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Immunochemical Studies of Hemoglobin and Myoglobin and Their Globin Moieties

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Rabbit antisera to porcine hemoglobin and horse muscle myoglobin have been characterized. Antihemoglobin was identified by immunodiffusion analysis and constant specific immunologic activity during chromatography. Similar techniques were used to identify the antigen-antibody system being measured by C′ fixation as myoglobin-antimyoglobin. Both antihemoglobin and antimyoglobin detected specific conformational differences between the heme proteins and their globin moieties. Addition of heme to the globin moieties restored full serologic capacity to the heme proteins. Restoration of specific antigenic competence by addition of heme to globin permitted measurement of heme transfer from heterologous hemoglobin to the globin moieties of porcine hemoglobin and horse muscle myoglobin.

Precipitating antibody to hemoglobin was first conclusively demonstrated by Heidelberger and Landsteiner in 1923. In subsequent years reports confirming both the antigenicity and the narrow species specificity of the hemoglobins have appeared (Hektoen and Boor, 1931; Johnson and Bradley, 1935). The study of globin, the apoprotein of hemoglobin, received little immunochemical characterization because, until recently, isolated globins were heterogeneous (Gralen, 1939; Moore and Reiner, 1944; Havinga and Itano, 1953) and their reconstitution products, on addition of heme, were different from native hemoglobins (Gralen, 1939). Rossi-Fanelli *et al.* (1958) have recently described the preparation of homogeneous human globin preparations and have reconstituted native human hemoglobin, as measured by physical variables, by addition of stoichiometric amounts of heme.

The present report is concerned with the immunochemical characterization of hemoglobin and myoglobin and their globin moieties. Our objective was to examine the possibility that conformational changes occurring during conversion of hemoglobin could be detected by antibodies to the heme proteins, and to use this sensitive method to measure transfer of the hemes to their respective globin moieties.

MATERIALS AND METHODS

Antigens: Porcine Hemoglobin.—Either crystalline porcine hemoglobin (purchased from Pentex Corp.) or porcine hemoglobin twice crystallized from fresh red blood cells by the method of Drabkin (1946) was used. The hemoglobin was crystallized from both 2.8 M PO_4, *p*H 6.8, and 65% saturated $(NH_4)_2SO_4$ adjusted to *p*H 6.8. The crystals were washed and suspended in either strong salt solution at 4°. Under these conditions the hemoglobin was maintained as oxyhemoglobin for 2–3 months as judged by periodic spectral determinations. Methemoglobin was prepared from oxyhemoglobin by treatment with ferricyanide, followed by dialysis as described by Austin and Drabkin (1935).

Porcine globin was prepared only from freshly crystallized hemoglobin and the method of Rossi-Fanelli *et al.* (1958) was used. All experiments with globin were performed within 48.0 hours of its preparation. Porcine globin has properties similar to human globin and will be described under Results. All preparations used had less than 1 mole% unsplit heme as judged by spectral analysis.

Horse Muscle Myoglobin.—Initially horse heart myoglobin, twice crystallized (purchased from Pentex Corp.), was used for immunization. This material was found subsequently to contain 3% hemoglobin, an unidentified nonheme protein, and also the chromatographically separable myoglobins described by Akeson & Theorell (1960). The immune sera prepared from this antigen was heterogeneous and will be described in detail under Results.

Crystalline horse muscle myoglobin was also prepared by a modification of Bowen's method (1948). This material was recrystallized three times and was uniformly crystalline microscopically. The dissolved crystals were dialyzed exhaustively against 0.005 M PO_4, *p*H 6.4, and chromatographed on carboxymethylcellulose previously equilibrated with the same buffer. Five peaks were separated by this procedure and identified spectroscopically and ultracentrifugally as myoglobin. The major fraction, which constituted about 80% of the myoglobin, eluted sharply between *p*H 6.9 and 7.2. It was reapplied to carboxymethylcellulose equilibrated with 0.02 M PO_4, *p*H 6.4.

Under these conditions a major fraction separated as a sharp band and was preceded by a faster component. The resin was gently blown from the column by compressed air, cut out, and eluted with 0.2 M Na_2HPO_4

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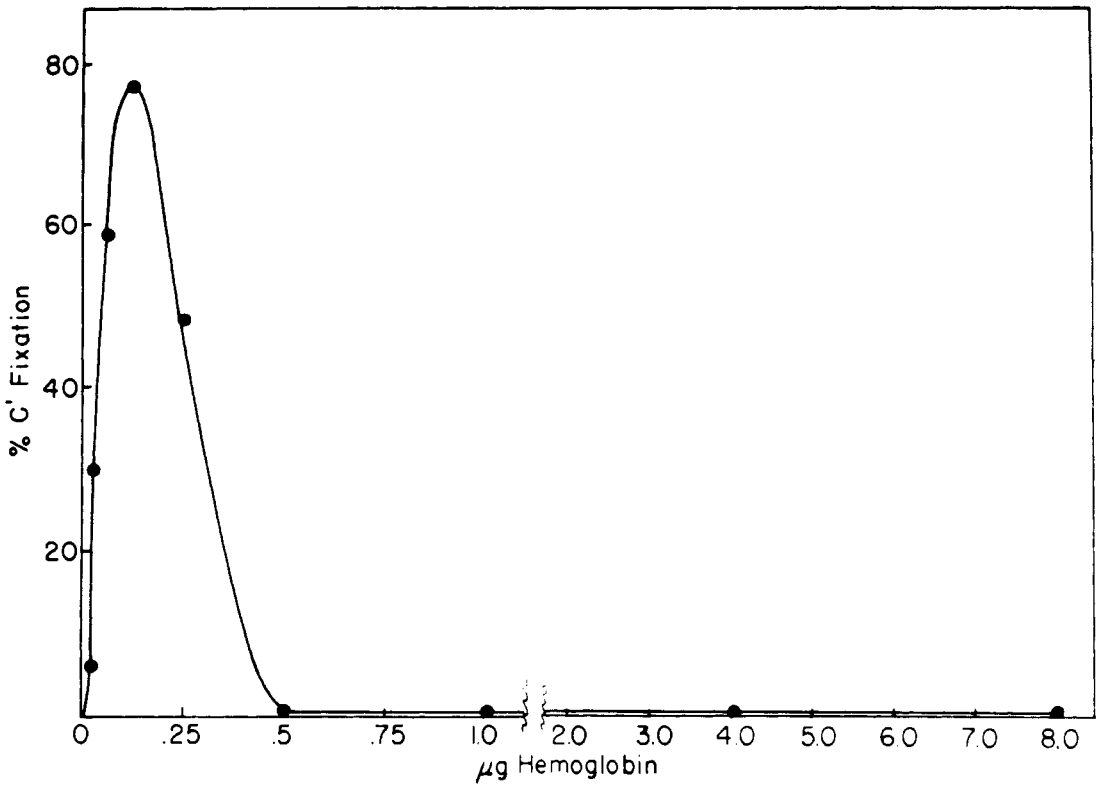

FIG. 1.—Fixation of C′ by increments of porcine hemoglobin and antiporcine hemoglobin (Ra-87, 1/350).

as described by Akeson and Theorell (1960). This material, thought to be identical with Mb_1 myoglobin, was dialyzed against 0.01 M PO_4, *p*H 7.0, 0.15 M NaCl.

