and native horse methemoglobin at pH 10.0.

Theoretical curves given for:

A. Native horse methemoglobin - $K_{12} = 250$, $K_{24} = 0.0$, $\Delta\epsilon_{12} = 20{,}000$.

B. Guanidinated horse methemoglobin - $K_{12} = 2400$, $K_{24} = 0.0$, $\Delta\epsilon_{12} = 20{,}000$.

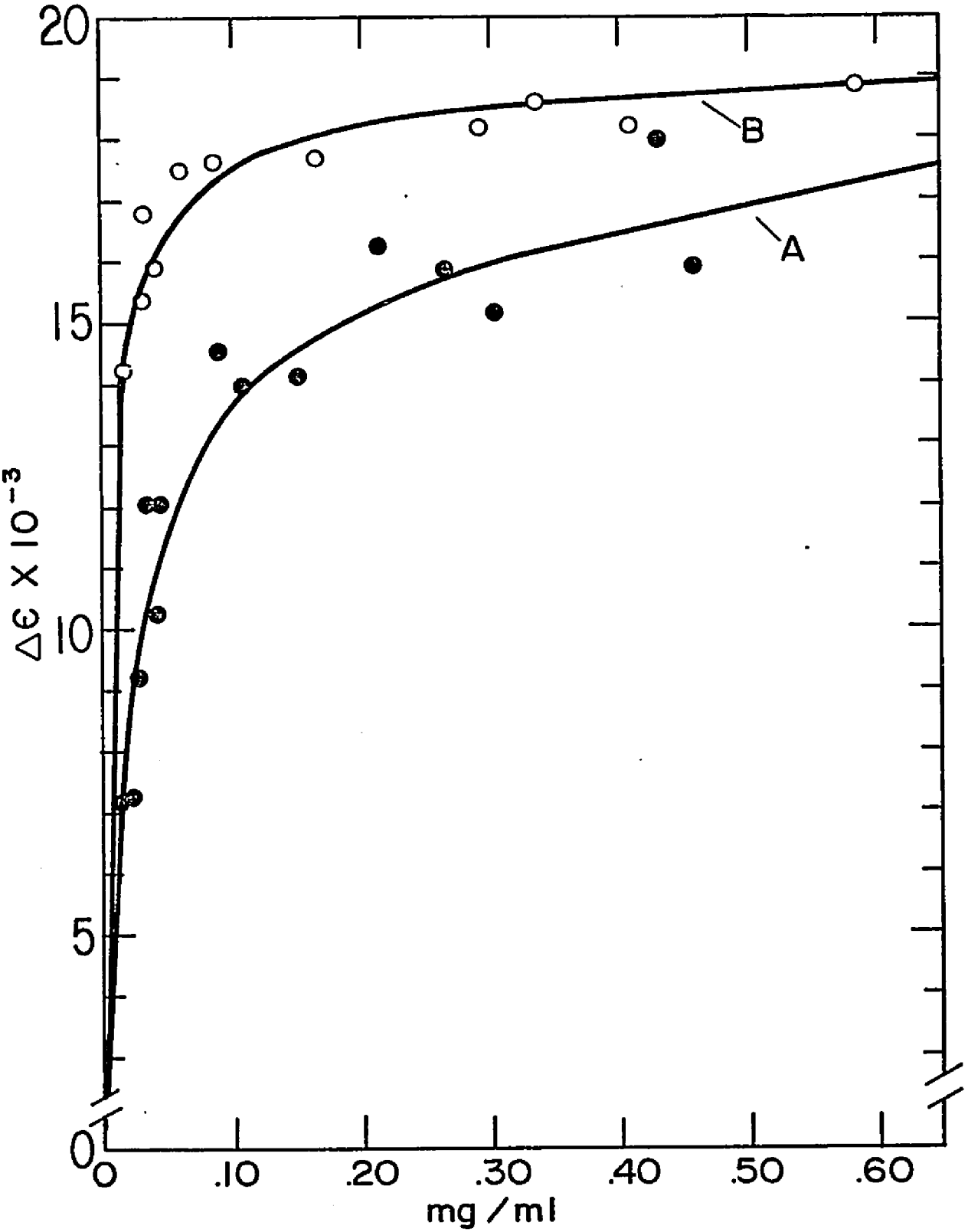


Figure 18. Concentration difference spectra data.

Figure 19. Concentration difference spectra data for horse methemoglobin in 1.0 M NaCl and 2.0 M NaCl, pH 7.0.

