Comparative Binding Studies of the Hemoglobin–Haptoglobin and the Hemoglobin–Antihemoglobin Reactions†

Patricia Cohen-Dix,‡ Robert W. Noble, and Morris Reichlin*,§

ABSTRACT: The species specificity of the reactions of human haptoglobin and antihuman hemoglobin antibodies with different animal hemoglobins was studied with respect to equilibrium behavior and reaction kinetics. These studies indicate that while the reaction of human haptoglobin with hemoglobin has a very broad species specificity, very distantly related hemoglobins (frog and carp) do exhibit weaker binding than those more closely related, most of which appear indistinguishable from the human protein in these tests. In contrast, antihuman hemoglobin antibodies show a very strict species specificity reacting most strongly with human hemoglobin. In addition, data are presented which demonstrate that the sites on hemoglobin where haptoglobin binds and the sites of antibody binding are completely independent. The proposed explanation is that antibodies are directed against regions on hemoglobin that have varied during evolution. In contrast, it is concluded that the site on hemoglobin for binding haptoglobin is a region of the hemoglobin molecule that has been conserved in evolution. On the basis of these studies and other known facts about the hemoglobin–haptoglobin reaction, it is suggested that the binding site on hemoglobin for haptoglobin is situated in the $\alpha^1\beta^2$ contact region of the hemoglobin molecule.

Haptoglobin, Hp,[1] is a serum α_2 glycoprotein (Jayle and Boussier, 1954; Jayle *et al.*, 1956) which binds hemoglobin, Hb (Polonovski and Jayle, 1940). There are many similarities between Hp and antibody, Ab. Like Ab, Hp type 1-1 is thought to be composed of two light (α) chains and two heavy (β) chains linked together by disulfide bonds (Connell *et al.*, 1962). Sequence homology between Hp and immunoglobulins has been discovered by Black and Dixon (1968) who sequenced the α chain of human Hp and noted the homology between this polypeptide chain and the light chain of immunoglobulins. They have further postulated that the α chain of Hp and the light chain of immunoglobulins may have derived from the same ancestral gene. At the present time the physiological function of Hp is controversial, but it has been suggested (Malchy and Dixon, 1970) that Hp may be a preformed Ab directed toward Hb.

However, there are also major differences between the two proteins. The β chain of Hp has now been partially sequenced and exhibits no homology with known sequences of immunoglobulin polypeptide chains (Barnett *et al.*, 1972).

In the present study a comparison has been made between the binding to Hb of Hp and antibodies to human Hb. Both types of proteins are directed strongly and specifically toward certain parts of the Hb molecule. Although much information is already available on the location of antigenic sites on human Hb (Reichlin, 1972), little is known about the site of binding of Hp to Hb. It was thought that a comparison of the species specificity of binding of these two types of proteins to animal Hbs might provide information that would help to localize the Hp binding site of Hb. In fact, these data plus those obtained by other investigators, combined with a consideration of sequence data in regions exposed by dimer formation, have led to a hypothesis of the binding site on Hb for Hp.

Hp binds so tightly to Hb that some have termed the reaction irreversible. Nagel *et al.* (1965), Noyes and Laurell (1961), and Chiancone *et al.* (1966) were all unable to measure significant complex dissociation under ordinary conditions. However, such negative results can only establish an upper limit for the rate of dissociation, and it was hoped that more sensitive methods as well as an extension of the measurements to longer time periods might produce evidence of dissociation, if not for the human Hb–human Hp complex then at least for the complex of some other animal Hb with human Hp.

Methods and Materials

Purification of Hp. Haptoglobin was purified from the plasma of normal persons of Hp type 1-1. The first step was a modification of the procedure of Waks and Alfsen (1966). Plasma, 100–250 ml, was adjusted to pH 4.7 with HCl and dialyzed against 0.01 M sodium acetate–acetic acid buffer (pH 4.7). After centrifugation at 15,000*g* for 20 min, the plasma supernatant was chromatographed at 4° on a 2.5 × 30 cm DEAE-cellulose column equilibrated with 0.01 M acetate buffer (pH 4.7). The column was washed exhaustively with 0.02 M acetate buffer (pH 4.7). The Hp-rich fraction was eluted from the column with 0.08 M acetate buffer (pH 4.7).

The Hp solution was concentrated and gel filtered at 4° on a 2.5 × 70 cm Sephadex G-200 column equilibrated with 0.05 M Tris buffer (pH 7.7). The Hp peak was concentrated and judged to be at least 85% pure by starch gel electrophoresis. The Hp was stored at −30° with no apparent loss of Hb binding ability.

The pure Hp–Hb complex was obtained by combining pure Hp with excess HbA and separating the Hp–Hb complex

† From the Departments of Medicine and Biochemistry, State University of New York at Buffalo, and the Veterans Administration Hospital, Buffalo, New York 14215. *Received February 12, 1973.* This work was supported by Grant HE 12524 from the National Heart and Lung Institute (to R. W. N.), Grant AM 10428 from the National Arthritis Institute (to M. R.), and designated research funds from the Veteran's Administration to R. W. N. and M. R.

‡ This work was submitted in partial fulfillment for the degree of doctor of philosophy from the State University of New York at Buffalo. Present address: Department of Medicine, Yale University School of Medicine, New Haven, Conn.

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[1] Abbreviations used are: Hb, hemoglobin; Hp, haptoglobin; Ab, antibody.

from free Hb at 4° on a 2.5 × 70 cm Sephadex G-100 column equilibrated with 0.05 M phosphate–borate buffer (pH 8.0).