The globin of myoglobin was again prepared by the method of Rossi-Fanelli *et al.* (1958). Experiments were conducted within 48 hours of the preparation of the globin.

Immunization.—For production of rabbit antisera, 20.0 mg of antigen in a volume of 1.0–1.5 cc mixed with an equal volume of complete Freund's adjuant was injected into the toepads and intramuscularly. Animals were bled three weeks after the initial injection and were boosted with 10.0 mg of the antigen intravenously. Bleedings were taken 7 days following the intravenous injection. Thereafter intraveneous injections were given about once a month. Sera were tested for presence of antibody by double diffusion in agar. Complement C′ fixation was performed by the method of Wasserman and Levine (1961).

Estimation of Protein.—The concentrations of the hemoglobin and myoglobin were measured as cyanmethemoglobin and metmyoglobin using millimolar extinction coefficients of 11.5 at 540 mμ and 160.0 at 410 mμ, respectively. The extinction coefficient for pork globin was determined by Kjeldahl analysis and found to be $E'^{1\%}_{280\ m\mu}$ 1.0 cm = 8.0. A value of $E^{1\%}_{280\ m\mu}$ 1.0 cm of 7.0 was used for the horse muscle myoglobin globin.

Analytical Methods.—Determination of S values were carried out in a Spinco Model E analytical ultracentrifuge. Starch gel electrophoresis was performed according to Smithies (1955). Runs were for 24 hours at 4° at voltages of 6.0 v/cm. Double diffusion experiments were performed in 1% agar at 20° and 4°, and plates were observed for 2 weeks before discarding. mmunoelectrophoresis was performed in 1% agar at IH 8.6, 0.05 M Veronal for 4 hours at 4° at 10 v/cm. ter the electrophoresis, antiserum as added to a 1.0 mm trough and the plate developed as an ordinary double diffusion experiment. Determinations of the diffusion coefficient by immunodiffusion were performed as described by Allison and Humphrey (1959).

RESULTS

A. Identification of the Antihemoglobin Sera.—Studies were carried out to establish the immunochemical purity of the system and to identify the reaction being measured as hemoglobin-antihemoglobin.

Data indicating immunochemical homogeneity were obtained by observation of a single sharp pink precipitin line in double diffusion in agar. A single pink arc was also observed by immunoelectrophoresis at *p*H 8.6, and this arc corresponded exactly in position to the visibly migrating hemoglobin band.

Determination of the diffusion coefficient by the rectangular trough method of Allison and Humphrey (1959) yielded an average value of 6.5×10^7 cm^2 sec^{-1} for the antigen. The value of the diffusion coefficient of hemoglobin measured by free diffusion techniques is 6.9×10^7 cm^2 sec^{-1} (Tanford, 1961). C′ fixation set up over a wide range of antigen concentrations revealed a single curve with maximum fixation occurring at 0.1 μg of the antigen (Fig. 1).

Finally the pork hemoglobin was chromatographed on carboxymethylcellulose utilizing a *p*H gradient for elution of the protein. The CMC and the hemoglobin solution containing 39 mg oxyhemoglobin were equilibrated with 0.01 M PO_4 buffer, *p*H 6.4. To establish the gradient, a reservoir containing 500 cc 0.01 M PO_4, *p*H 8.3, was connected to a mixing vessel containing the equilibrating buffer and the flow rate was adjusted to 60 cc/hour. The data in Figure 2 demonstrate the elution pattern and the quantitative estimation of antigen as measured by C′ fixation. More than 95% of the protein eluted in a narrow range of the

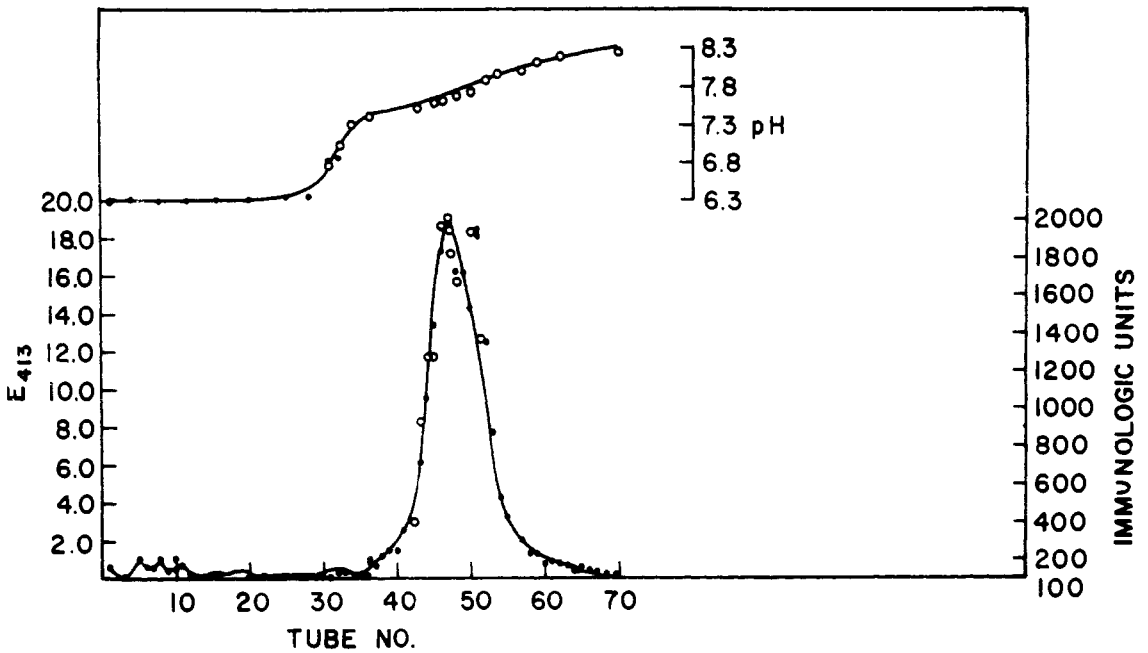

FIG. 2.—Chromatography of porcine hemoglobin on carboxymethylcellulose utilizing *p*H gradient for elution. Absorbance 413 (●); antigenic activity (○). Absorbance is total color yield per tube. Immune serum for C′ assay, Ra-87, 1/350.

