

Subunit Dissociation of Certain Abnormal Human Hemoglobins

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ABSTRACT The extent of dissociation of various hemoglobins into subunits was estimated from their elution volumes (V_e) on G-100 Sephadex. Under the same controlled conditions carboxyhemoglobins A, A3 (A_1), F, S, and C all had the same elution volumes. The carboxy and cyanmet derivatives of hemoglobin Kansas (a variant with very low oxygen affinity) had a relatively high V_e , indicating a decreased mean molecular weight and therefore an increased tendency to form dimers and even monomers. Conversely, the liganded derivatives of hemoglobin Chesapeake (a variant with high oxygen affinity) had a relatively low V_e , suggestive of an impaired degree of subunit dissociation. Deoxyhemoglobin Chesapeake had a V_e identical with that of deoxyhemoglobin A. Cat hemoglobin, known to have an unusually low oxygen affinity, was found to have a higher V_e than human, dog, rabbit, rat, or guinea pig hemoglobins.

Haptoglobin is thought to bind $\alpha\beta$ dimers in preference to the $\alpha_2\beta_2$ -tetramer. The comparative haptoglobin affinities of the human hemoglobins were measured by competition between the test hemoglobin and radioactive reference hemoglobin for haptoglobin binding sites. Hemoglobins A, F, S, and C all seemed to bind equally readily, but hemoglobin Kansas and cat hemoglobin showed a higher affinity, and hemoglobin Chesapeake a lower affinity.

These results are in accord with recently proposed models which predict that hemoglobins which have an increased degree of subunit dissociation will have a low oxygen affinity, and vice versa.

INTRODUCTION

Most human hemoglobin variants generally have a single amino acid substitution in either the α - or β -polypeptide chains. Such a structural alteration will com-

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monly confer a difference in net surface charge allowing the abnormal protein to be detected and isolated by electrophoretic and chromatographic methods. Many of these hemoglobins have no unusual chemical or physical properties and appear to be functionally normal. A smaller number of human hemoglobin variants are associated with clinical features which lead to their detection. These hemoglobins almost always have unusual properties which are readily demonstrable in vitro.

A few human hemoglobin variants are characterized by marked abnormalities in oxygen affinity (Table I). A mother and son with cyanosis and a normal hematocrit were found by Reissman, Ruth, and Nomura to be heterozygous for a hemoglobin variant, designated as Kansas (1). The cyanosis was due to the low oxygen affinity of the whole blood. Recently Bonaventura and Riggs have determined the structure of isolated hemoglobin Kansas and have confirmed that this hemoglobin has a markedly reduced oxygen affinity and low heme-heme interaction, but a normal Bohr effect (2). Five hemoglobin variants: Chesapeake (3), Yakima (4, 5), Kempsey (6), and Rainier (7) and Hiroshima (8) have now been found to be associated with familial erythrocytosis (Table I). In each instance the affected individuals have been heterozygous for a hemoglobin with a markedly *high* oxygen affinity. In addition, hemolysates containing hemoglobin J-Cape Town have been found to have high oxygen affinity (9). However affected heterozygotes do not have erythrocytosis.

The way in which hemoglobin reversibly binds oxygen has eluded precise understanding. Most puzzling has been the mechanism by which the oxygen affinity increases upon the stepwise addition of ligand: so called heme-heme interaction. The conformation of hemoglobin is known to be altered considerably upon oxygenation (10). Furthermore, ligand binding enhances the tendency of the tetramer to dissociate symmetrically into dimers: $\alpha_2\beta_2 \rightleftharpoons 2\alpha\beta$ (11, 12). These experimental observations have formed the basis of a model proposed

TABLE I
Hemoglobin Variants Associated with
Abnormal Oxygen Affinity

Hemoglobin variant		Clinical features in heterozygote	Oxygen affinity	Heme- heme inter- action
Kansas	$\alpha_2\beta_2^{102}$ Thr	Cyanosis Normal hct	Low	Low
Chesapeake	α_2^{92} Leu β_2	Erythro- cytosis	High	Low
Yakima	$\alpha_2\beta_2^{99}$ His			
Kempsey	$\alpha_2\beta_2^{99}$ Asn			
Rainier	$\alpha_2\beta_2^{145}$ His			
Hiroshima	$\alpha_2\beta_2^{145}$ Asp			
J-Cape Town	α_2^{92} Gln β_2	Normal hct	High	?

Thr, threonine; Leu, leucine; His, histidine; Asn, asparagine; Asp, aspartic acid; Gln, glutamine.

by Benesch, Benesch, and MacDuff for the reaction of ligands with hemoglobin which postulates that the $\alpha\beta$ dimer is the primary functioning unit (13). Stepwise oxygenation is thought to involve rapid exchange between oxygenated and deoxygenated dimers. It is of great interest to examine those hemoglobins with abnormal ligand affinity in the light of this model. It predicts that if a hemoglobin variant dissociates more readily than normal from a liganded tetramer to dimer it will have a low oxygen affinity, and vice versa. It is noteworthy that the amino acid substitution in all of the hemoglobins shown in Table I except Rainier and Hiroshima is in the nonhelical FG segment, or adjacent G helix, regions which are in the interface between adjoining $\alpha\beta$ -dimers (14). The substitution in hemoglobins Rainier and Hiroshima is in the H helix which is contiguous with the FG segment. A structural alteration in these areas might well effect a marked change in the equilibrium between dimer and tetramer.

In these experiments the extent of subunit dissociation of various normal and abnormal human and animal hemoglobins was studied by measuring their relative elution volumes on G-100 Sephadex, a cross-linked dextran which serves as a molecular sieve. In addition, the relative haptoglobin binding of these hemoglobins was tested.

METHODS

Preparation of hemoglobin samples. Blood specimens were collected in acid citrate dextrose (ACD). Hemolysates were prepared from blood specimens as previously described (15), and unless stated otherwise, were gassed with carbon monoxide and stored at 4°C. Hemolysates were dialysed against the appropriate eluting buffer before hemoglobin purification by column chromatography. Isolated hemoglobins were concentrated by ultrafiltration and again gassed with carbon monox-

ide. Blood specimens from an individual heterozygous for hemoglobin Chesapeake were mailed by Air Express. Hemoglobin Chesapeake was separated from hemoglobin A by chromatography on carboxymethylcellulose as described by Charache, Weatherall, and Clegg (3). Experiments on hemoglobin Chesapeake were performed within 1 wk after blood samples were obtained. Hemoglobins Kansas and A were isolated from the hemolysate of a heterozygote by chromatography on carboxymethylcellulose by Drs. Joseph Bonaventura and Austen Riggs (2) and air-shipped in the carboxy form packed in ice. In like manner hemoglobins Yakima and A from a heterozygote were isolated by chromatography on diethylaminoethyl (DEAE)-Sephadex by Dr. Demetrios Rigas (4) and air-shipped. Hemoglobin F was isolated from a fresh sample of cord blood on Amberlite (CG-50) by the method of Allen, Schroeder, and Balog with the use of developer 2 (16). With this procedure hemoglobin A3 (A₁)¹ was isolated from a hemolysate of a normal adult. Hemoglobins C and S were isolated from the blood of a patient with hemoglobin SC disease by chromatography on carboxymethylcellulose (17). Hemoglobin S from the hemolysate of a homozygote was also tested without further purification.