Antisera. The method of purification of HbA and immunization schedules for Hb have been reported previously (Reichlin *et al.*, 1964). Antisera against pure Hp–Hb were prepared by injecting 1 mg of Hp–Hb complex in 1 ml of 0.05 M phosphate–borate buffer (pH 8.0) in complete Freunds adjuvant into hind toe pads and multiple sites. After 2 weeks, the animals were injected in the thigh muscle with the same mixture of antigen and adjuvant originally used. Six weeks later the animals were injected with 1 mg of Hb–Hp complex intravenously. They were bled from the ear on 3 successive days beginning 5 days after the last injection. Anti-Hp–Hb sera were rendered specific by absorbing out the contaminating antibodies.

Immunological Methods. Double diffusion tests in agar were performed according to the technique of Ouchterlony (1949) and quantitative precipitin tests were performed by the method of Heidelberger and Kendall (1935). The method of Stavitsky (1954) as modified by Sharp *et al.* (1971) was followed in hemagglutination experiments except that most of the test antisera were not absorbed with sheep red blood cells.

Binding Techniques. (A) GENERAL. The various binding techniques used here are modifications of the method of Farr (1958). Hemoglobins were labeled with ^{131}I by the chloramine T method (Hunter and Greenwood, 1962). The labeled Hb was usually diluted until 0.05 ml of solution gave ~30,000 counts in 10 min in a Nuclear-Chicago γ-ray spectrometer. In each experiment, 0.05 ml of trace labeled Hb solution was used with 0.05 ml of the inhibitor or competitor solution. The source of Hp was 0.1 ml of a normal human plasma of Hp type 1-1 which was diluted 1:15. The source of antihuman Hb antibody was 0.1 ml of rabbit antiserum 286 bleeding 3, diluted 1:15. This bleeding of serum 286 contained 0.9 mg of antibody protein/ml as determined by quantitative precipitin analysis. The precipitating antibody was standard in all Hp or in all Ab experiments. In Hp experiments, the precipitating antibody was 0.2 ml of rabbit anti-Hp–Hb serum (serum 280) that had been completely absorbed with human Hb so that it could only bind Hp. It was used in a 1:4 dilution. This serum contained 1.08 mg of antibody protein/ml with Hp as antigen by quantitative precipitin analysis. In experiments involving Ab, instead of Hp, 0.2 ml of a sheep antirabbit γ-globulin serum was the precipitating antiserum. The amount of precipitating Ab was always sufficient to quantitatively precipitate all the rabbit γ-globulin or human Hp present. At least 18 hr at 4° was allowed for the precipitation. Then all precipitates were centrifuged at 2000*g* in a PRII refrigerated centrifuge, washed three times, and counted in a Nuclear-Chicago γ-ray spectrometer.

(B) COMPETITION EXPERIMENTS. In the competition experiments, trace labeled human Hb competed with an equal concentration of unlabeled test Hb for binding sites on either human Hp or rabbit antihuman Hb antibody. ^{131}I-Labeled human Hb was diluted into an unlabeled human Hb solution of 110 μg/ml. An equal volume of the trace labeled Hb solution and the unlabeled test Hb solution (also at 110 μg/ml) were mixed and Hp or Ab was added. After 1 hr at 4°, the appropriate precipitating antiserum was added. In all experiments, unlabeled human Hb was made to compete with the labeled human Hb as a control and all other test hemoglobins were then compared to the unlabeled human Hb.

(C) REPLACEMENT EXPERIMENTS. In the replacement experiments, dissociation of ^{131}I-labeled Hb from Hp or Ab, in the presence of a large excess of unlabeled human Hb, was followed with time. The Hb to be tested was labeled and diluted into a 25-μg/ml solution of unlabeled Hb. The trace labeled Hb was allowed to react with an excess of Hp or Ab, usually for 1 hr at 4°. (Trace labeled turtle Hb, however, was incubated at 28° for 3 hr since it binds Hp so slowly.) A 50-fold excess of unlabled human Hb was added and the solutions were incubated for various times at 4 or 35°. After this, they were cooled to 4° and the precipitating Ab was added.

(D) BLOCKING THE BINDING OF Hb TO Hp. Attempts to inhibit the binding of Hb to Hp by rabbit antihuman Hb Fab fragments were carried out by mixing trace labeled human Hb, 25 μg/ml, with an excess of Fab fragments. After waiting for 5 min at 4°, the Hp solution was added and, again, tubes were left for 5 min at 4° before precipitating antiserum was added.

Preparing Fab Fragments. The Fab fragments were prepared by the method of Nisonoff *et al.* (1960) except that whole serum was digested instead of purified γ-globulin. Cysteine in a final concentration of 0.01 M was used so that 3.5S Fab fragments rather than 5.0S $(Fab)^2$ fragments were generated. Such digested sera no longer precipitated with antigen but effectively inhibited precipitation of intact antibody with antigen as shown by double diffusion in agar.