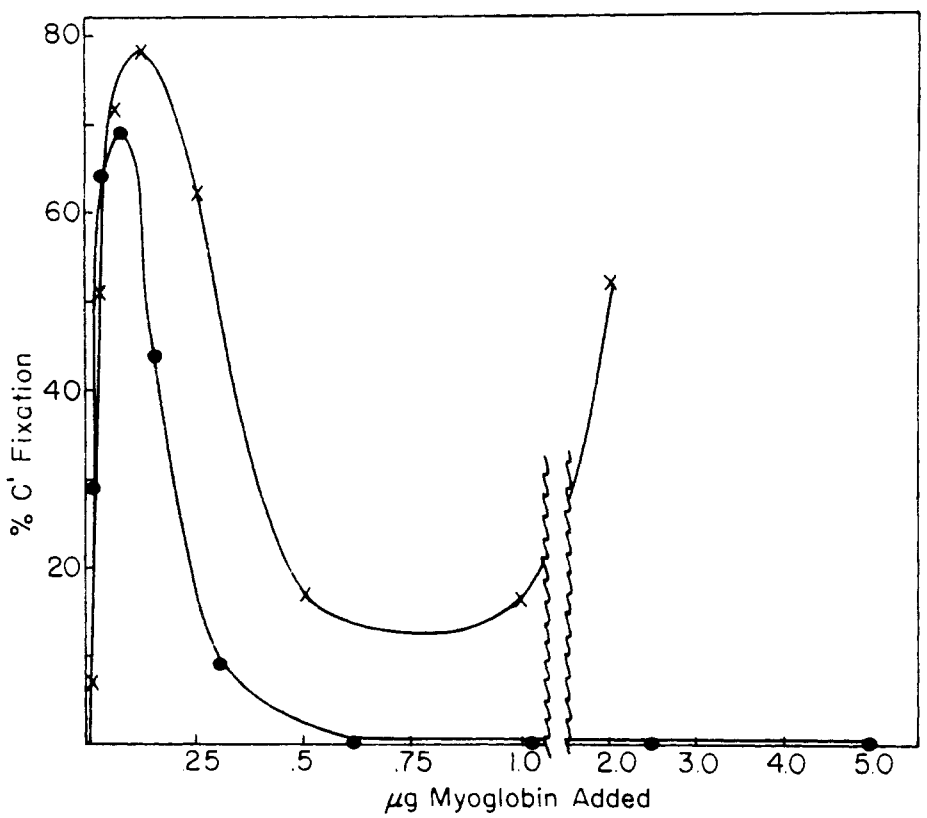

FIG. 3.—Fixation of C′ by increments of horse muscle myoglobin and antihorse-muscle myoglobin (Ra-172, 1/100). Two × crystalline horse heart myoglobin (Pentex) (×); chromatographically purified Mb_1 myoglobin from crystalline horse muscle myoglobin, (●). Immune serum for C′ fixation Ra-172, 1/100.

gradient (0.3 *p*H unit), yielding a symmetrical curve with excellent correspondence between absorbance at 413 mμ and immunologic activity.

B. Identification of the Antimyoglobin Sera.—Whereas the immunochemical homogeneity of the system was demonstrated with the antihemoglobin serum, the horse heart myoglobin antiserum was immunochemically heterogeneous. This undesired heterogeneity was circumvented by extensive purification of the antigen to be used in the C′ fixation experiments.

One of the nonmyoglobin antigen-antibody systems was identified as horse hemoglobin-antihemoglobin and the others were not identified. Crystalline myoglobin was chromatographed on carboxymethylcellulose utilizing a *p*H gradient as described under Materials and Methods. The major component of the second chromatographic run (designated Mb_1 by Akeson and,

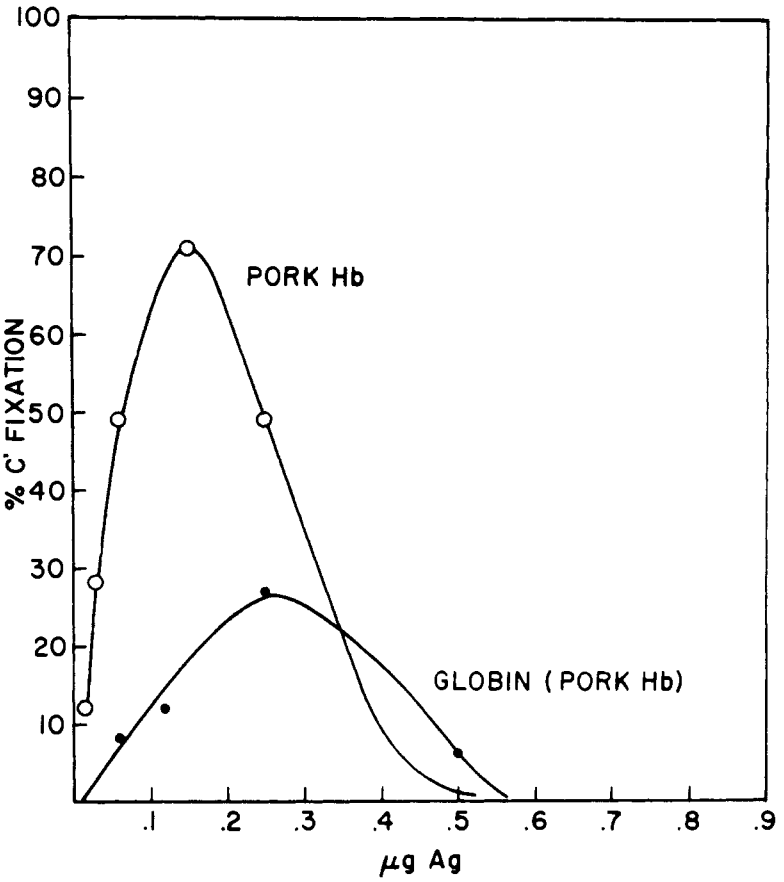

FIG. 4.—Fixation of C′ by increments of porcine hemoglobin (O) and porcine globin (●). Serum for C′ fixation, Ra-87, 1/350.