In these experiments hemoglobins-⁵⁹Fe were used as markers. Rabbit hemoglobin was labeled as previously described (18). Human hemoglobin A-⁵⁹Fe was prepared by the exchange of heme between unlabeled ferrihemoglobin A and ⁵⁹Fe-labeled methemalbumin. This method, described in detail elsewhere (15), allowed much higher specific activity than is possible by the incubation of human reticulocytes with ⁵⁹Fe. In brief hemin-⁵⁹Fe was crystallized from rabbit hemoglobin-⁵⁹Fe and was then bound to human serum albumin.² A mixture containing methemalbumin-⁵⁹Fe and a heme equivalent amount of unlabeled ferrihemoglobin A was incubated for 100 min at 37°C and then dialyzed against developer 2 at 4°C. Under the experimental conditions employed there was no net loss of heme from the hemoglobin. The labeled cyanmethemoglobin A was separated from the methemalbumin-⁵⁹Fe on Amberlite CG-50 (16).

Elution volumes of hemoglobins on G-100 Sephadex. A column of G-100 Sephadex³ measuring 95.5 × 2.4 cm was used in all these experiments. When oxy- or carboxy-hemoglobins were studied the column was equilibrated with a solution consisting of two parts isotonic NaCl and one part isotonic phosphate buffer, pH 7.4 (buffered saline). During the chromatograms of carboxyhemoglobin, it was not practical to keep the buffer saturated with carbon monoxide, so that some of the ligand may have been lost or replaced by oxygen. When ferrihemoglobin cyanide was tested, the buffer contained cyanide (20 mmoles/liter). When deoxyhemoglobin was applied to the column the buffer contained sodium dithionite (1.5 mmoles/liter), and 100% nitrogen was bubbled slowly into the buffer in the supply bottle to exclude as much oxygen as possible from the system. Most of the experiments were done at room temperature. Before running the column at 4°C, it was repacked to its original dimensions in the cold. Samples of 1.5 ml were applied to the column and unless stated otherwise consisted of 8 mg/ml of hemoglobin in buffered saline. In some experiments human albumin labeled with ¹²⁵I⁴ served as a

¹ A3 or A₁ hemoglobin refers to the minor fractions which are more electronegative than hemoglobin A and comprise about 8% of the adult human hemolysate.

² Nutritional Biochemicals Corporation, Cleveland, Ohio.

³ Pharmacia Fine Chemicals Inc., Piscataway, N. J.

⁴ Squibb Radio-iodinated (¹²⁵I) Human Serum Albumin USP.

marker. 1.5 mg of albumin-¹²⁵I, having an activity of 0.1–0.5 μ c, was mixed with the hemoglobin solution immediately before application. A flow rate of 20–30 ml/hr was maintained. Fractions of equal volume (3.62 ml) were obtained by a Gilson linear fractionator (Model VL). The hemoglobin concentration of successive fractions was obtained from the absorbance of an appropriate dilution in Drabkins solution, measured at 540 $m\mu$ or, with more dilute solutions, at 415 $m\mu$. Radioactivity of an aliquot of successive fractions was measured in a well scintillation counter.

The relative Sephadex mobility of a given substance can be expressed by the constant K_{av} which is independent of the column dimensions and the extent of packing (19).

$$K_{av} = \frac{V_e - V_0}{V_t - V_0}$$

The elution volume V_e of a substance was determined by the volume of effluent between its point of application and its peak concentration during elution. The void volume V_0 was determined by the elution volume of Dextran Blue,³ a high molecular weight polymer which is entirely excluded by the gel matrix and therefore has maximal mobility. V_t was the total bed volume of the column.

The determination of V_e for hemoglobin was subject to experimental error such as slight variability in the initiation of collection and in the fraction volumes. This uncertainty could be minimized by the use of albumin-¹²⁵I as a marker. Chromatography of a mixture of hemoglobin and albumin-¹²⁵I allowed sharp resolution of the two components despite some degree of overlap since their respective concentrations were measured by different means. In the data reported in Table II and Fig. 1, mixtures containing the test hemoglobin and a small amount of albumin-¹²⁵I were chromatographed. The V_e of hemoglobin was corrected for slight variations in the V_e of the albumin-¹²⁵I.

The resolution of hemoglobins on G-100 Sephadex was greatly increased by chromatographing a mixture containing equal amounts of the test hemoglobin and a ⁵⁹Fe-labeled reference hemoglobin. In these experiments the applied

samples contained a total of 8 mg/ml of hemoglobin in either the carboxy or cyanmet form and were chromatographed at 25°C, since under these conditions subunit dissociation appeared to be enhanced (see Results). The specific activity of a given fraction SA_f (cpm/mg) was calculated from its radioactivity A_f (cpm/ml) and its hemoglobin concentration C_f (mg/ml).

$$SA_f = \frac{A_f}{C_f}$$

The concentration of the labeled reference hemoglobin C_r and that of the unlabeled test hemoglobin C_t in each fraction was calculated as follows:

$$C_r = \frac{A_f}{SA_0}$$

where SA_0 is the specific activity of the original labeled hemoglobin.

$$C_t = C_f - C_r$$

In these and other experiments, the hemoglobin A isolated from the same hemolysate as the abnormal hemoglobin was always tested for direct comparison.

Measurement of haptoglobin binding. The relative binding affinities of various human hemoglobins to haptoglobin were tested by a competition approach described in detail elsewhere (18). In brief, a mixture of equal amounts of the carboxy derivatives of unlabeled test hemoglobin and rabbit hemoglobin-⁵⁹Fe was added in excess to serum of known haptoglobin phenotype. The bound hemoglobin was then separated with G-100 Sephadex. The binding of the test hemoglobin relative to the reference labeled hemoglobin was calculated as follows:

$$\% \text{ Hp bound by test Hb} = 100 - \frac{50 SA_{HbHP}}{SA_{\text{mixture}}}$$

Miscellaneous procedures. Carboxyhemoglobin was converted into oxyhemoglobin as recommended by Riggs and

TABLE II
Elution Data for Hemoglobins and Other Substances on G-100 Sephadex

Substance	Number of determinations	Concentrations of		$\frac{V_{e,alb}}{V_0}$	K_{av}
		applied sample	Temperature		
		mg/ml	°C		
Dextran blue	2	1.0	25	1.42	0.000
Human albumin	12	1.0	25	(1.00)	0.224
Oxyhemoglobin A	1	1.6	25	0.869	0.359
Oxyhemoglobin A	4	8.0	25	0.873 (0.870–0.876)*	0.353
Oxyhemoglobin A	1	100	25	0.890	0.339
Carboxyhemoglobin A	1	8.0	25	0.874	0.352
Cyanmethemoglobin A	1	8.0	25	0.874	0.353
Deoxyhemoglobin A	3	8.0	25	0.888 (0.884–0.891)*	0.338
Cyanmethemoglobin Chesapeake	1	8.0	25	0.882	0.349
Cyanmethemoglobin Kansas	1	8.0	25	0.80†	0.436
Dextran blue	2	1.0	4	1.43	0.000
Human albumin	4	1.0	4	(1.00)	0.225
Oxyhemoglobin A	3	8.0	4	0.885 (0.883–0.887)*	0.348
Oxyhemoglobin A	1	100	4	0.891	0.338

* Mean and range.

