

ON THE ANTIGENIC PROPERTIES OF HEMOGLOBIN.*

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The antigenic properties of hemoglobin¹ are of interest because there is available in the form of the oxy-compound a crystalline substance which, unlike most other animal proteins, appears to be a chemical individual and can be tested for purity by chemical methods. For these reasons much work has been done on the subject, but the conclusions reached have been conflicting.

Leblanc,² Ide,³ and Demees⁴ were the first to report the immunization of animals with hemoglobin. Ide's antiserum was hemolytic in addition, but his pupil Demees, by purifying his hemoglobin more thoroughly, succeeded in obtaining a serum which precipitated hemoglobin solutions and did not cause hemolysis. Thorough purification is important since the blood corpuscles contain substances of stronger antigenic power (globulins, stromata) than hemoglobin (Ide). The precipitins obtained by Demees were not bound by intact red cells, and were produced only by intensive treatment of the animals.

The question of the species-specificity of hemoglobin was investigated by Thomsen⁵ with the aid of the anaphylactic reaction. Most of his work was carried on with dissolved corpuscles, though he made a few experiments with the crystalline hemoglobin of two species. Similar experiments were made by Bradley and Sansum.⁶ Guinea pigs sensitized with dog hemoglobin reacted strongly (but not

* Seventeenth paper on antigens.

¹ The word hemoglobin, in this communication, is used in the generic sense, including oxyhemoglobin and its immediate derivatives.

² Leblanc, A., *La Cellule*, 1901, xviii, 337.

³ Ide, M., *La Cellule*, 1902, xx, 263.

⁴ Demees, O., *La Cellule*, 1907, xxiv, 423.

⁵ Thomsen, O., *Z. Immunitätsforsch., Orig.*, 1909, iii, 539. Cf. Schittenhelm, A., and Weichardt, W., *Z. Immunitätsforsch., Orig.*, 1912, xiv, 609. Pfeiffer, H., and Mita, S., *Z. Immunitätsforsch., Orig.*, 1910, vi.

⁶ Bradley, H. C., and Sansum, W. D., *J. Biol. Chem.*, 1914, xviii, 497.

with acute death) to solutions of dog red cells, less markedly to those of the cat, pig, and turtle, and not at all to the blood of a number of other animals.

As there is no reason to believe that the heme in the hemoglobin molecule of various species is different, and it is at least certain that there are not many different hemes, the differences in the antigenic properties of the different hemoglobins would appear to be due to the globin, or the protein part of the molecule. The serological behavior of globin was studied by Browning and Wilson;⁷ also by Gay and Robertson.⁸ The former obtained, in addition, an antihemoglobin serum and found that an immune serum against guinea pig globin fixed the complement strongly with the corresponding antigen, and reacted only weakly with rabbit globin and not at all with ox globin. In a second paper Browning and Robertson report that ox globin antiserum reacts, in addition, with the globins of the goat, guinea pig, and duck, but not with that of the rabbit. They write: "Thus while evidence of species-specificity exists in certain cases, there is also a wide, though not universal, community of antigenic properties shared by the globin of widely separate animal species."

In contrast to the positive results cited above, Ford and Halsey⁹ were unable to produce either antibody formation or anaphylaxis by injecting repeatedly recrystallized hemoglobin. Similar negative results with both hemoglobin and globin were reported by Schmidt and Bennett,^{10, 11} using hemoglobin which had been carefully purified by various methods. They therefore concluded that hemoglobin is a non-antigenic substance and that the positive findings of previous workers were possibly due to the impurity of the hemoglobin or globin used. Subsequent to their work positive findings were again reported by Fujiwara.¹²

Differences between the various antigens contained in the blood corpuscles have been investigated by Klein, Leers, Levene, Chodat, Steward, and Fujiwara.