Theorell, 1960) migrated as a single heme-protein at *p*H 8.6 and 6.0 in starch gel electrophoresis. This protein was considered homogeneous. It gave a single C′F curve over a wide Ag concentration (Fig. 3) and a single pink precipitin line in agar diffusion, and was the material utilized in all the following experiments.

C. Immunochemical Relationship of Hemoglobin and Its Globin Moiety.—In Figure 4 are seen C′ fixation curves obtained when hemoglobin and globin reacted with antihemoglobin. Globin was less effective antigenically as judged by the decrease in maximum fixation and the increased quantity of protein required for this maximum. The extent of C′ fixation (at peak) with globin varied with different preparations but was always significantly less than that of hemoglobin. Furthermore, peak fixation was always reached with higher concentrations of globin.

That the globin and hemoglobin were reacting with the same population of antibody was demonstrated by inhibition experiments in which a large excess of globin completely inhibited the C′ fixation reaction of hemoglobin-antihemoglobin. The possibility that hematin was an antigenic determinant is unlikely since hematin in 30,000 molar excess failed to inhibit the reaction of hemoglobin-antihemoglobin.

D. Reconstitution of hemoglobin from globin.—Globin was prepared according to Rossi-Fanelli *et al.* (1958) and had the following properties: It sedimented in the ultracentrifuge as a single symmetrical boundary with an $s_{20,w}$ = 2.62 at a concentration of 5 mg/ml. For reconstitution experiments, increments of hematin were added to globin solutions (100 μg/ml) at *p*H 7.0, 0.1 M PO_4 buffer. The reaction mixtures were equilibrated at 0° for 24 hours. Spectral analyses were performed on the reaction mixtures while C′ fixation was performed on appropriate dilutions. As can be seen in Figure 5, the globin hematin reaction had a stoichiometry of 4.0 moles of heme bound per 66,000 g of proteins.

Physical parameters measured on the fully reconstituted product (4.0 moles heme bound/66,000 g protein) included ultracentrifugation and starch gel electrophoresis. The fully reconstituted product had an $s_{20,w}$ value of 4.26 at a concentration of 0.5% and sedimented as a single symmetrical boundary. Intact hemoglobin has an $s_{20,w}$ of 4.3. Globin with 4.0 moles of heme bound had the same electrophoretic mobility on starch gel as native pork hemoglobin at *p*H 8.6.

The immunological reactivity of the globin solutions with integral numbers of moles of heme bound is seen in Figure 6. An increase in reactivity was observed with every mole of heme bound and complete reactivity was achieved with only 3.0 moles.

In all reconstitution experiments, essentially the same results were observed, i.e., complete reconstitution occurred with addition of 3.0 moles of heme. In free solution, when the reaction mixture containing 3.0 moles of heme was examined electrophoretically, a minor heme-containing slower component was observed. In the same reaction mixture, a minor lighter component was observed in ultracentrifugal analysis. The reaction of globin with antihemoglobin, however, varied quantitatively with each globin preparation We have attempted to account for this variation in globin reactivity but have not been successful. For comparison with a globin preparation which reacted poorly with antihemoglobin (Fig. 6) is seen a reconstitution experiment in which the globin preparation was least discriminated from hemoglobin by the antihemoglobin (Fig. 7). With this globin preparation, re-

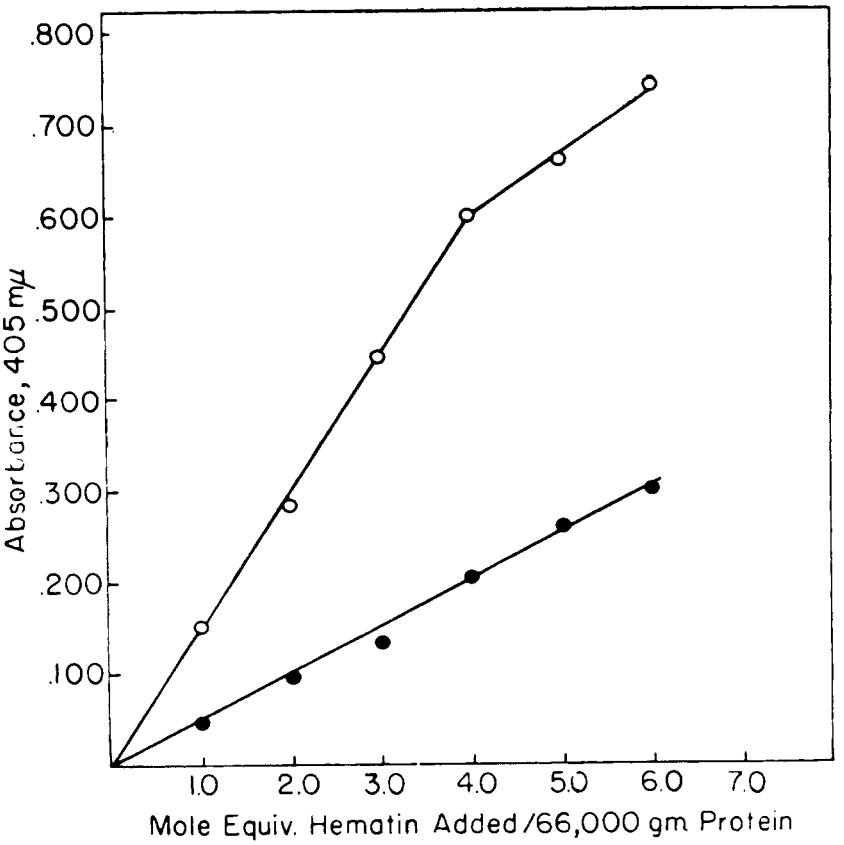

FIG. 5.—Spectral titration of procine globin with hematin. Hematin added in increments 1.0 μg/100 μg pork globin. Hematin alone (●); hematin and porcine globin, 100.0 μg/ml in all tubes (○).