† Estimated from data in Fig. 2.

Herner (20). Cyanmethemoglobin was prepared from oxyhemoglobin as previously described (15). Methemoglobin was determined by the method of Evelyn and Malloy (21). Alkali denaturation was tested by the procedure devised by Huisman and Meyering (17). One part of 12 N NaOH was added to 100 parts of oxyhemoglobin dissolved in 0.01 M phosphate buffer, pH 6.75. The final pH of this mixture was 11.7. Under these conditions the rate of denaturation of hemoglobin A was slow enough (half-time 3 min) to detect subtle changes in other hemoglobins with which hemoglobin A was compared. Auto-oxidation of oxyhemoglobin Chesapeake and A (3 mg/ml) was measured at 37°C in 0.01 M phosphate buffers at pH 6.6 and 7.4.

RESULTS

Experiments were first done to confirm that the K_{av} of hemoglobin on G-100 Sephadex was useful as an indicator of the extent of subunit dissociation. Conditions which enhance the subunit dissociation of hemoglobin will result in a lower mean statistical molecular weight and therefore an increase in K_{av} . Conversely, a decreased K_{av} would be expected in any hemoglobin which has an impaired degree of dissociation. Human albumin was found to have a considerably lower elution volume (and therefore a lower K_{av}) than all the hemoglobins tested (Table II), in agreement with the findings of Andrews (22). Only part of this difference could be due to the slight difference in molecular weight between human albumin and hemoglobin (69,000 vs. 64,500). The elution volume of hemoglobin would be further increased

by the extent to which it dissociates into dimers during the chromatography. Oxyhemoglobin, cyanmethemoglobin, and carboxyhemoglobin all had very similar K_{av} s (Table II). Guidotti, using osmotic pressure measurements, found that the first two forms had identical degrees of subunit dissociation, but that of carboxyhemoglobin was somewhat less (12). In contrast, the elution volume of deoxyhemoglobin was consistently less than that of the liganded forms (Fig. 1, Table II). It was not technically possible to run the column under the strictly anaerobic conditions necessary to maintain hemoglobin in its deoxygenated form. Therefore the deoxyhemoglobin was chromatographed in the presence of dithionite. There is evidence that this reducing agent may cause certain denaturative effects which could alter its physical and chemical properties (23). Nevertheless, the relatively low K_{av} of deoxyhemoglobin suggests a lower degree of subunit dissociation in agreement with the gel filtration data of Merrett (24), the sedimentation data of Benesch and associates (11), and the osmotic pressure measurements of Guidotti (12). Elution volumes of oxyhemoglobin were measured over a wide concentration range. Mass action law applied to self-associating systems would dictate that dilute solutions have a greater degree of subunit dissociation than concentrated ones. The K_{av} of oxyhemoglobin was found to vary inversely with its concentration (Table II) in

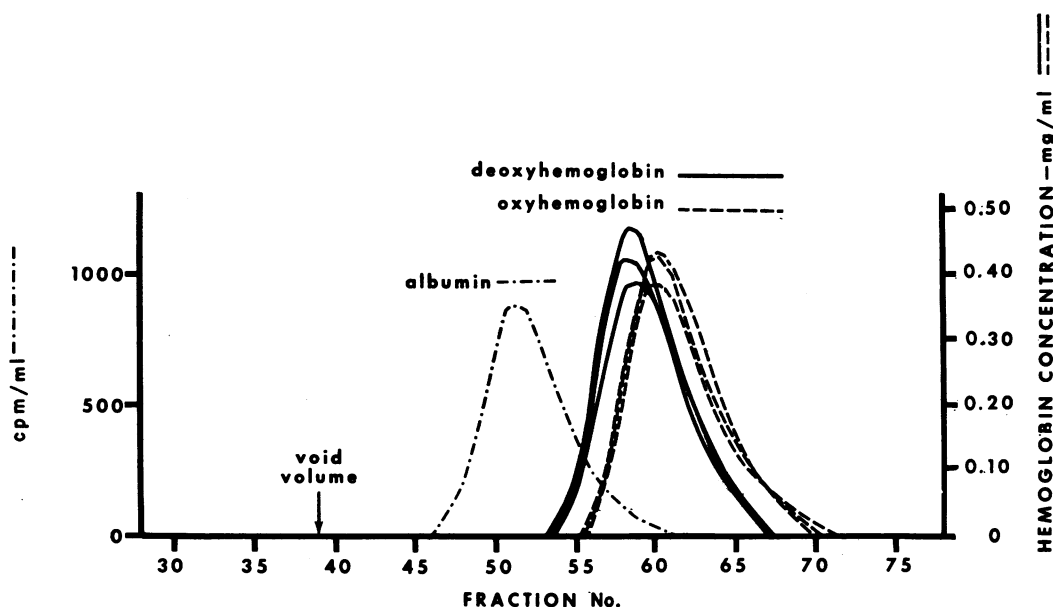


FIGURE 1 Elution profiles of oxyhemoglobin A, deoxyhemoglobin A, and human serum albumin on G-100 Sephadex. Chromatograms of oxyhemoglobin (---) and deoxyhemoglobin (—) were each run in triplicate. The resolution of these hemoglobins was enhanced by chromatographing a mixture containing the hemoglobin and a small amount of human albumin- 251 I. The elution profile of the albumin (-·-·-) was determined from radioactivity of successive fractions.