A. 2.0 M NaCl - $K_{12} = 600$, $K_{24} = 0.0$, $\Delta\epsilon_{12} = 20{,}000$.

B. 1.0 M NaCl - $K_{12} = 1050$, $K_{24} = 0.0$, $\Delta\epsilon_{12} = 20{,}000$.

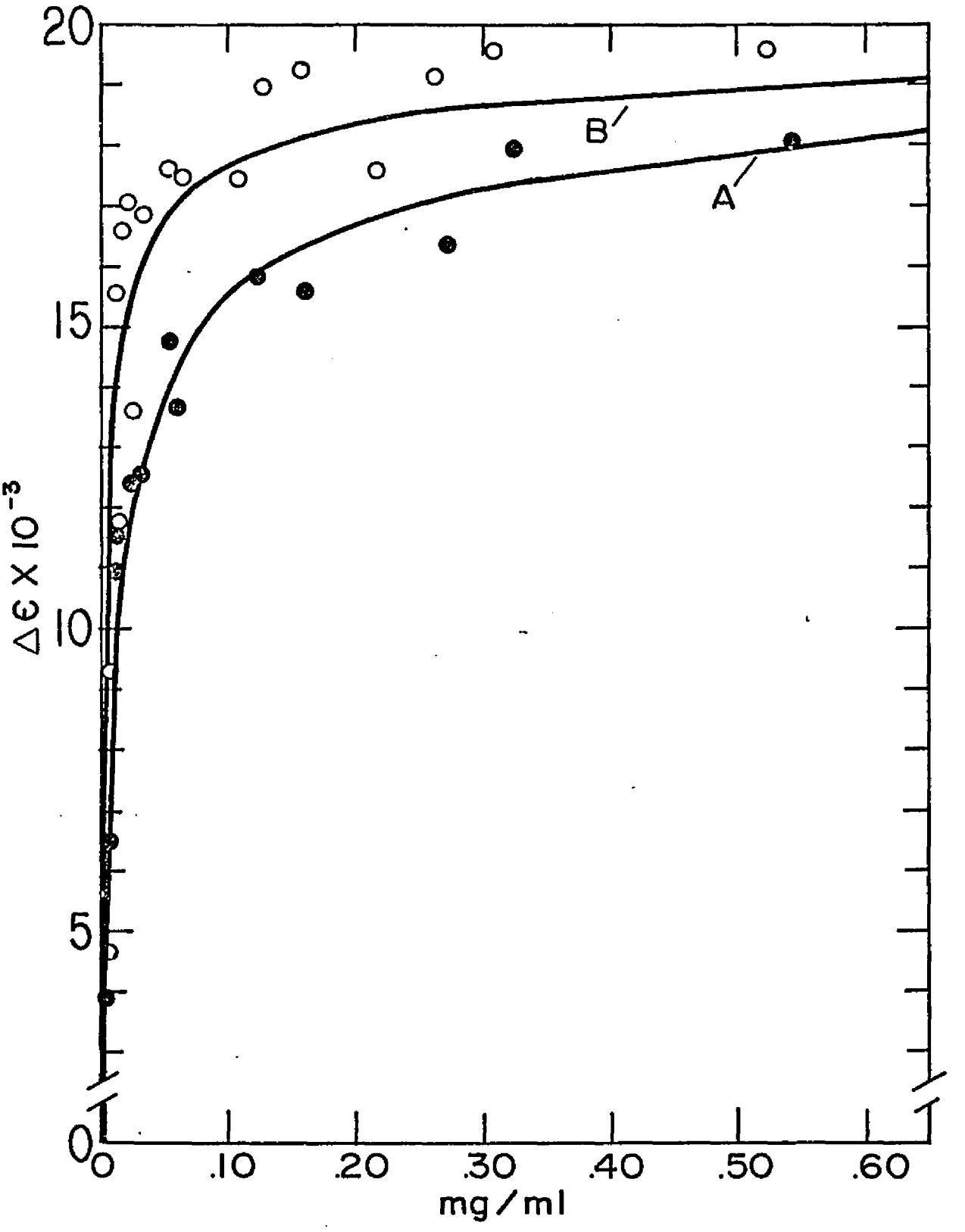


Figure 19. Concentration difference spectra data for horse methemoglobin in 1.0 M NaCl and 2.0 M NaCl, pH 7.0.

K_{12} and K_{24} fixed at the Gilbert values (columns E - G and Figure 15) showed good fit with the smaller K_{12}, expected since estimates K_{12} (columns A and B) were close to this number. Table 14 and Figure 16 shows that horse methemoglobin does not significantly differ in association behavior from the human protein. To summarize, K_{12} is 500-3000 1/gm, i.e., 10-100 times larger than K_{24}, and $\Delta\epsilon_{12}$ is probably near 20,000 and $\Delta\epsilon_{24}$ near zero.

Table 15 summarizes association properties of several hemoglobins under various conditions. In all trials K_{24} was fixed at zero, in accord with the conclusi n from Table 14 that a non-zero value did not significantly improve fits. Columns I and III give values of K_{12} for $\Delta\epsilon_{12}$ = 20,000 and 10,000, respectively. Column II for comparison gives the quality of fit with K_{12} set at the value for native horse methemoglobin at pH 7 and ionic strength, μ = 0.22. Guanidinated and native horse methemoglobin did not differ at pH 7 (compare also Figures 16 and 17). At pH 10, horse hemoglobin showed considerably smaller K_{12} than at pH 7, but the guanidinated protein behaved as at pH 7 (Figure 18). This accords with the sedimentation data of Table 7 and literature reports (Itano et al. 1964). Horse methemoglobin in 1 or 2 M NaCl showed slightly weaker association (K_{12}) than in dilute salt (Figure 19). Since these measurements reflect behavior at low protein concentrations, in particular the monomer-dimer equilibrium, it is not clear how they relate to literature reports of dissociation to the dimer in ca. 2 M NaCl (see Table

13). The small spectrum changes obtained with human cyanmethemoglobin allow only the conclusions that this derivative does not differ greatly from the met form.