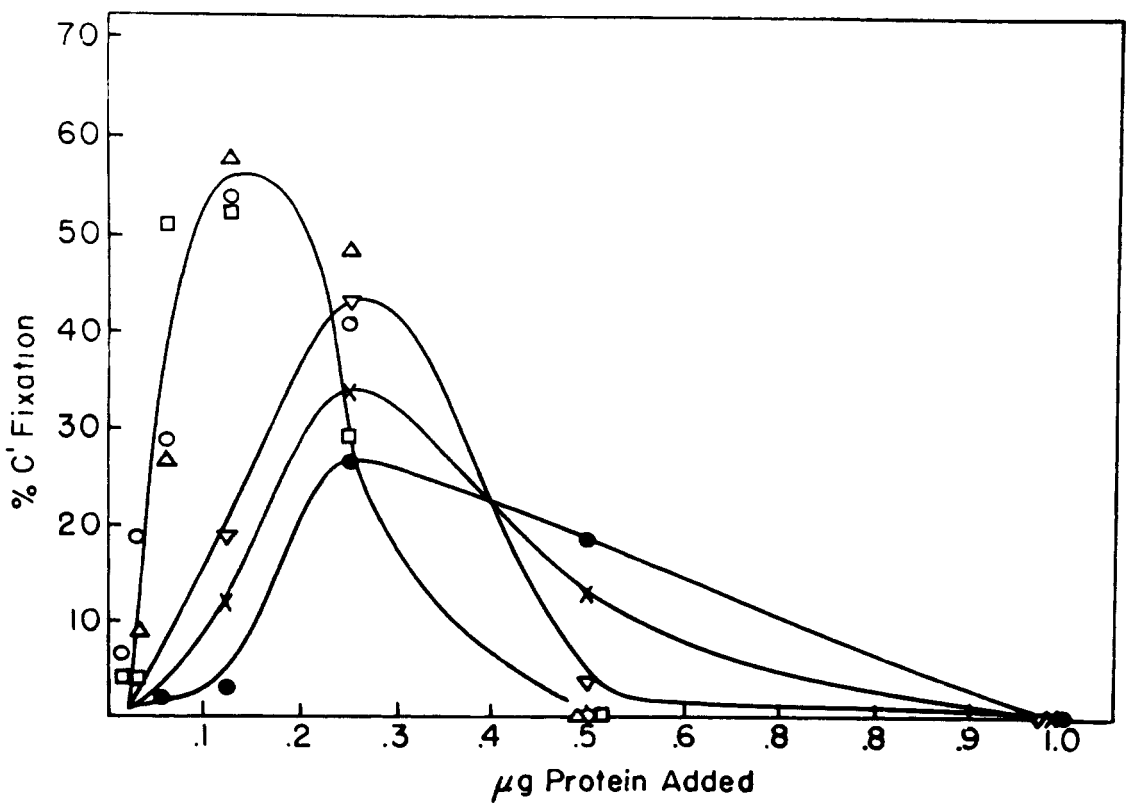

FIG. 6.—Fixation of C′ by increments of porcine globin, hemoglobin, and partially reconstituted hemoglobins. Globin (●); 1.0 mole hematin added (×); 2.0 moles hematin added (▽); 3.0 moles hematin added (△); 4.0 moles hematin added (□); intact hemoglobin (○). Immune serum for C′ fixation, Ra-87. 1/350.

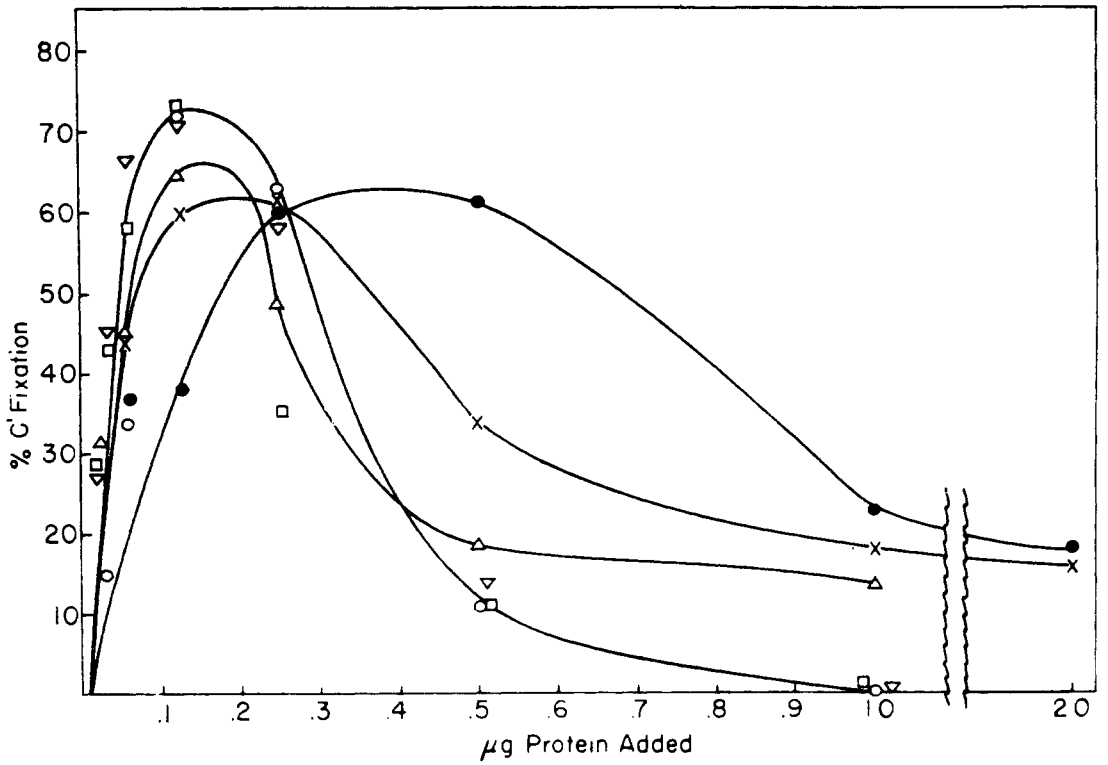

FIG. 7.—Fixation of C′ by increments of porcine globin, hemoglobin, and partially reconstituted hemoglobins. Globin (●); 1.0 mole heme added (×); 2.0 moles heme added (△); 3.0 moles heme added (▽); 4.0 moles heme added (□); intact hemoglobin (○). Immune serum for C′ fixation, Ra-87, 1/350.

constitution was observed by a lateral displacement of C′ fixation to lower antigen concentrations. In Figure 6, however, reconstitution was observed not only by this lateral displacement but also by increases in maximal fixation. Globin solutions more than 72 hours old failed to exhibit complete reconstitution either by spectral or immunologic analysis.