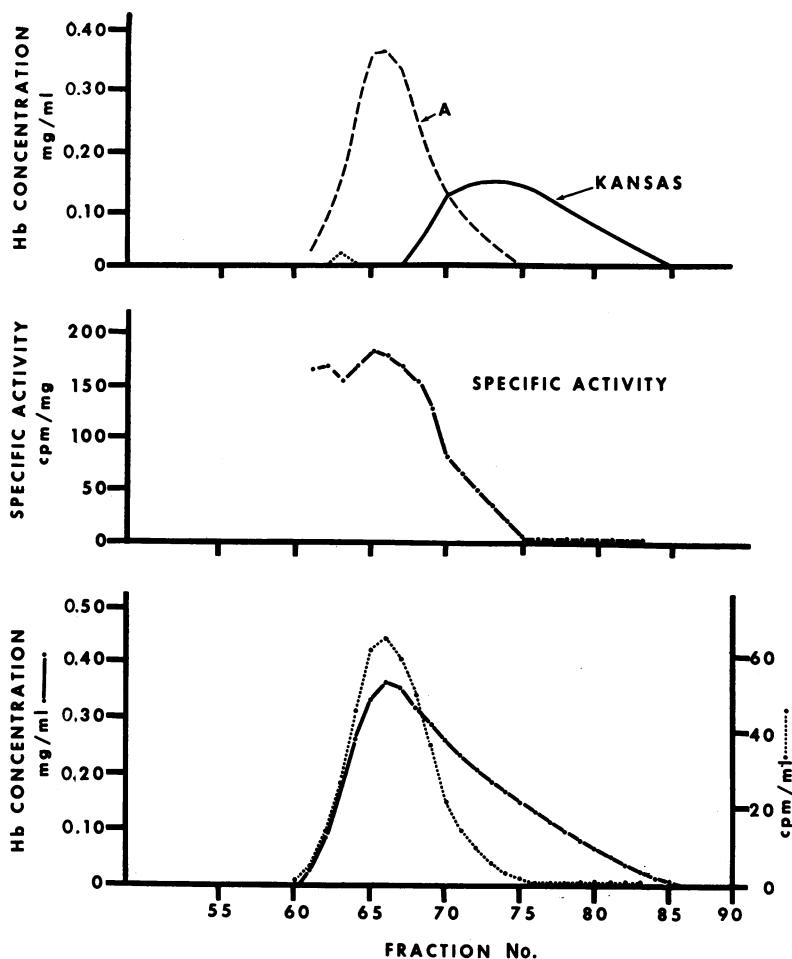


FIGURE 2 Elution profile of cyanmethemoglobin Kansas and cyanmethemoglobin A-⁵⁹Fe. A mixture containing equal amounts of the two hemoglobins was run on the G-100 Sephadex column. From the optical density and radioactivity measurements of successive fractions (lower panel), the specific activities of these fractions were calculated (middle panel). The top panel shows the resolution of the two hemoglobins, as calculated from the experimental data (see Results). The small peak (.....) under the hemoglobin A peak (---) represents unlabeled hemoglobin of unknown identity and questionable significance.

agreement with the data of Andrews (22) and Guidotti (25). Concentration dependence of hemoglobin dissociation has also been found from osmotic pressure measurements (12) and in recent sedimentation data (26). The elution volumes of oxyhemoglobin solutions were greater at 25° than 4°C. This change is unlikely to be due to a significant alteration in the distribution of water inside and outside the gel matrix, since the mobility of albumin was unaffected by the temperature change (Table II). Obrink, Laurent, and Rigler have shown that the K_{av} of Ficoll fractions on G-200 Sephadex varied only slightly over a wide temperature range (9°–60°C) (27). Our results suggest that the subunit dissociation equilibrium of oxyhemoglobin increases slightly with temperature. Benesch and associates have some indirect experimental evidence supporting this conclusion (13). However Kirschner and Tanford found that the sedimentation of human carboxyhemoglobin was independent of temperature (28).

The elution volumes of various abnormal human hemoglobins were compared. Chromatography of a mixture

of labeled reference hemoglobin allowed much greater resolution than was possible by measurement of the K_{av} of the test hemoglobin alone. If the test hemoglobin has the same degree of subunit dissociation as the reference hemoglobin, then they should have identical elution volumes on G-100 Sephadex, and the specific activities of successive fractions should be identical. This was found to be true for carboxyhemoglobins A3, F, S, and C. The ion-exchange capacity of G-100 Sephadex appeared to be insignificant since these hemoglobins have widely different surface charges and yet had elution volumes identical with carboxyhemoglobin A-⁵⁹Fe, and therefore to each other. When carboxyhemoglobin Kansas was tested, a marked fall in specific activity of successive fractions indicated that the unlabeled test hemoglobin had a greater elution volume than the reference hemoglobin, and therefore a lower mean statistical molecular weight indicative of an increased degree of subunit dissociation. Identical results were obtained when the cyanmet derivatives of hemoglobin Kansas and A-⁵⁹Fe were tested (Fig. 2). The K_{av} of Kansas was

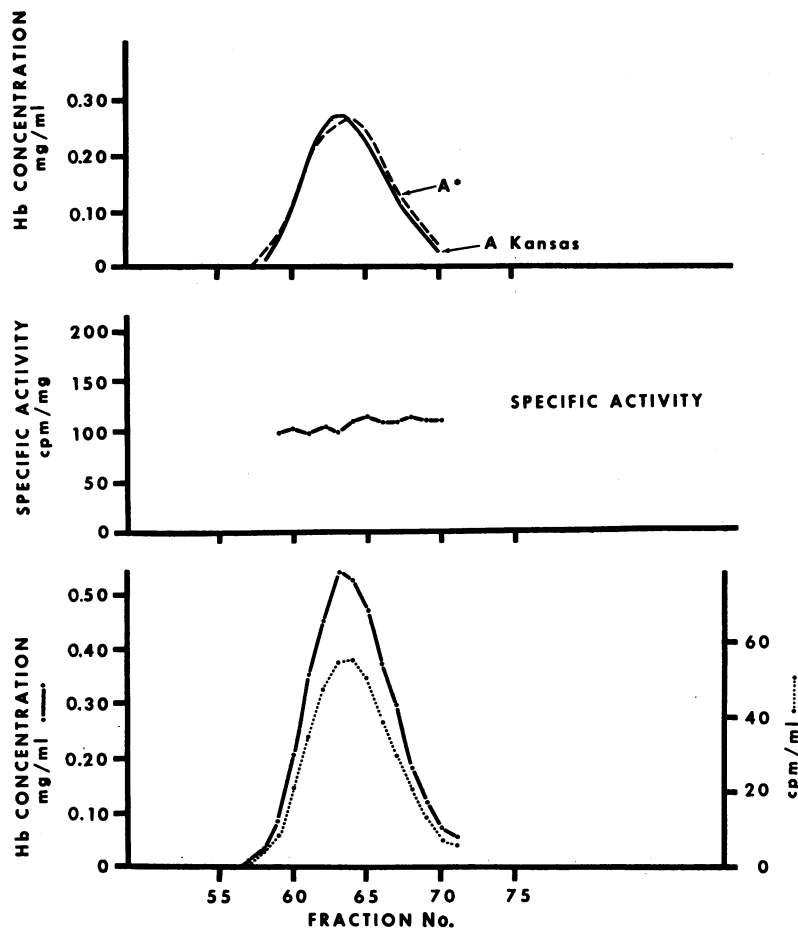


FIGURE 3 Elution profile of cyanmethemoglobin A, isolated from the Kansas hemolysate, and cyanmethemoglobin A-⁵⁹Fe.

higher than that of any of the other hemoglobins tested (Table II). When the most dilute solution of hemoglobin A was chromatographed, its initial concentration at the point of application was 1.6 mg/ml, and its mean concentration upon elution was 0.073 mg/ml. Under these conditions, during the run most of the hemoglobin would be expected to be in the form of dimers, as estimated from recently derived equilibrium constants (12, 28, 29). The fact that the elution volume of hemoglobin Kansas was far greater suggests that dissociation had extended to the formation of monomers. These findings are in agreement with the sedimentation velocity data of Bonaventura and Riggs (2). They found that carboxy- and oxy- but not deoxyhemoglobin Kansas had low sedimentation coefficients indicating that Kansas dissociated into dimers at concentrations in which hemoglobin A remained as an intact tetramer. The abnormal Sephadex mobility of hemoglobin Kansas was not likely to be due to any alterations resulting from its purification since the hemoglobin A isolated from the same column showed an elution volume identical with the A-⁵⁹Fe reference hemoglobin (Fig. 3).