DISCUSSION

Guanidination

Two approaches to chemical modification of proteins are: (1) reaction of one or a small number of residues and (2) complete modification of all groups of a given class. In the former case, changes in physical properties can be associated with the specific group; in the latter, the absence of a change rules out the participation of the type of group modified. Guanidination of proteins fit in the latter class in that extensive conversion of lysine to homoarginine has been observed for myoglobin (Banaszak et al. 1963), lysozyme (Geschwind and Li 1957), and ribonuclease (Klee and Richards 1957). Similarly, we have prepared modified hemoglobin in which all lysyl residues have been converted to homoarginyl residues with properties similar to native at neutral pH.

If lysyl groups were involved in subunit contact sites, and/or buried within the molecule, guanidination should be difficult and should disrupt the protein structure. This was not observed with hemoglobin. Perutz et al. (1968) suggest two possible lysyl interactions between like chains (see Table 3). We find guanidinated hemoglobin does not dissociate as does the native protein in the pH region of lysyl ionization. This accords with either specific participation of one or more ϵ-amino residues in the contact site or with a general electrostatic contribution to association. Apparently the function, if any, of the ϵ-amino groups can be carried out by the bulkier and more basic

guanido group. This suggests that any lysyl side chains within subunit contacts have considerable freedom, because replacement by guanido displaces the charge by more than one angstrom from the lysyl amino location.

Spectrum Change

Concentration difference spectra and recombination experiments show an increase in Soret extinction with association of methemoglobin subunits. The maximum change occurs at 407nm, near the absorption maximum (406nm). We estimate its value to be $\Delta\epsilon/\epsilon$ = 10,000-20,000/150,000. Association of dimer to tetramer produced no further change in spectrum.

Geraci and Li (1969) have reported changes in spectrum for human met and deoxyhemoglobin and other derivatives. Antonini et al. (1968) measured the difference spectrum between free and recombined deoxy chains, finding a value of $\Delta\epsilon/\epsilon$ = 15,000/127,000 similar to the value we report for methemoglobin. Chain recombination also produces changes in circular dichroism (Geraci and Li 1969); the effect of association is greater for deoxyhemoglobin than for oxy, met, and cyanmet forms. In every case, the molar ellipticities and areas under the circular dichroism bands of the β chains are smaller than those for the α chains. Thus, it is likely that, for all hemoglobin forms, chain combination produces spectrum changes. For met and cyanmethemoglobin these changes come through dimer formation. Considering the strong (ΔF° ca. -10 kcal) and presumably highly specific association of monomer to dimer, the spectrum changes for other derivatives probably develop similarly.

Perutz et al. (1968) suggest that the tetramer is the fundamental unit of hemoglobin. This conclusion is based upon an analysis of the contacts seen in crystallographic data. The structure about the heme appears more closely linked to the $\alpha_1\beta_2$ than the $\alpha_1\beta_1$ contacts; i.e., dissociation of the tetramer to dimer would be expected to change heme environment. Because a change in environment and chemistry of the heme should be reflected in its spectrum, our data do not entirely support the above suggestion. Since formation of the dimer produces the spectrum change, the dimer can be described as the fundamental structural unit. It must be emphasized that association of dimer to tetramer can affect ligand binding without producing additional changes in subunit structure. This conclusion follows since the liganded and unliganded dimers have different structures. Although association to the dimer likely affects ligand binding and develops some cooperativity, only the tetramer exhibits the full functional properties of hemoglobin.

Association Constants

The formation constants (K_{12}) for human and horse methemoglobin dimers (ca. 1200 to 2500 $l \cdot gm^{-1}$) obtained from concentration difference spectra agree with literature results for human oxyhemoglobin (Chiancone et al. 1968, Edelstein 1967). This is expected since crystals of met and oxy forms are isomorphous, with presumably similar subunit contact sites. Estimates of K_{24} (Table 13) show that it is 100 times smaller than K_{12}; thus dissociation occurs in two distinguishable steps.

The concentration for half-dissociation of methemoglobin is 1-10 μgm/ml. A number of problems arose in connection with handling of

dilute proteins. We attribute these difficulties to surface effects. Corrections (myoglobin blanks) were applied to the data, which introduced ca. 50% uncertainty in $\Delta\epsilon_{12}$ and consequently in K_{12} calculated from the spectrum changes. In accord, the standard deviation in $\Delta\epsilon$ of 1000-2000 is larger than the 450-900 expected from considering only other sources of experimental error. Surface effects are likely to be of general importance in a study of strongly associating systems, and handling of dilute proteins must be kept to a minimum.

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