Static Flourescence Quenching. The method for the measurement of the fluorescence quenching of Hp by Hb was described by Chiancone *et al.* (1968). A solution of pure Hp (2 ml at 0.1 mg/ml) was titrated by small additions of a Hb solution of ~2 mg/ml using an Agla syringe (Burroughs Welcome Inc.). Leopard frog Hb, however, was used at 4 mg/ml and carp Hb at 50 mg/ml since greater concentrations of these hemoglobins were required to completely bind the Hp. The measurements were made with an Aminco-Bowman spectrophotofluorometer equipped with a parabolic mirror attachment. The excitation wavelength was 285 nm and fluorescence was measured at 350 nm. Experiments were performed at room temperature in 0.05 M Tris buffer (pH 7.7). The concentration of Hp solution used was determined by its optical density (OD) at 280 nm using an extinction value of 1.2 OD units for a 0.1% solution. The extinction value used for Hb was 0.837 OD unit at 540 nm for a 0.1% solution.

Kinetics of the Reaction of Hp with Hb. The method of measuring the kinetics of the reaction of Hp with Hb by fluorescence quenching was described by Nagel and Gibson (1964). The sources of illumination and the optical filters used were the same as those described by these authors. The modification of the Gibson–Durrum stopped flow apparatus for fluorescence measurement was described by Gibson *et al.* (1966). The Hp for these experiments had been purified only by chromatography on DEAE-cellulose and was used at concentrations of 0.2 mg/ml. The Hb solutions before mixing were approximately 0.65×10^{-5} M in Hb tetramer.

Hb Hemolysates. Hemolysates of animal hemoglobins were prepared after the manner of Drabkin (1946). Sheep, goat, bovine, horse, and chicken bloods in Alsevers solutions were obtained from Gibco Diagnostics Microbio Lab, Madison, Wis. Human, rabbit, and sometimes chicken bloods were drawn from individual normal animals into a sodium citrate solution. Leopard frogs (northern *Rana pipiens*) and painted turtles (*Chrysemies picta*) were obtained from Mogul-Ed, Oshkosh, Wis., anesthetized with ether, and bled from the heart into Alsevers solution. Washed carp red blood cells were donated by Anna Tan of the Biochemistry Department, State University of New York at Buffalo, School of Medicine. Bloods were centrifuged and red blood cells washed at least three times with isotonic saline before preparation of hemolysates.

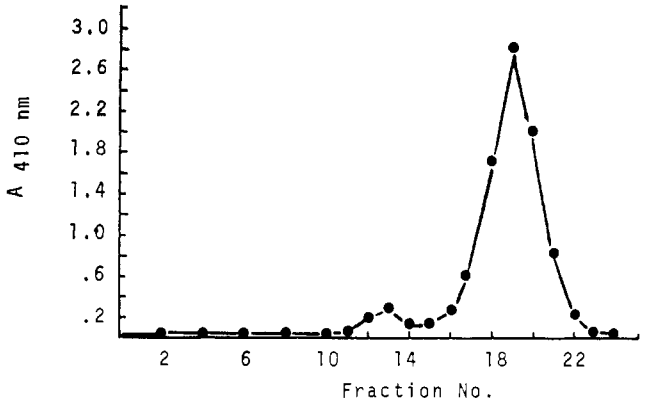

FIGURE 1: Sephadex G-100 filtration profile of human serum Hp type 1-1, combined with carp Hb. The column (2.5 × 70 cm) was eluted with 0.05 M phosphate–borate buffer (pH 8.0) at 4°, and optical density was monitored at 410 nm. Fractions of 8.0 ml were collected.

Results

Species Specificity of the Reaction of Human Haptoglobin with Animal Hemoglobins. EQUILIBRIUM MEASUREMENTS. With the exception of carp Hb, all species of animal Hb investigated (cow, sheep, rabbit, horse, chicken, painted turtle, and leopard frog) have an extra heme staining (guaiacol positive) band on starch gel electrophoresis when added to human serum of Hp type 1-1. Each of these new bands appeared to have the same mobility on starch gel electrophoresis as the human Hb–human Hp complex.

The binding of carp Hb to human Hp could be demonstrated utilizing gel filtration on Sephadex G-100 columns. When a large excess of carp Hb was added to human plasma of Hp type 1-1 and gel filtered on Sephadex G-100, a very small amount of Hb was in the excluded volume (Figure 1). The Hb in this peak was identified as carp Hb, and not contaminating human Hb, by its specific reactivity with anticarp Hb serum utilizing double diffusion in agar.

Human Hp was titrated with the various animal hemoglobins using the quenching of the fluorescence of Hp as an assay. When the percentage of fluorescence quenched was plotted against the amount of Hb added, rabbit, sheep, and chicken hemoglobins all gave straight titration curves (Figure 2). The end points of the titrations, which indicated the amount of Hb required to bind all of the Hp present and define the stoichiometry of the reaction, were the same for these three animal Hbs and for human Hb. Turtle Hb also gave an identical titration curve with a sharp end point but its rate of

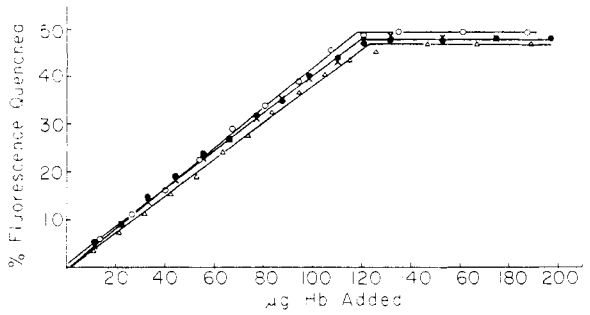

FIGURE 2: Fluorescence quenching titration of pure Hp (1.15×10^{-6} M) with human (●), rabbit (×), sheep (○), and chicken (△) hemoglobins in 0.05 M Tris buffer (pH 7.7) at room temperature.