Hemoglobin Chesapeake had a lower elution volume than the labeled hemoglobin A (Fig. 4). Both the carboxy- and ferrihemoglobin cyanide derivatives gave identical and reproducible results on a total of six chromatograms prepared from hemoglobins isolated from two separate blood samples. Hemoglobin A isolated on the same preparative column as the Chesapeake A was also tested each time and found to have an elution volume identical with the A-⁵⁹Fe reference hemoglobin (Fig. 5). When cyanmethemoglobin Chesapeake was run alone with the albumin-¹²⁵I marker, its K_{av} was not very much less than that of cyanmethemoglobin A obtained from the same column (Table II). This small difference is probably a reflection of the much higher resolution offered by the chromatography of hemoglobin mixtures. In contrast to the carboxy and cyanmet derivatives, deoxyhemoglobin Chesapeake showed an elution volume identical with that of deoxyhemoglobin A-⁵⁹Fe. The results from this experiment very closely resembled those shown in Figs. 3 and 5. Finally when a mixture of the cyanmet derivatives of Chesapeake and A-⁵⁹Fe was run at 4°C, only a slight increase in specific activity of

successive fractions was noted indicating less of a difference in elution volumes than at 25°C. Perhaps this is because subunit dissociation of hemoglobin A appears to be reduced at the lower temperature (Table II). These results indicate that the liganded but not the deoxy form of hemoglobin Chesapeake have decreased elution volumes and therefore a reduced degree of subunit dissociation when compared to hemoglobin A.

Under the same conditions used for hemoglobin Chesapeake (Fig. 4), no difference between the elution volume of hemoglobin Yakima (and its companion A hemoglobin) and hemoglobin A-⁵⁹Fe could be demonstrated.

The elution volumes of various animal hemoglobins were measured by chromatographing a mixture of the animal hemoglobin and human hemoglobin A-⁵⁹Fe, under the same conditions employed in the previous experiments (Figs. 2-5). The cyanmet and carboxy derivatives gave similar results. Dog and guinea pig hemoglobins showed elution volumes identical with human. The results for these experiments were very similar to the data shown in Figs. 3 and 5. Cat hemoglobin had a larger elution volume than human hemoglobin (Fig. 6). The elution volumes of rabbit and rat hemoglobins were slightly greater than the human, dog, and guinea pig hemoglobins but less than the cat hemoglobin.

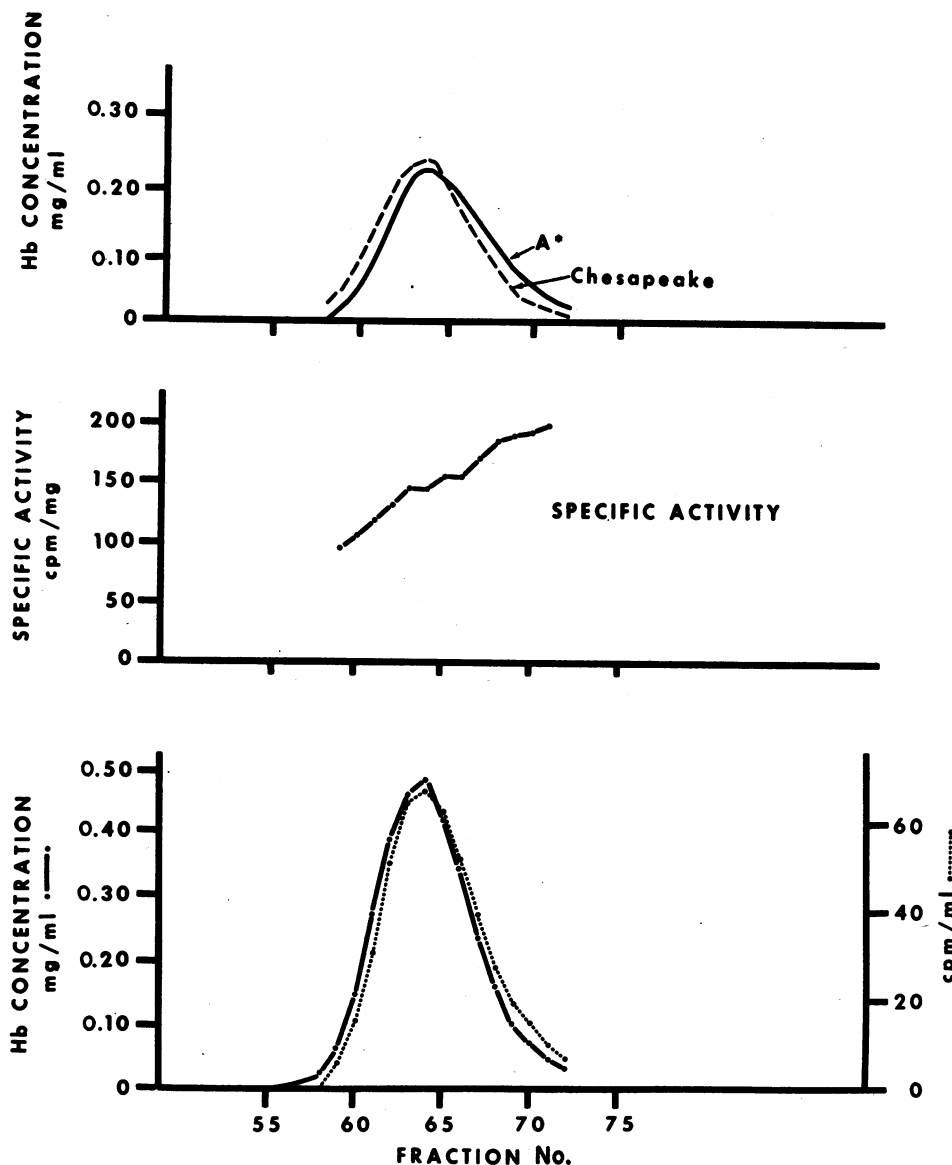


FIGURE 4 Elution profile of cyanmethemoglobin Chesapeake and cyanmethemoglobin A-⁵⁹Fe.