Reconstitution of hemoglobin was also observed by double-diffusion experiments. Globin gave only faint bands with antihemoglobin. The same addition of heme as used previously (Fig. 5) resulted in strong bands of precipitation with identity patterns with themselves (2, 3, and 4 moles of heme addition) and hemoglobin.

In order to evaluate the possibility of heme transfer from either the rabbit hemoglobin in the whole antiserum or guinea pig hemoglobin in the guinea pig serum, the following experiments were performed. Hemoglobin-free gammaglobulin was prepared by cyclic 0–37% $(NH_4)_2SO_4$ precipitation of the immune serum and a hemoglobin-poor (<10.0 γ/ml) preparation of G.Pig C′ obtained by heart puncture of a guinea pig with rapid separation of the serum from the cells. This gamma globulin preparation and C′ gave the same results as unfractionated rabbit serum and commercial C′. Three moles of heme reconstituted the complete antigen.

E. Immunochemical Relationship of Myoglobin and Its Globin Moiety.—Studies similar to those performed on hemoglobin were also carried out with myoglobin. As pointed out in the section on characterization of the sera, 172-B-1, the antimyoglobin serum was heterogeneous, and therefore all studies were carried out with a highly purified myoglobin antigen.

The difference in reactivity of myoglobin and its apoprotein, globin, is shown in Figures 9 and 10. The same characteristics are exhibited in the myoglobin systems as in the hemoglobin system. Myoglobin globin fixes less C′ at peak and requires a higher antigen concentration to reach peak fixation. As observed with globin prepared from hemoglobin, the globin of myoglobin also exhibited variation in reactivity with antimyoglobin. Heme was not inhibitory when tested with 100,000 molar excess whereas globin in high excess did completely inhibit the homologous system.

F. Reconstitution of Myoglobin from Globin.—Titration of the globin from myoglobin revealed that 17,000 g of protein bound 1.0 mole of heme as measured by hyperchromicity at 405 mμ (Fig. 8). Immunological experiments on the same reaction mixtures revealed that full serologic reactivity was not reached until each monomeric myoglobin chain had bound an equivalent quantity of heme (Fig. 9). It should be noted that a small difference between reconstituted myoglobin and myoglobin is discernible in the region of antigen excess. The reason for this small difference remains unexplored.

G. Heme Transfer from Rabbit Hemoglobin to Globin of Hemoglobin or Myoglobin.—The serological specificity of the hemoglobin and myoglobins as well as the differences in reactivity of their globin moieties suggested a procedure for measurement of heme transfer from hemoglobin or myoglobin to globin. Rossi-Fanelli and Antonini (1959) had demonstrated transfer but only between proteins that had marked spectral or electrophoretic differences.

For measurement of heme transfer, the globin moiety of porcine hemoglobin and the globin of horse heart myoglobin were used as the acceptor and rabbit methemoglobin was used as the heme donor. Transfer was observed by the reconstitution of the appropriate heme protein.

Rabbit hemoglobin (100 μg) was incubated in 0.1 M PO_4 at *p*H 7.0 with porcine hemoglobin-globin or myoglobin-globin (20 μg), respectively. The mixtures were allowed to incubate 1.0 hour at room temperature and were then assayed for immunological reactivity.

The data in Figure 10 illustrate the reaction of anti-

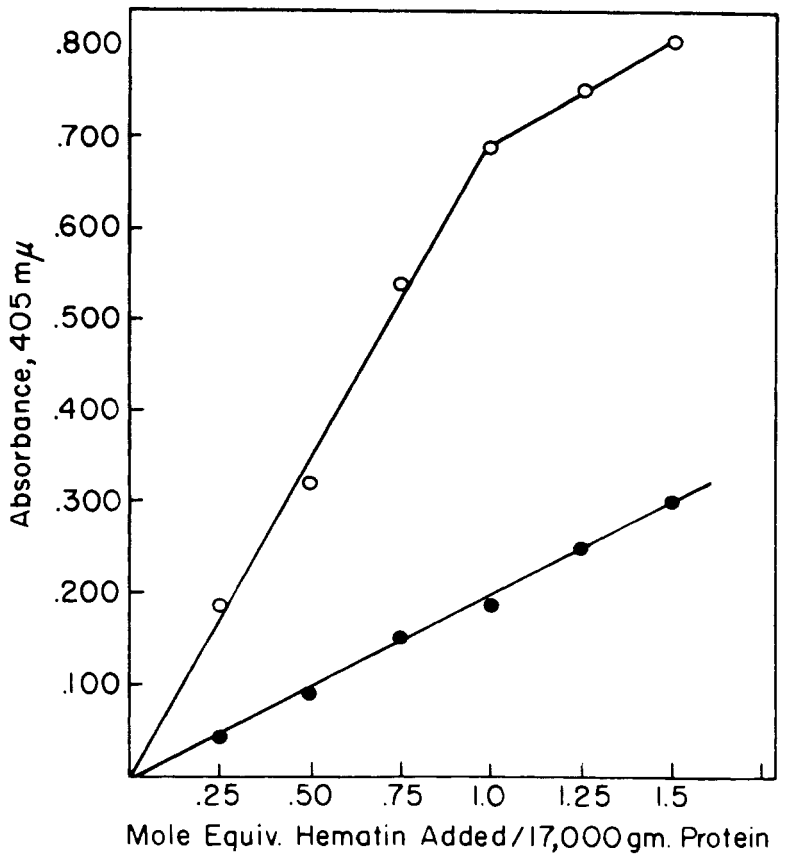

FIG. 8.—Spectral titration of Mb_I muscle myoglobin globin with hematin. Hematin added in increments of 1.0 μg/100 μg myoglobin globin. Hematin alone (●); hematin and Mb_I globin, 100.0 μg/ml in all tubes (○).