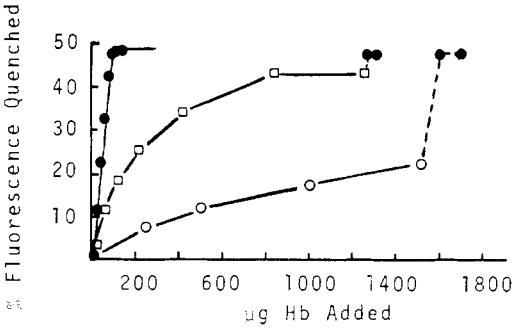

FIGURE 3: Fluorescence quenching titration of pure Hp (0.991×10^{-6} M) with human (●), northern leopard frog (□), and carp (○) hemoglobin, pH 7.7, at room temperature. Dotted line represents titration with human hemoglobin after titration of Hp with frog and carp Hbs, respectively.

reaction was so slow that 30 min was required after each Hb addition before the reaction was complete.

Leopard frog and carp Hbs bound human Hp less well than the other animal Hbs studied as shown by the fact that higher concentrations of frog and carp Hbs were needed in order to achieve a given level of fluorescence quenching (Figure 3). These data points were taken 30 min after the addition of Hb to the Hp solution. The reaction of frog Hb with Hp was completed after 30 min. However, it is not known whether the reaction of carp Hb with human Hp had been completed since an attempt to investigate this point was inconclusive. A Hill plot of the reaction of frog Hb with human Hp showed a straight line with a slope of 1. The equilibrium association constant for the reaction as determined from the Hill plot is 4.6×10^{5} M^{-1}.

Association of Hp with Various Animal Hbs. Relative rates of combination of animal hemoglobins with human Hp were investigated by a competitive binding technique in which animal Hbs competed with radioactive human Hb for binding sites on human Hp. The larger the percentage of the total counts precipitated by anti-Hp serum, the poorer the test animal Hb was in competing with the labeled human Hb for binding sites on Hp. In each experiment, unlabeled human Hb was also a competitor with the labeled human Hb for binding sites on Hp and could therefore be compared to each animal Hb. This is reported by subtracting the per cent of the labeled Hb precipitated (bound to Hp) with human Hb as competitor from the per cent of the labeled Hb precipitated with the animal Hb as competitor. Therefore, an animal Hb with a positive value competed less well than human Hb while a Hb with a negative value competed better than human Hb. A value close to zero indicates the animal Hb and human Hb competed similarly.

In Table I are presented the results of competitive binding experiments carried out at pH 9.2 and 7.7. Bovine, horse, chicken, and rabbit Hbs behaved much like human Hb. Sheep and goat Hbs competed better than human Hb while turtle, frog, and carp Hbs competed less well for binding sites on human Hp. In general, the same results were obtained at both pH values except that turtle Hb competes less well at pH 7.7 than at pH 9.2.

To verify these results by a direct binding technique, the kinetics of the reactions of the animal Hbs with human Hp were measured by fluorescence quenching in a stopped flow apparatus. The combination velocity constants (Table II) for the reaction of Hp with human and rabbit Hb were quite similar at pH 9.1 and 7.7 while the reaction with sheep Hb was always about three times faster than that with human Hb.

TABLE I: Unlabeled Animal Hbs Competing with Trace Labeled Human Hb for Binding Sites on Human Hp.[a,b]

Competing Unlabeled Animal Hb	pH 9.2 Competitive Difference[c]	No. of Determinations	No. of Hb Preparations	pH 7.7 Competitive Difference[c]
Sheep	−9.7	4	3	−8.9
Goat	−10.6	2	1	
Bovine	5.3	1	1	
Horse	−3.0	1	1	
Human	0			0
Chicken	0.9	7	3	−2.0
Rabbit	4.0	2	1	−1.0
Human ($^1/_2$ concn)	13.6	6	3	11.5
Painted turtle	16.3	3	1	31.4
Leopard frog	49.0	3	1	55.0
Carp	68.8	1	1	

[a] In whole human plasma, Hp type 1-1. [b] Total tetramer Hb concentration after mixing was 8.46×10^{-7} M and Hp concentration was $\sim 4.2 \times 10^{-7}$ M. [c] The competitive difference is obtained by subtracting the per cent ^{131}I-labeled Hb bound to Hp with unlabeled human Hb competing for the Hp from the per cent ^{131}I-labeled Hb bound to Hp with the unlabeled test Hb competing for the Hp.

Painted turtle Hb reacted very slowly with human Hp. Leopard frog Hb exhibited a biphasic reaction with Hp. There was an initial large homogeneous reaction followed by a very slow reaction. All hemoglobins, with the exception of frog, gave homogeneous reaction kinetics. The results also showed that the rate of reaction of human Hb with human Hp decreased with increasing pH.