The solution of the question of the antigenic or non-antigenic nature of hemoglobin is of considerable general interest, since if it were really impossible to produce antibodies with hemoglobin, doubts might arise as to the antigenic properties of absolutely pure proteins in general. It would seem far more reasonable, however, to view such behavior as pointing to a relationship between the con-

⁷ Browning, C. H., and Wilson, G. H., *J. Path. and Bact.*, 1909, xiv, 174; *J. Immunol.*, 1920, v, 417.

⁸ Gay, F. P., and Robertson, T. B., *J. Exp. Med.*, 1913, xvii, 535.

⁹ Ford, W. W., and Halsey, J. T., *J. Med. Research*, 1904, xi, 403.

¹⁰ Schmidt, C. L. A., and Bennett, C. B., *J. Infect. Dis.*, 1919, xxv, 207. Schmidt, C. L. A., *Univ. Calif. Pub.*, 1916, ii, 157.

¹¹ Bennett, C. B., and Schmidt, C. L. A., *J. Immunol.*, 1919, iv, 29. Cf. Gay, F. P., and Robertson, T. B., *J. Exp. Med.*, 1913, xvii, 535.

¹² Fujiwara, K., *Mitt. med. Ges. Tokyo*, 1920, xxxiv, No. 23.

stitution of individual proteins and their antigenic action. Moreover, the determination of the species-specificity of hemoglobin antibodies, if they really exist, would have importance as throwing light on the general problem of how species-specificity is manifested in the individual proteins of the animal organism. It is from this standpoint that we have taken up the matter, and a preliminary note has been published in which this phase of the problem is discussed more thoroughly.¹³ It has developed that antibodies can be obtained with hemoglobin, although less readily than with most other proteins, and that these antibodies are species-specific to a high degree.

After the publication of the preliminary note, and after the completion of the present work, a communication by Higashi¹⁴ was received in which the author's conclusions coincide essentially with our own. On the other hand, negative results were published by Depla.¹⁵

Recently, Hektoen and Schulhof¹⁶ reported the production of antibodies with blood extracts and hemoglobin solutions, concluding that hemoglobin is a species-specific antigen. Contradictory in a certain measure, however, is their finding that there was no appreciable diminution of precipitable substance after fission of the hemoglobin into hematin and globin and removal of most of the split products. Their conclusion is: "While the precipitinogens in extracts of red corpuscles and in hemoglobin may exist independently of hemoglobin after treatment with acids, they ordinarily are attached closely to the hemoglobin molecule, not being removed or diminished in proportion to the amount of hemoglobin by repeated crystallization or by treatment with aluminium cream, the antigen being apparently either closely adsorbed to the hemoglobin molecule or forming a part of it which can be split off by acids." We shall discuss this conclusion further on in our paper.

Preparation of Oxyhemoglobin and Immunization of the Animals.

In the work reported in this paper horse hemoglobin only was used for immunization.

The preliminary experiments referred to above were carried out with a preparation (A) made as follows, according to the methods of Ide³ and Demecs.⁴

¹³ Landsteiner, K., *Kon. Akad. van Wetensch. te Amsterdam*, 1921, xxix, 1029.

¹⁴ Higashi, S., *J. Biochem. (Japan)*, 1923, ii, 315. Cf. *J. Tokyo Med. Soc.*, 1921, xxxv, No. 9.

¹⁵ Depla, H., *Compt. rend. Soc. biol.*, 1922, lxxxvii, 383. Cf. Chodat, F., *Compt. rend. Soc. biol.*, 1921, lxxxv, 735.

¹⁶ Hektoen, L., and Schulhof, K., *J. Infect. Dis.*, 1922, xxxi, 32.

Defibrinated horse blood was centrifuged, washed five times, and dissolved in a volume of water equal to twice the amount of blood used. Ether was added, and the mixture allowed to stand in the ice box for 1 or 2 days, with occasional stirring. After decantation from any sediment present, the solution was treated with an equal volume of saturated ammonium sulfate solution, and filtered through folded filters to remove precipitated globulin and stromata. Saturated ammonium sulfate was then added until the hemoglobin separated in the cold. The product was filtered off, washed with ammonium sulfate solution, and dialyzed after the addition of a little ether. Finally, 1 per cent of sodium chloride was added.