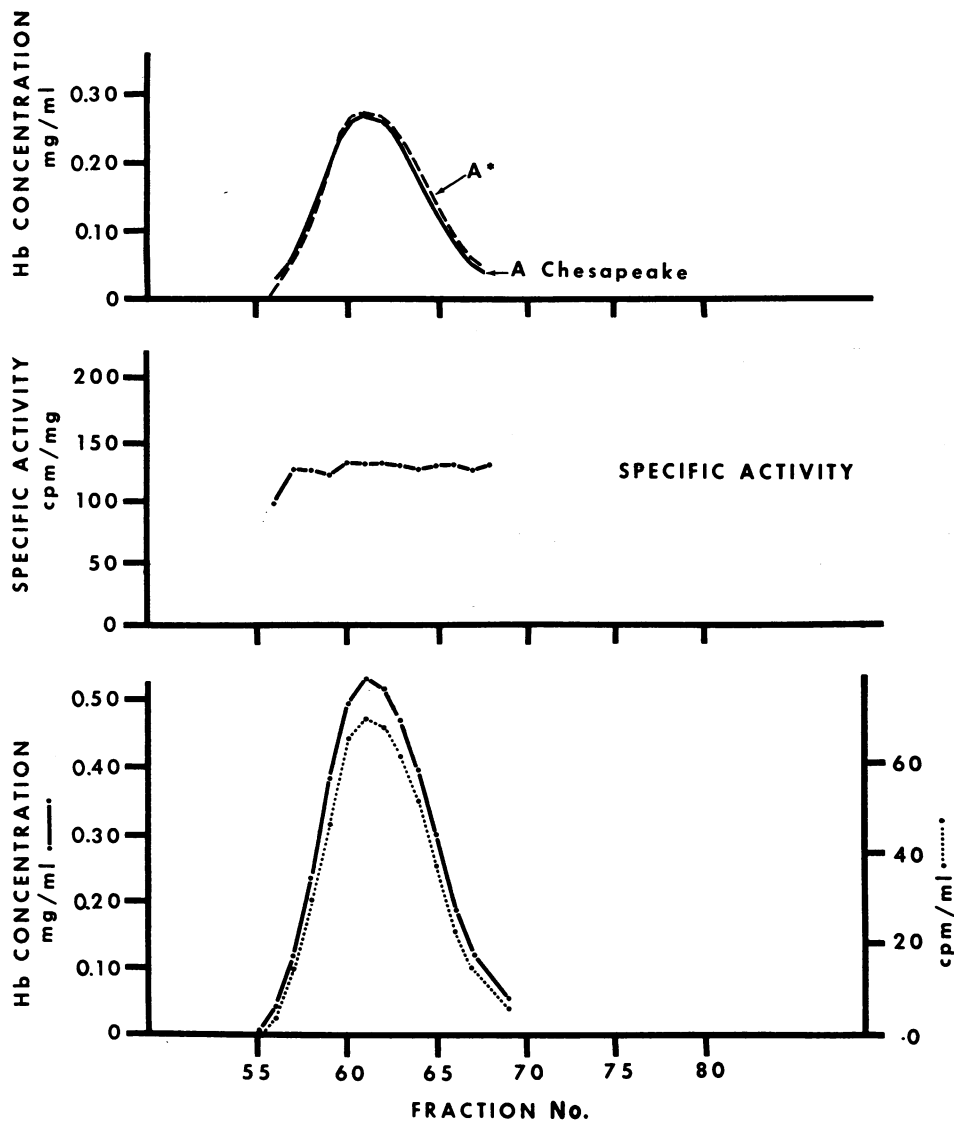


FIGURE 5 Elution profile of cyanmethemoglobin A, isolated from the Chesapeake hemolysate, and cyanmethemoglobin A-⁵⁹Fe.

The relative affinities of the various human hemoglobins for haptoglobin are shown in Fig. 7. If the test hemoglobin had precisely the same affinity as the rabbit hemoglobin-⁵⁹Fe, then the specific activity of the isolated Hb-Hp complex would be identical with that of the original hemoglobin mixture. Exactly 50% of the haptoglobin would be bound by the test hemoglobin. In fact most of the test hemoglobins showed a slightly greater affinity than the labeled rabbit hemoglobin, forming complexes with about 52-55% of the haptoglobin. These results agree well with earlier data on hemoglobin A (18). In contrast to hemoglobins A, F, S, and C, hemoglobin Kansas showed a considerably greater affinity for haptoglobin, whereas hemoglobins Chesapeake and Yakima showed somewhat decreased affinity.

Haptoglobin phenotype had no effect on its binding to the various test hemoglobins. In a parallel experiment, a mixture containing equal amounts of unlabeled cat hemoglobin and ⁵⁹Fe-labeled human hemoglobin was added in excess to human serum (type 1-1 haptoglobin). 62% of the haptoglobin complexed with the cat hemoglobin, 38% with the human.

Oxyhemoglobin Chesapeake and its companion oxyhemoglobin A (isolated from the same ion-exchange column) both showed equal rates of alkali denaturation. Furthermore these hemoglobins both auto-oxidized at the same rate.