Dissociation of Hp from Various Animal Hbs. The tightness of binding of Hp to Hb was investigated by a replacement reaction. As described under Methods and Materials, ^{131}I-labeled animal Hb was incubated with an excess of human Hp, after which a large excess of unlabeled human Hb was added and replacement was allowed to occur for different lengths of time at 4 or 35°. In Table III, part A, it can be seen that for human, rabbit, chicken, bovine, and turtle hemoglobin the amount of labeled Hb precipitable by anti-Hp antisera remained constant from 1 to 5 days after addition of excess unlabeled Hb when incubated at 4° at pH 9.2. In Table III, part B, it is seen that, for human and rabbit Hb, 24 hr at 37° following 2 days at 4° at pH 9.2 produced no effect. The loss of precipitable labeled Hb that occurred during the first 24 hr of incubation is apparently due to a fraction of the Hb being damaged during the labeled procedure. As can be seen in Table III, part C, if the Hp was mixed with an excess of labeled Hb and the mixture incubated for 24 hr prior to addition of unlabeled Hb, no subsequent dissociation of the labeled Hb from the Hp could be detected.

Species Specificity of the Reaction of Rabbit Antihuman Hemoglobin Sera with Animal Hemoglobins. Three rabbit antihuman Hb sera were allowed to react with the hemoglobins of human, monkey, horse, cow, sheep, goat, and chicken in double diffusion in agar tests. With the possible exception of monkey Hb, all animal Hbs reacted much more poorly with the antisera than did human Hb. The human Hb line gave large spurs over the lines of horse and cow Hbs, while sheep and goat Hbs gave only very weak lines. (One antiserum gave no precipitin line at all with goat or sheep Hb.) Chicken Hb precipitated with only one out of the three antisera. Therefore, as would be expected, antihuman Hb sera reacted best with human Hb.

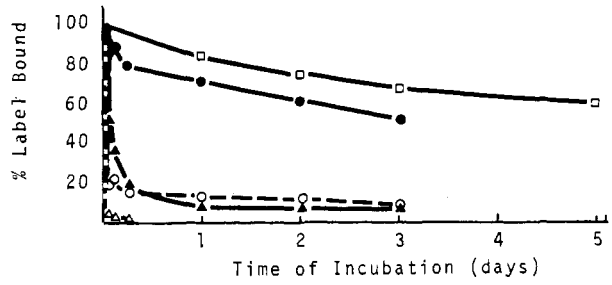

FIGURE 4: Dissociation of trace labeled human Hb from antihuman Hb antibody at pH 8.5 and at 4° (□) and at 4 (●) and 35° (○), and from trace labeled bovine Hb at 4 (▲) and 35° (△).

Association of Rabbit Antihuman Hemoglobin Antibody with Animal Hemoglobins. The species specificity of the reaction of antihuman Hb serum with various animal Hbs was investigated by a competitive binding measurement. As described under Methods and Materials, unlabeled human Hb and other animal Hbs were allowed to compete with ^{131}I-labeled human Hb for binding sites on antihuman Hb antibodies. No heterologous animal Hb (at the same concentration as trace labeled human Hb) competed effectively with the labeled human Hb. That is, none were able to decrease the amount of labeled Hb bound to antibody (Table IV). Only unlabeled human Hb competed favorably with labeled human Hb for sites on antihuman Hb antibodies.

Dissociation of Human and Bovine Hb from Antihuman Hemoglobin Sera. The dissociation of Hb from anti-Hb antibody was investigated by measuring the displacement of ^{131}I-labeled Hb from the Ab by unlabeled Hb. Unlike the Hp–Hb complex, Ab–Hb complexes showed progressive dissociation for a 5-day period at 4° (Figure 4). In comparing the

TABLE II: Kinetics of the Fluorescence Quenching of Human Hp by Various Animal Hbs at 20°.

Animal Hbs[a]	Buffer	Rate[b] of Combination $\times 10^{-5}$ (M^{-1} sec^{-1})
Human	0.05 M Tris (pH 7.7)	0.93
Rabbit	0.05 M Tris (pH 7.7)	0.89
Sheep	0.05 M Tris (pH 7.7)	2.71
Painted turtle	0.05 M Tris (pH 7.7)	0.18
Leopard frog	0.05 M Tris (pH 7.7)	0.08
Human	0.05 M phosphate–borate (pH 9.1)	0.50
Rabbit		0.32
Sheep		1.47
Human	0.05 M phosphate (pH 8.0)	0.99
	0.05 M phosphate (pH 7.1)	2.83

[a] After mixing tetramer Hb concentrations were approximately 6.5×10^{-6} M and Hp concentrations were 1×10^{-6} M. [b] Where more than one determination was made, average values are listed.

TABLE III: Trace Labeled Test Hb Displaced by Excess Unlabeled Human Hb from Human Hp (Normal Human Plasma, Hp Type 1-1) with Time.[a]

Trace Labeled	A. % ^{131}I-Labeled Hb Still Bound to Hp at Alkaline pH and 4° After[b]				
	1 Day	2 Days	3 Days	4 Days	5 Days
Human	73.4	72.1	70.1	77.7	72.0
Rabbit	73.7	69.0	68.4	68.0	66.8
Chicken	43.1	41.0	45.7	43.3	
Bovine	62.9	64.1	61.7	59.8	
Turtle	58.7	66.5	64.2	74.3	67.7

	B. % ^{131}I-Labeled Hb Still Bound to Hp at Alkaline pH and 4 and 35° After[b]				
	4°		35°		
	1 Day	2 Days	2 hr	8 hr	24 hr
Human	75.0	67.0	74.5	73.7	75.3
Rabbit	68.6	70.5	66.5	66.9	73.5

Trace Labeled Hb	Trace Labeled Human Hb Concn[c]	C. % ^{131}I-Labeled Hb Still Bound to Hp at Neutral pH and 4 and 35° After[b]				
		4°				4° (3 Days) + 37° (1 Day)
		1 Day	2 Days	3 Days	5 Days	
Human	Underatursating	85.7	87.0	79.0		
Human	Oversaturating	100.2	87.8	100.0	97.5	98.5