Preparation B, a solution of oxyhemoglobin, was obtained according to the method recently published by one of us.¹⁷

The oxyhemoglobin was recrystallized three times as outlined in the method referred to, and washed several times with water saturated with carbon dioxide and oxygen. The crystals were ground to a thin paste with 0.85 per cent sodium chloride solution, diluted further with saline, dissolved with the minimum amount of normal sodium hydroxide solution, and diluted with saline to a total hemoglobin content of 8 to 10 per cent. The solution was first filtered through a Berkefeld V filter, then through a sterile Berkefeld N filter, and was preserved under sterile conditions.

Preparation C, also a solution of oxyhemoglobin, was prepared by following the method used for Preparation A as far as the addition of an equal volume of saturated ammonium sulfate solution.

It was found that if the ether used was repeatedly washed with water, and then dried first over calcium chloride and then over stick potassium or sodium hydroxide, the formation of methemoglobin was avoided. In fact, crystallization of the oxyhemoglobin occurred so rapidly after addition of the ammonium sulfate solution that it was rarely possible to filter the mixture without losing much of the pigment. The filtrate from the globulin and stromata was allowed to stand overnight in the ice box and the solution was then decanted from the heavy deposit of oxyhemoglobin crystals. These were filtered on a Buchner funnel and washed, and were recrystallized once and dissolved according to the methods used for Preparation B, omitting the preliminary filtration through a Berkefeld V filter.

8 to 10 per cent hemoglobin solutions prepared as above were used for injection. Seventeen rabbits in the three groups each received seven to eleven intraperitoneal injections of 8 to 15 cc. at weekly intervals. Only with a small proportion of the animals did we obtain strongly reacting sera, that is, sera which gave a heavy precipi-

¹⁷ Heidelberger, M., *J. Biol. Chem.*, 1922, liii, 31.

tate with 0.01 per cent hemoglobin solution within a few minutes. In the case of Preparation A one out of five rabbits gave a strongly reacting serum and two gave weaker sera; with Preparation B, two out of five rabbits gave strongly reacting sera, while with Preparation C, in spite of the use of seven rabbits, only weak sera were obtained, that is sera which, under the conditions given below, gave readily perceptible, but relatively weak turbidities or slight precipitates.

Thus we have found, as did Demees⁴ and Browning and Wilson,⁷ that hemoglobin is a rather weak antigen, a fact which may account for the negative results of the experiments of others. Possibly, however, a greater formation of antibodies could be achieved through intravenous injection as was done by Hektoen and Schulhof. It is also not impossible that some of the impurities associated with hemoglobin in the cell may actually facilitate antibody formation by the hemoglobin. By the use of the especially sensitive ring test, as employed by Hektoen and Schulhof¹⁶ and Higashi,¹⁴ other sera would undoubtedly have been found positive.

The Specificity of Antihemoglobin Sera.

The tests were carried out as follows: 2 to 3 capillary drops (1 drop = 0.04 cc.) of the serum were added to 0.2 cc. of 0.01 per cent solutions of the different hemoglobins. In Table I is given the intensity of the turbidity or precipitate after the tubes were shaken and had stood for various periods at room temperature. Solutions of crystalline horse and dog oxyhemoglobin were used in the tests, while in the case of other animals, red cells were laked with water and ether, centrifuged, and the resulting solution was filtered through asbestos or kieselguhr paper (Macherey) and made up to a definite hemoglobin concentration. Serum 1 was obtained with Preparation A, the others, with Preparation B.