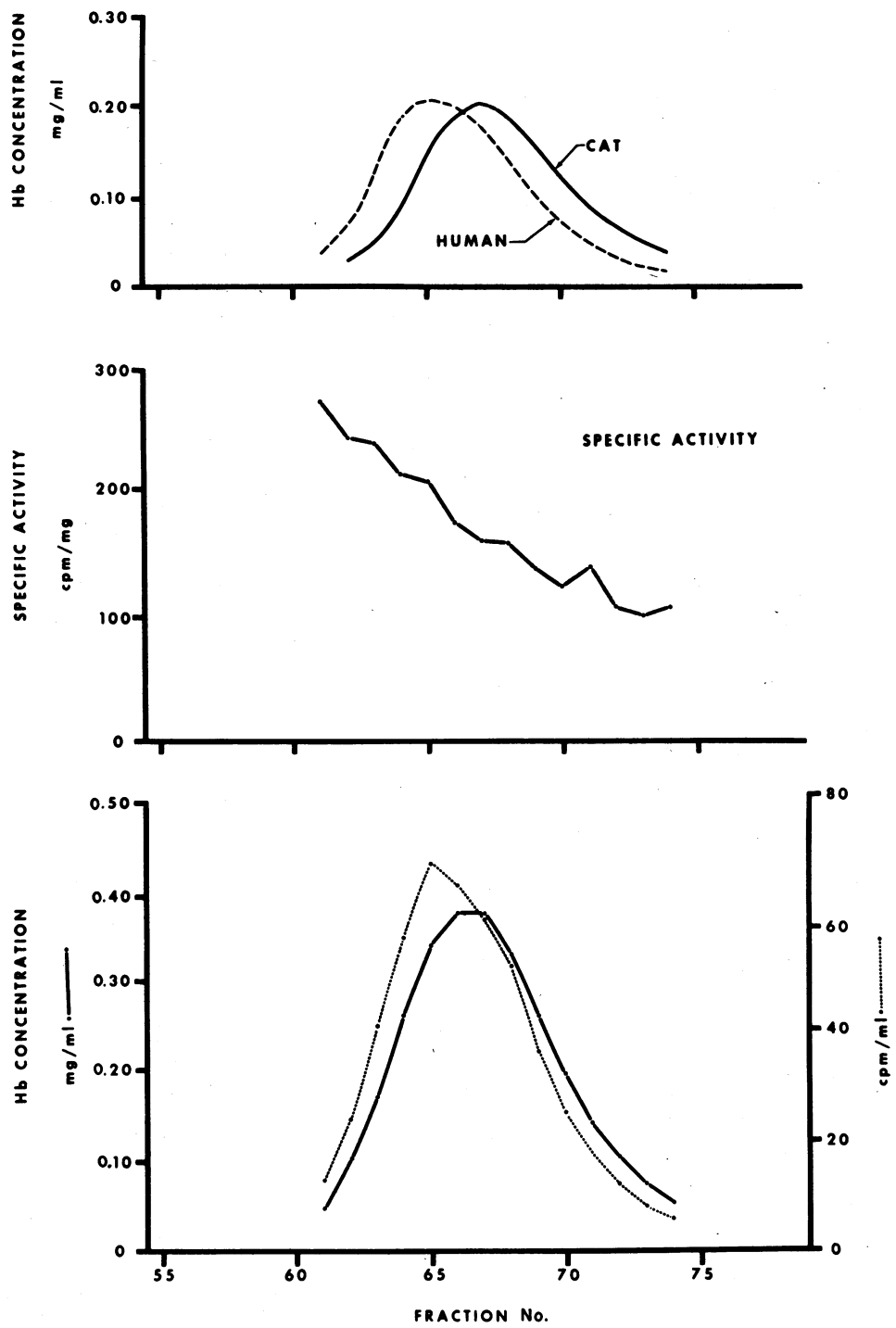


FIGURE 6 Elution profile of the cyanmet derivative of cat hemoglobin and cyanmethemoglobin A-⁵⁹Fe.

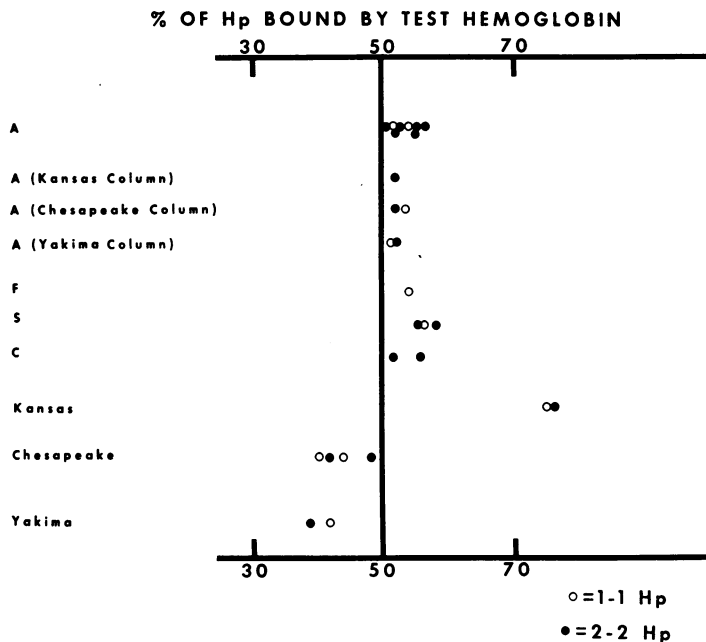
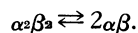


FIGURE 7 Relative haptoglobin binding of various human hemoglobins. To a mixture containing equal amounts of carboxy-⁵⁶Fe rabbit hemoglobin and the carboxy hemoglobin to be tested was added a small amount of normal serum, of haptoglobin phenotype 1-1 (○), or 2-2 (●). The per cent of the haptoglobin bound to the test hemoglobin was calculated from the specific activity of the isolated hemoglobin-haptoglobin complex (see Results).

DISCUSSION

One of the long-standing interests in hemoglobin chemistry has been the dissociation of the molecule into smaller fragments. Subunit formation was readily seen after subjecting hemoglobin to various stresses such as extremes of pH or very high ionic strength (30). As the protein structure was elaborated it appeared likely that subunit dissociation involved disruption of linkages between the component α and β polypeptide chains. Vinograd and Hutchinson presented convincing evidence that the tetramer split symmetrically (31):



Experiments along several different lines indicated that this equilibrium existed under physiologic conditions of pH and ionic strength (12, 13, 32) and therefore might be an important determinant of hemoglobin function.

Subunit dissociation has been approached by a variety of methods, all of which involve the measurement of average molecular weight. The observed molecular weight can be related to that of the intact molecule and serves as an index of the extent of dissociation. Osmotic pressure measurements (12) and sedimentation velocity data (28) have been used to calculate an equilibrium constant for hemoglobin dissociation under physiologic conditions. Ackers and Thompson (29), and more recently Chiancone, Gilbert, and Kellest (33), have used G-100 Sephadex to study hemoglobin dissociation and have calculated equilibrium dissociation constants for human oxyhemoglobin. In order to obtain these quantitative results it was necessary to load the

column with a large enough volume of hemoglobin solution to achieve a plateau type elution profile. In the experiments reported here and in those of Andrews (22), small volumes were applied, resulting in the formation of sharp peaks. Although this experimental approach provided qualitative data, it does not permit the calculation of equilibrium constants. However, the data shown in Table II confirm that it can be useful, since conditions known to affect hemoglobin dissociation are reflected in expected alterations in hemoglobin mobility.

In these experiments the elution volume of single hemoglobin solutions was not sensitive enough to detect subtle changes in subunit dissociation. When liganded forms of hemoglobin Chesapeake were run in a mixture with an equal amount of hemoglobin A-⁵⁶Fe, a consistent rise in specific activity of successive fractions indicated that the Chesapeake had a lower elution volume and therefore a decreased tendency to dissociate into dimers. Exchange of intact heme groups, a phenomenon which proceeds quite readily in mixtures containing methemoglobin, was very unlikely to affect the results cited above. Carboxy and cyanmet derivatives were run at 25°C, conditions under which heme exchange does not occur (15). However, another source of error may be more significant. Guidotti has osmotic pressure data indicating that in mixtures of two different hemoglobins, say ($\alpha_2\beta_2$) and ($\alpha'_2\beta'_2$), the hybrid $\alpha\beta\alpha'\beta'$ escapes detection by any method dependent on charge differences for resolution (34). In the chromatographic runs containing mixtures of hemoglobin A and Chesapeake, the extent that

the hybrid $\alpha^A\alpha^{Ches}\beta_A$ was present would attenuate the resolution of the two parent molecules into separate peaks. The extent of this phenomenon cannot be measured directly, but it was unlikely to be a major source of error since there was little difference between the Sephadex mobility of hemoglobin Chesapeake when measured alone (Table II) as compared to its mobility in a mixture with the labeled reference hemoglobin (Fig. 4). The failure to observe a difference between the elution volumes of hemoglobin Yakima and the reference hemoglobin does not support the contention that Yakima like Chesapeake has a decreased tendency to dissociate. This possibility is not entirely ruled out, however. The extent of formation of the interspecies hybrid ($\alpha_2\beta^A\beta^{Yakima}$) may have been great enough to preclude resolution of the two hemoglobins.