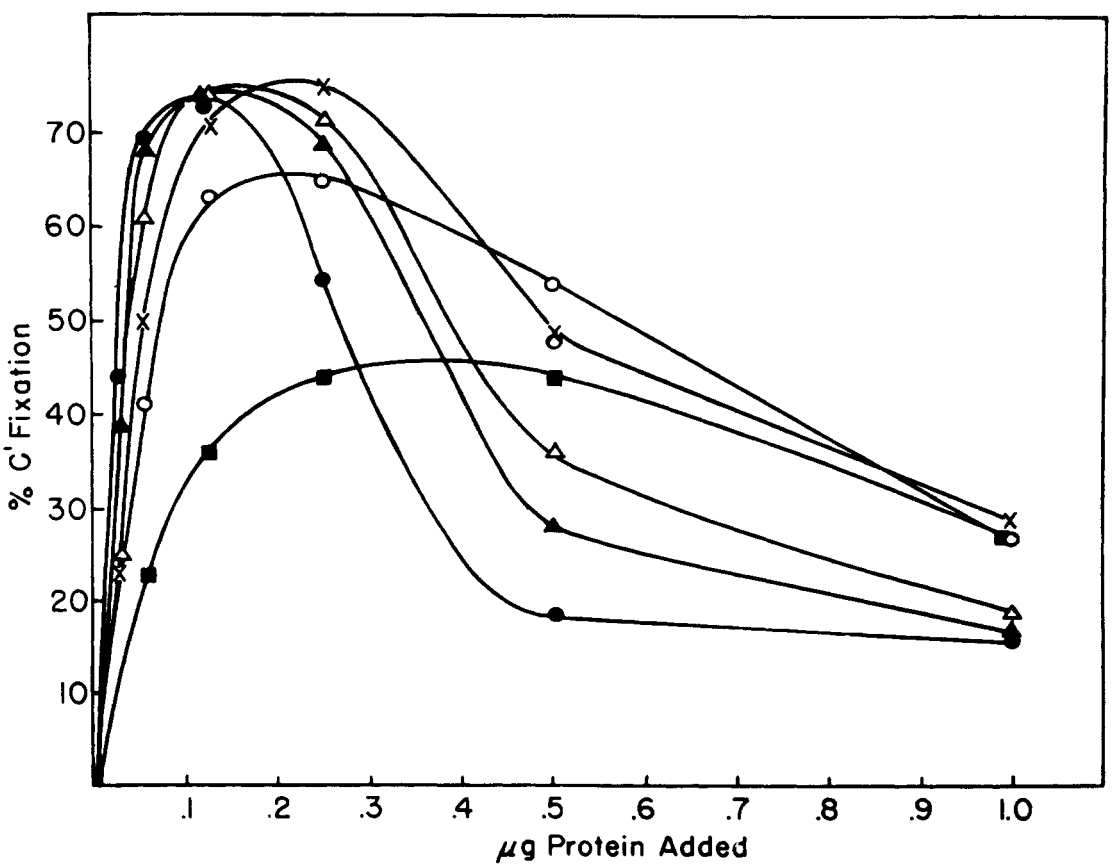

FIG. 9.—Fixation of C′ by increments of Mb_I globin, Mb_I myoglobin, and partially reconstituted myoglobin. Mb_I globin (■); 0.25 mole hematin added (○); 0.50 mole hematin added (×); 0.75 mole hematin added (△); 1.0 mole hematin added (▲); Mb_I myoglobin (●). Immune serum for C′ fixation, Ra-172, 1/100.

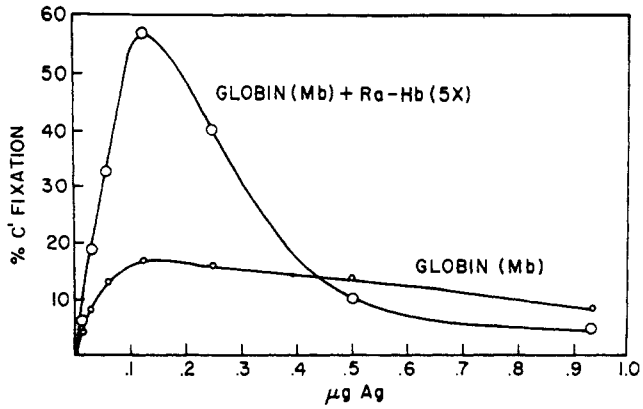

FIG. 10.—Fixation of C′ by increments of Mb_1 myoglobin globin (●) and Mb_1 myoglobin globin incubated 1.0 hour in 0.1 M phosphate buffer, *p*H 7.0, with rabbit methemoglobin (○) in the weight ratio of 1:5. Immune serum for C′ fixation, Ra-172, 1/100.

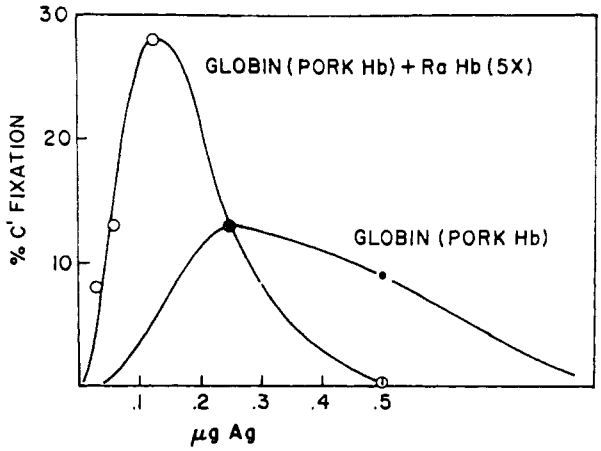

FIG. 11.—Fixation of C′ by increments of porcine globin (●) and porcine globin incubated 1.0 hour in 0.1 M phosphate buffer, *p*H 7.0, with rabbit methemoglobin (○) in the weight ratio of 1:5. Immune serum for C′ fixation, Ra-87, 1/350.

myoglobin sera with myoglobin-globin and myoglobin-globin incubated with rabbit methemoglobin. The reactivity of the myoglobin-globin was increased markedly; heme was transferred from the rabbit methemoglobin to the myoglobin-globin. (Rabbit hemoglobin itself neither reacts with this antiserum directly nor inhibits the homologous system). Similarly the data in Figure 11 illustrate the partial reconstitution of porcine globin after incubation with a 5-fold weight excess of rabbit methemoglobin. Quantitative aspects of heme transfer with different donors and acceptors is presently under investigation.

DISCUSSION

It has long been appreciated that immunochemical specificity, differences in absorption spectra, and function with respect to oxygen and carbon monoxide equilibria of mammalian hemoglobins resided in the globin moieties, since in all mammalain hemoglobins the heme is identical. Thus, globin conformation can and does measurably influence the functional and spectral properties of the heme group. Viewed from the other perspective of this relationship, it seems quite likely that the heme group influences the structure of the globin.