[a] After mixing, Hp concentrations were approximately 4.2×10^{-7} M, tetramer concentrations of the trace labeled Hbs were 0.96×10^{-7} M, and the unlabeled human Hb concentrations were 7.7×10^{-5} M. [b] The trace labeled test Hbs and human plasma were mixed, excess unlabeled human Hb was added, and mixtures were incubated for various time at 4° (A), or 4 and 37° (B). [c] After mixing, the tetramer concentrations of trace labeled Hbs were either undersaturating (0.96×10^{-7} M) or oversaturating (8.6×10^{-7} M), while Hp concentrations were approximately 4.2×10^{-7} M and unlabeled Hb concentrations were 7.7×10^{-5} M.

dissociation of labeled Hb from antibody at 4° on two different days, it could be seen that, while the initial slopes of the curves might be different, the slopes after 24 hr were quite similar (Figure 4). Incubation at 35° increased the rate of dissociation of labeled Hb from Ab. Figure 4 also shows that labeled bovine Hb dissociated much faster from antihuman Hb antibodies than did human Hb. Only 20% of the labeled bovine Hb capable of binding antihuman Hb antibodies was still bound after 90 min at 4° and less than 10% was still bound after 3 days. When the bovine Hb–antibody complex was incubated at 35° with cold human Hb, almost all of the bovine Hb had dissociated from the Ab after only 90 min.

TABLE IV: Unlabeled Animal Hbs Competing with Trace Labeled Human Hb for Binding Sites on Antihuman Hb Antibody.[a]

Competing Unlabeled Animal Hb[b]	% Labeled Hb Bound to Ab
Human	66.0
Human (0.5 concn)	77.8
Bovine	101.0
Horse	101.4
Chicken	99.8
Carp	103.6
None	100.0

[a] Rabbit antihuman Hb serum 286. [b] The total tetramer Hb concentration after mixing was 8.46×10^{-7} M and the antibody concentration was $\sim 6.2 \times 10^{-7}$ M. [c] After the labeled and unlabeled Hbs were mixed and combined with antiserum, the Hb–Ab complexes formed were precipitated with anti-rabbit γ-globulin serum. All values, except that for carp Hb, are the averages of two experiments.

Distinction between the Antibody Binding Sites on Hemoglobin and the Site of Haptoglobin Binding. In quantitative precipitin tests with two rabbit antisera and with a goat antiserum, Hp–Hb and free Hb precipitated an equivalent amount of Ab (Table V). This result suggests that Hp bound to Hb blocks no antigenic region(s). To rule out a small amount of nonprecipitating antibody specific for Hb and unreactive with Hp–Hb, the sera were studied for hemagglutinating activity against Hb-coated red blood cells after absorption with the Hb–Hp complex. No antibody activity was found in the supernatants by the hemagglutination assay. Both rabbit sera 286 and 340 agglutinated Hb-coated red blood cells to a titer of 1/36,450 before absorption. After absorption of these sera with the equivalent amount of Hp–Hb, the hemagglutination titer was less than 1/15 in both cases. These experiments clearly point to the independence of the Hp and Ab binding regions on the surface of the Hb molecule.

A second approach to this problem was to attempt inhibition of the Hb–Hp reaction by an excess of Fab fragments prepared from rabbit sera containing known amounts of antibody to human Hb. In this experiment Fab fragments and Hb were mixed at 4° and then Hp was added. (At 4°, the $t_{1/2}$ of dissociation of Hb and Ab though heterogeneous is at least 12 hr.) Despite the premixing and the presence of a great excess of Fab fragments relative to Hb and Hp, there was no inhibition of Hp binding to Hb. The two rabbit anti-Hb sera

tested had 4.5 and 1.7 mg/ml of precipitating antibody, respectively. The number of antigenic sites on Hb is between 6 and 7 per dimer (Noble *et al.*, 1969). If pepsin digestion destroyed no antibody-combining sites then the Fab/Hb ratio was 84 and 30 for the two rabbit sera on a molar basis. Since there are six sites per dimer this means that the ratio of Fab per site (assuming equal distribution of the various Ab populations) would be 14 and 5, respectively. Even if 50–75% of the Ab sites was destroyed by pepsin a good excess of Fab per site would still be available. Qualitatively the pepsin digested sera were potent inhibitors of the precipitin reaction of undigested serum with antigen in diffusion experiments in agar. Thus, the second approach like the first points to complete independence of the binding sites on Hb for Ab and Hp, respectively.

TABLE V: Precipitin Data on Reactions of Rabbit and Goat Antihuman Hb Sera with Hb and Hp–Hb.

Antiserum[b]	Max μg of Ab Protein Pptd with 1 ml of Serum		% Homologous Reaction Achieved with Hp
	Hb	Hp–Hb[a]	
Ra 286-B-2[c]	916	921	100.5
Ra 340-B-1	1730	1750	101
Goat 6[c]	1150	1187	103

[a] The source of the complex was human serum of Hp type 1-1. Excess human HbA was added to serum and the Hp–Hb complex and free Hb were separated completely by Sephadex G-100 gel filtration. [b] Values reported are averages of two determinations, one using an HbA_1 solution separated from Hp–Hb by gel filtration and the other using pure HbA. [c] Hb of the identical animal was added to each animal antiserum to ensure that the antisera contained no free Hp.