It was pertinent to compare the affinities of the various hemoglobins to haptoglobin. This plasma protein binds specifically and irreversibly to hemoglobin. 1 mole of type 1-1 haptoglobin is known to combine stoichiometrically with 1 mole of hemoglobin (35). Since type 1-1 haptoglobin is a symmetrical molecule (36), it is likely to have two binding sites. Evidence from several different experimental approaches indicates that haptoglobin binds separate $\alpha\beta$ dimers rather than the intact hemoglobin tetramer (18, 37-39). If so, a hemoglobin which has an increased tendency to dissociate into half molecules would have a relatively high affinity for haptoglobin, and vice versa. When viewed in this way, the relatively high haptoglobin affinity of hemoglobin Kansas and cat hemoglobin and the low haptoglobin affinity of hemoglobin Chesapeake are in good agreement with the Sephadex mobility data. However, it is also possible that these hemoglobins have alterations in tertiary structure which affect their affinities for haptoglobin. Comparable data for hemoglobin A treated with various sulfhydryl agents offer an interesting comparison (18). *P*-mercuribenzoate-treated hemoglobin, known to have an enhanced degree of dissociation into subunits, had a relatively high haptoglobin affinity. Conversely hemoglobin reacted with bis-(*N*-maleimidomethyl) ether (BME) has a markedly impaired degree of dissociation (40) and a low affinity for haptoglobin. The G-100 Sephadex mobility of BME hemoglobin was considerably greater than that of untreated hemoglobin-⁵⁹Fe with which it had been mixed,⁵ a finding similar to that obtained for hemoglobin Chesapeake (Fig. 4). The similarities between hemoglobin Chesapeake and BME hemoglobin extend even further. Of all the sulfhydryl reagents, BME appears to effect the greatest increase in oxygen affinity (40-42).

Benesch and associates have proposed a model (13) which is based on two important properties of hemo-

globin: its change in conformation upon reaction with ligand and its reversible dissociation into dimers. More recently Guidotti has derived a comprehensive series of equations which relate subunit dissociation and ligand binding in quantitative terms (43). When hemoglobin concentration is large relative to the values of the dissociation equilibrium constants, a condition certainly existing in the red cell, the following simplified equation can be written (43):

$$P_{50} = \left(\frac{K_0}{K_3^2 K_{deo}} \right)^{1/4}$$

where P_{50} is the partial pressure at which half of the hemoglobin is bound by ligand (such as oxygen); K_0 and K_{deo} are the dissociation equilibrium constants for the oxygenated and deoxygenated tetramers respectively, and K_3 is the association equilibrium constant for the reaction of the $\alpha\beta$ dimer with oxygen. It can be readily appreciated from this equation that if the ratio of the dissociation constant of liganded hemoglobin over that of unliganded hemoglobin is abnormally high, as is true for hemoglobin Kansas, there will be an increase in P_{50} , or a decrease in oxygen affinity. It is of interest to apply the equilibrium constant derived by Bonaventura and Riggs for oxyhemoglobin Kansas to this equation. Riggs' and Bonaventura's sedimentation data indicate that $K_{deoKansas} = K_{deoA}$. If $K_{3Kansas} = K_{3A}$, an assumption that as yet has no experimental basis, then, from experimental values for K_{oA} ($1.0-1.4 \times 10^{-6}$ mole/liter [44, 45]) and $K_{oKansas}$ (200×10^{-6} [2]), the following ratio can be calculated: $P_{50A}/P_{50Kansas} = 0.28$. The ratio $P_{50A}/P_{50Kansas}$ derived from the oxygen dissociation curves of Riggs and Bonaventura is 0.30 (2). This good agreement lends support for the models proposed by Benesch et al. and by Guidotti. Further support is provided by a comparison of oxygen affinity and subunit dissociation of certain animal hemoglobins. Cat hemoglobin, for example, has a much lower oxygen affinity than that of other mammals (46). Early sedimentation data suggested that cat hemoglobin dissociated readily in dilute solution, although the data are difficult to interpret because of variability in experimental conditions (47). Sullivan recently reported that cat hemoglobin eluted after human hemoglobin on G-100 Sephadex but presented no data (48). The results shown on Fig. 6 confirm that cat hemoglobin has a large elution volume and therefore an increased degree of subunit dissociation. The adult heterozygous sheep has two hemoglobins, types A and B, which are readily separated by electrophoresis. The "slow" hemoglobin, type B, has a much lower oxygen affinity than the "fast" type A hemoglobin (49). Gilbert, using a highly sensitive gel filtration approach, similar in principle to the one used in the present studies, has recently shown that type B sheep hemoglobin dissociates more readily than type A (50).

⁵ Unpublished observation.

The abnormally high oxygen affinity seen in certain hemoglobin variants (Table I) may be due to a low degree of subunit dissociation of the liganded form. The data on hemoglobin Chesapeake which have been presented here suggest this explanation, but other factors may also be contributory. It may be that the amino acid substitution in Chesapeake (and similar variants) confers subtle conformational changes which per se could alter ligand affinity. Nagel, Gibson, and Charache have shown that deoxyhemoglobin Chesapeake differs from deoxyhemoglobin A in reactivity to iodoacetamide and the ionization of tyrosine residues (51). In contrast only the liganded forms of the two hemoglobins differ in their Sephadex elution volumes. Furthermore, Nagel and colleagues showed that carbon monoxide reacted with Chesapeake twice as rapidly as A, but this observation does not exclude the postulated importance of subunit dissociation as a determinant of ligand affinity.

Hemoglobin Chesapeake had a normal rate of alkali denaturation. It is noteworthy that hemoglobin Rainier, which resembles Chesapeake in its clinical features and functional properties (Table I), is the first adult human hemoglobin found to be alkali resistant (7). This phenomenon which is so striking in hemoglobin F is not likely to be due to a decreased tendency to form dimers, since hemoglobin F appears to have a normal mobility on G-100 Sephadex. Furthermore, hemoglobin Chesapeake and BME hemoglobin,⁶ both of which appear to dissociate less readily into half molecules, have rates of alkali denaturation similar to hemoglobin A. Finally the very high pH at which denaturation is measured would be expected to favor dimer formation in all hemoglobins. What seems more plausible is that resistance to alkali denaturation is due to decreased dissociation of dimer to monomer. Hemoglobin F forms interspecies hybrids much less readily than hemoglobin A (52), a phenomenon dependent upon the formation of monomer. Furthermore, hemoglobin Kansas has a greater rate of alkali denaturation than hemoglobin A (2). The data shown in Table II and Fig. 2 suggest that Kansas has an increased tendency to form monomers as well as dimers.

All the hemoglobin variants depicted in Table I have a low n value, indicative of decreased "heme-heme" interaction. This fact is not predictable from Guidotti's model merely on the basis of alterations in the extent of subunit dissociation and point to conformational changes in the $\alpha\beta$ -dimers themselves, which would affect cooperative interactions. The findings of Nagel and coworkers (51) indicate that this is probably true for hemoglobin Chesapeake. Furthermore, the apparent tendency for hemoglobin Kansas to form monomers may underlie a decrease in cooperative interactions.

⁶ Unpublished observation.

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