Physiochemical studies on globin have indicated a different structure than in the conjugated protein. Rossi-Fanelli *et al.* (1959a,b) have shown human globin to be smaller and roughly half the size of hemoglobin, to have a different shape with significantly more assymmetry, to be more labile to heat, and to have a different electrophoretic mobility than the intact heme-protein. Doty's optical rotatory dispersion studies of the globins of myoglobin and hemoglobin indicate a lower helical content than in the conjugated proteins. Breslow (1962) has described the appearance of 2–3 groups in the globin of sperm whale myoglobin which are in hydrogen ion equilibrium in the neutral *p*H range as compared to the conjugated protein.

Data presented in this paper strongly support the concept of the different conformation of globin, be it derived from myoglobin or hemoglobin, and a dependence of globin structure on its interaction with heme. In the case of myoglobin this heme-globin interaction results in a conformation of the protein in which antigenic determinants (and presumably the entire surface of the molecule) are held in their specific secondary and tertiary structures.

In the case of hemoglobin, the situation is more complicated since there is also quarternary structure to consider; that is, the relationship between the subunit α and β chains. The transition from globin to the intact tetramer hemoglobin may involve an intermediate. That is, can one or two moles of heme combine with a half molecule resulting in a stable structure different from both globin and hemoglobin? Or alternatively does heme when added in less than the stoichiometric amount lead to the formation of a tetrameric hemoglobin molecule?

The two alternatives can be restated in another way. On addition of 1 mole of heme per 66,000 g of protein, does 25% of the population of protein react with this 1 mole to form tetrameric hemoglobin, or does it distribute itself randomly among the proteins to form more than 25% of a heme protein complex? These two alternatives would appear distinguishable by our immunologic techniques, but the inhibitory competence of globin makes interpretation of our data, after one heme addition, difficult. Physical measurements to distinguish these alternatives are equally difficult. We have seen an intermediate electrophoretic band between globin and hemoglobin under these conditions in some experiments. Ultracentrifugal analysis has not been sufficiently discriminating in our hands to definitely establish the presence or absence of an intermediate complex.

The demonstration of heme transfer at neutral *p*H by immunochemical techniques open up the possibility of studying the transfer of heme between any two heme proteins which possess high immunochemical specificity and in which the heme is not covalently linked to the protein. Both these conditions are fulfilled in a large number of hemoglobins and myoglobins.

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Affinity Labeling of the Active Sites of Antibodies to the 2,4-Dinitrophenyl Hapten*

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The method of affinity labeling provides a general method for attaching a covalently bound label to the active sites of antibody and enzyme molecules. Previous studies with antibodies directed to the benzenearsonate hapten were entirely in accord with the predictions of the method. In the present studies, antibodies to the 2,4-dinitrophenyl (DNP) hapten were investigated. The labeling reagent *p*-nitrophenyldiazonium fluoborate was found to react irreversibly with an excess of the unprotected antibody: (a) at a much more rapid rate than with the antibody whose active sites were protected with an excess of *N*-DNP-ε-aminocaproic acid; (b) to give a product with essentially a pure azotyrosine spectrum; (c) at a rate which was first-order in the concentration of the reversible complex formed initially between the reagent and the antibody sites, in accord with the mechanism proposed for affinity labeling; and (d) to produce a loss of antibody binding sites corresponding closely to the number of azotyrosine groups formed. Related but less extensive studies were carried out with DNP-diazonium and *p*(carboxy)-benzenediazonium fluoborates and with other reagents. The results closely follow the predictions of the method, and provide strong evidence that a tyrosine residue is present in anti-DNP antibody sites. The results also indicate that there is a broad distribution of rates at which the antibody sites are labeled, and therefore that the sites are heterogeneous. These studies provide the basis for an attempt to isolate and analyze labeled peptide fragments from the antibody active sites.

In a previous paper (Wofsy *et al.*, 1962), we proposed and discussed a general method, called affinity labeling, to attach a covalently bound label to the active sites of antibody and enzyme molecules. For an antibody (Ab)[1] specifically directed to a given hapten (Hp) a labeling reagent HpX is used, where X is a chemical group which is constantly attached to Hp and is capable of reacting with one or more kinds of amino acid residues to form stable covalent bonds. By virtue of the initial reversible complex formed between HpX and the active site of the specific Ab, the local concentration of X in the site is greatly increased, thereby favoring covalent bond formation between X and a group in the site.

The proposed mechanism is summarized in equation (1):

$$Y + HpX \underset{k_2}{\overset{k_1}{\rightleftharpoons}} C \xrightarrow{k_3} L \qquad (1)$$

where Y is a group in the active site, C is the specific reversible complex, L is the desired covalently labeled product, and the k's are (assumed single-valued) specific rate constants for the reactions indicated. Competing with Y for the reagent HpX are a number of residues y elsewhere on the Ab molecule,

$$y + HpX \xrightarrow{k_4} M \qquad (2)$$

which can form the undesired covalently labeled product M. For simplicity, the groups y are here considered identical. If the rate of formation of L, $\dot{L}_c$, is substantially greater than the rate of formation of M, $\dot{M}$, then specific labeling of the active site can be achieved. It was shown that in the highly probable event that the complex C is in equilibrium with Y and HpX, the ratio $\dot{L}_c/\dot{M}$, which is termed the *enhancement*, is given by:

$$\dot{L}_c/\dot{M} = \frac{k_3 K_A [Y]_e}{k_4 [y]} \qquad (3)$$

where $K_A = k_1/k_2$ is the intrinsic association equilibrium constant for the formation of C, $[Y]_e$ is the equilibrium molar concentration of free Y, and $[y]$ is the molar concentration of any single unreacted residue y outside the site.

Experimental studies were also reported in the previous paper with antibodies directed to the benzenearsonate group which strongly supported the proposed mechanism, and which provided substantial evidence that a tyrosine residue was present in antibenzenearsonate Ab active sites. In order to test further the proposed mechanism of affinity labeling, and to explore the generality of its application, we have now carried out studies with antibodies to the DNP hapten, which are described in this paper.

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[1] Abbreviations used: Ab, antibody; Hp, hapten; DNP, 2,4-dinitrophenyl; DNPDF, 2,4-dinitrophenyl diazonium fluoborate; PNPDF, *p*-nitrophenyl diazonium fluoborate; CDF, *p*-(carboxyl)benzenediazonium fluoborate; DNPNS, 2-(2,4-dinitrophenylazo)-1-naphthol-3,6-disulfonic acid, disodium salt.