Discussion

It has been reported by a number of authors (Liang, 1957; Jayle and Moretti, 1962; Sasazuki, 1970) that human Hp is capable of binding various animal Hbs. While gel electrophoresis of human serum and animal Hbs can give evidence of Hp binding, it cannot give information of the relative affinities of the Hbs for Hp. It was hoped that more sensitive and quantitative techniques as well as the study of more hemoglobins (painted turtle, leopard frog, and carp) would yield information on relative affinities and that such data, in turn, might suggest the location of the Hp binding site on Hb.

Fluorescence quenching titration at the concentrations used here (1×10^{-6} M Hp) is a technique that should permit the comparison of Hbs whose equilibrium association constants for Hp are less than about 10^7 M^{-1}. Such Hbs would not give the typical straight titration curve with a sharp end point as seen in Figure 2 but would give curves that deviate from a straight line in the direction of higher Hb concentrations. Hbs that bind Hp with association constants greater than 10^7 M^{-1} all appear indistinguishable in this test. The results of the fluorescence quenching studies indicate that human, sheep, rabbit, major chicken, and painted turtle Hbs bind Hp with association constants higher than 10^7 M^{-1} at 28°. However, northern leopard frog Hb binds with a much lower affinity ($K_a = 4.6 \times 10^5$ M^{-1}) and carp Hb binds even more poorly than frog. Kinetic studies of both the dissociation and association rates of the hemoglobin–haptoglobin reactions have extended these comparisons.

Using the sensitive displacement assay no dissociation of the complexes of human Hp with human, rabbit, bovine, chicken, or turtle Hb was detectable. This is in marked contrast to the situation with the antibody system where at 35° about 90% of human Hb dissociates from its antibody in 3 days and the complex of bovine Hb with antihuman Hb antibodies dissociates essentially completely in 2 hr at 35°. Antibodies are heterogenous, and the dissociation reactions exhibited the expected heterogeneity. Nonetheless, it was possible to estimate that at 4° the fastest of the dissociating complexes had an average $t_{1/2}$ of about 7×10^5 sec. It was also possible to set lower limits on the sensitivity of the technique and the longest measurable $t_{1/2}$ was estimated to be 2×10^6 sec. Even at 35°, from 5 to 10% of the Hb–antihuman Hb dissociated so slowly that the $t_{1/2}$ of this fraction is greater than 2×10^6 sec. We can by this same approach set a lower limit for the $t_{1/2}$ of the dissociation of the Hp–Hb complex as being 2×10^6 sec at 4°.

For Hbs that bind essentially irreversibly to Hp the comparison of their ability to compete with labeled human Hb for binding sites on Hp gives a measure of their relative rates of binding. For Hbs that can dissociate appreciably from Hp during 1 hr at 4°, the competition experiments would reflect the rate of dissociation as well as the rate of association. As expected from the results of fluorescence quenching titrations, it was found that frog Hb competes very poorly and carp Hb does not compete at all. In such tests, turtle Hb can be easily distinguished from the higher animal Hbs, since the turtle Hb binds human Hp much more slowly than these animal Hbs. Within the sensitivity of the method, rabbit, horse, bovine, and chicken Hbs all bind human Hp with the same rate as does human Hb. However, sheep and goat Hbs react with Hp at a faster rate than human Hb.

Many authors (Laurell and Grönvall, 1962; Shim *et al.*, 1965; Bunn, 1967; Chiancone *et al.*, 1968; Giblett, 1968; Nagel and Gibson, 1971) have shown that the unit of Hb that binds Hp is the α,β dimer, not the Hb tetramer. Differences in the tetramer–dimer equilibrium, then, might explain some of the differences in reactivity of the animal Hbs with human Hp. Another possible explanation, of course, is that the reduced affinity of an animal Hb dimer for Hp results from differences in amino acid sequence. Variations in sequence could result in alterations in three-dimensional structure of the Hp binding site or in amino acid substitutions of the Hp binding site itself. Since the studies of Perutz *et al.* (1968) have shown that the overall conformations of the α and β chains of hemoglobin are similar to the structure of myoglobin (Kendrew, 1962) despite extensive differences in amino acid sequence, it is not likely that the differences in sequence between the animal Hbs would be associated with gross variations in tertiary or quaternary structure.

Since Hb must dissociate to dimer before it can bind Hp, the simplest explanation for the faster rate of binding of goat and sheep Hbs as compared to that for human Hb is that goat and sheep Hbs form dimers more readily than human Hb. Gel filtration experiments by Gilbert (1966) indicate that sheep Hb is indeed more dissociated than human Hb at pH 7 supporting such an interpretation. The behavior of painted turtle Hb can also be explained in part on the basis of the tetramer–dimer equilibrium. Turtle Hb binds very slowly but with high affinity. It is possible, then, that turtle Hb is dissociated less

readily to dimer than human Hb, but that the dimer, once formed, can bind Hp as well as the human dimer. Cross-linking of tetramers to form large polymers has been reported for some turtle Hbs but it does not occur with painted turtle Hb.

The poor binding demonstrated by leopard frog and carp Hbs cannot be explained only on the basis of a poorer dissociation to dimer. These Hbs do not give stoichiometric binding in fluorescence quenching experiments with Hp although the reactions at least with frog Hb appear to have been completed. The reversible binding of these Hbs could be due to a reduced affinity of the dimer for Hp or a reduced affinity as well as reduced tetramer dissociation to dimer. There is some evidence that carp Hb does not form dimers easily.[2] Thus, the facts to be explained are that human, rabbit, sheep, and chicken Hbs bind human Hp at a rapid rate and irreversibly, turtle Hb binds to Hp slowly but irreversibly while leopard frog and carp Hbs bind slowly and reversibly to human Hp. Since the turtle, frog, and carp are in increasing evolutionary distance from the other animal species involved in this study it is reasonable to think that the differences between the amino acid sequences of their polypeptide chains and those of the chains of human Hb should have a similar relationship. That is, the turtle sequence should be most closely related to the human sequence, the frog sequence should be intermediate and the carp sequence most distantly related. It is tempting to think that the poorer binding to human Hp of these lower vertebrate Hbs is due at least in part to primary sequence changes in portions of the hemoglobin molecule which are more or less identical in all mammalian Hbs. We shall return to this matter again at the end of the discussion when considering the possible location of binding of Hp to Hb.

While Hp type 1-1 is a homogeneous population of molecules each of which is presumably directed against the same site on the Hb dimer, antibodies in antihuman Hb sera are heterogeneous and bind to many different sites on Hb. A typical rabbit antiserum may be directed against six–eight antigenic sites per Hb dimer (Noble *et al.*, 1969; Reichlin, 1972). None of the antibodies are able to bind rabbit Hb (Reichlin, 1970; Reichlin, 1972) which is not surprising since it is expected that a rabbit would be tolerant to its own Hb. Therefore, antigenic sites on human Hb must be regions of the molecule that differ from rabbit Hb. Amino acid sequence data (*cf.* Dayhoff and Eck, 1968) shows that human Hb differs from rabbit Hb in 25 residues of the α chain and 14 residues of the β chain. Variations in sequence between rabbit and human Hbs seem to control the antigenic sites and a single amino acid difference from the homologous antigen may be enough to prevent an antibody population from binding to heterologous antigen (Nisonoff *et al.*, 1970; Reichlin, 1972). We have shown by double diffusion in agar tests that antihuman Hb sera could not precipitate as well with nonhuman Hbs as it could with human Hb. Those antibodies that do bind apparently do so less well as shown by the competition experiments and measurement of rates of dissociation of antibodies from human and bovine Hbs.

Human Hb differs from other Hbs in a minimum of 13 residues per chain, the average number of differences with mammalian Hbs being about 20 residues (*cf.* Dayhoff and Eck, 1968). If an amino acid variation occurs in the region of an antigenic site, then it would be expected that antibodies directed toward this site would bind the modified antigen with reduced affinity or not at all. Changes that do not affect an antigenic site should not affect antibody binding. The results indicate that many of the sequence differences between human Hb and the other Hbs studied modify antigenic sites. These observations suggest that the antigenic sites on Hb and the Hp binding site are different. In addition, experiments presented here show that human Hb and complexes of human Hb–Hp precipitate equivalent amounts of antibody from rabbit antihuman Hb sera and an excess of antihuman Hb antibodies (as Fab fragments) does not block the human Hp–Hb reaction. These experiments provide further evidence for the independence of Hp and antibody binding sites on human Hb. These data are most consistent with the hypothesis that antigenic determinants are in topological regions on the hemoglobin surface which vary greatly in evolution while the binding site for haptoglobin is in a portion of the hemoglobin surfaces which has been relatively conserved in evolution.

Although assigning the Hp binding site to an invariant region on Hb does not identify the binding site, when combined with other kinds of information it can contribute to this identification. Since the Hb dimer binds Hp, there must either be a conformation change during tetramer dissociation that permits binding or the uncovering of a previously inaccessible site. The simplest theory is that the contact region between the two α,β dimers contains the Hp binding site. Other investigators have also suggested that (Nagel *et al.*, 1971; Makinen *et al.*, 1972). It is thought that the Hb tetramer dissociates to the α^1,β^1 and α^2,β^2 dimers (Rosemeyer and Huehns, 1967; Park, 1970). The amino acids in the contact region between the dimers appear to be quite constant. The points of contact between like chains are few in number and involve the α-amino and carboxyl-terminal residues (Perutz, 1969). Therefore, they are not pertinent to the discussion. The contact region between the α^1 and β^2 chains, however, is extensive. The ten residues of the α^1 chain that are thought to come in contact with the β^2 chains and the nine residues of the β^2 chains that came in contact with the α^1 chain (Perutz, 1969) are constant in all mammalian α and β chains that have been sequenced (*cf.* Dayhoff and Eck, 1968). The only known differences in the 19 contact residues of the Hbs used in this study are in the carp α chain where two of the ten contact residues are altered.

Thus, the contact regions between the α^1,β^1 and α^2,β^2 dimers would be a likely area for Hp binding. The amino acid positions of Hb implicated by Makinen *et al.* (1972) as possibly being involved in the binding site of Hp are in the contact region between α^1 and β^1 (Perutz, 1969) and, therefore, would not be expected to be available for Hp binding by the α^1,β^1 dimer.

In summary then we propose that Hp binds human Hb in the contact region of the two α^1,β^1 dimers which are exposed when such dimers are formed from tetramer.

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[2] Q. H. Gibson and R. W. Noble (unpublished results) have shown that even at a concentration of 0.2 μM in heme carp hemoglobin exhibits no concentration-dependent quickly reacting form, which in human hemoglobin is associated with dimer formation.

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