After the test mixture had stood overnight in the ice box, some further weakly positive reactions were noted. Precipitin reactions of equal intensity were obtained with methemoglobin, carbon monoxide hemoglobin, and cyanhemoglobin prepared from crystalline horse oxyhemoglobin.

The reactions in Table I show that the serological specificity of the antibodies produced by crystalline hemoglobin is no less developed than in the case of the serum proteins, which have been repeatedly and thoroughly studied from this standpoint (Nuttall¹⁸). Strong

¹⁸ Nuttall, G. H. F., Blood immunity and blood relationship, Cambridge, 1904.

TABLE I.
Specificity of Antihemoglobin Sera.

	Read af- ter	Human.	Dog.	Horse.	Donkey.	Pig.	Ox.	Sheep.	Goat.	Rabbit.	Guinea pig.	Rat.	Mouse.	Chicken.	Pigeon.
Serum 1	1 hour	0	0	+++	++±	0	0	0	0	0		0	+	0	0
" 2	5 min.	0	0	++±	++	0	0	0	0	0	0	0	0	0	0
" 2	20 "	M.Tr.	0	+++	++±	0	0	Tr.	0	0	0	0	0	0	0
" 2	1 hour	" "	0	+++	+++	0	M.Tr.	"	M.Tr.	0	0	0	M.Tr.	0	0
" 3	20 min.	0	0	++±	++	0	0	0	0	0	0	0	0	0	0
" 3	30 "	0	0	++±	++	0	0	0	0	0	0	0	0	0	0
" 3	1 hour	0	0	++±	++	0	0	0	0	0	0	0	M.Tr.	0	0
" 4	30 min.	0		+±	+		0						" "		
" 4	1 hour	0		++	+±		0						" "		

Tr. indicates trace; M.Tr., minimal trace.

reactions were given with the homologous horse hemoglobin and a somewhat weaker precipitation with the blood pigment of the closely related donkey, while the other red cell solutions gave little or no reaction. This marked precipitation of the hemoglobin of a closely related species parallels the findings with serum proteins, and shows that with hemoglobin as well, the serological or chemical relationship runs parallel to the morphological. In agreement with this conclusion, already stated in the preliminary paper,¹³ Higashi¹⁴ has likewise found a definite species-specificity in precipitin, complement fixation, and anaphylactic reactions. An exception was found by him in the case of closely related species. Moreover, anti-chicken and anti-goose hemoglobin sera gave reactions of equal or nearly equal intensity with the hemoglobin of the pigeon and sparrow.

The serological findings are manifestly a proof that the crystallographic differences found by Reichert and Brown¹⁹ in their comprehensive work are principally the result of chemical differences in the hemoglobin, a conclusion which has hitherto not been universally accepted,²⁰ since conditions of crystallization and impurities present undoubtedly do influence crystalline form.²¹

With respect to the serological differences between hemoglobin and other proteins, our immune sera, like those obtained by Demees and Higashi, had a very weak hemolytic action and either gave no reaction at all with horse serum albumin, or only a very faint trace on long standing. On the other hand, it was possible, by means of the inhibition reaction,²² to trace a relationship between hemoglobins of different origin. The observed effect was less, however, than that recorded in our preliminary publication.

To each of a number of tubes was added 0.2 cc. of 0.01 per cent horse hemoglobin solution and 0.1 cc. of a solution of red cells of the animal indicated and con-

¹⁹ Reichert, E. T., and Brown, A. P., *Carnegie Inst. Washington, Pub. No. 116*, 1909. Cf. Loeb, J., *Science*, 1917, xlv, 191.

²⁰ Cf. Robertson, T. B., *Principles of biochemistry*, Philadelphia and New York, 1920.

²¹ For additional evidence of chemical differences see Landsteiner, K., and Heidelberg, M., *J. Gen. Physiol.*, 1923-24, vi, 131.

²² (a) Camus, M. L., *Compt. rend. Acad.*, 1901, cxxxii, 215. (b) Landsteiner, K., and Halban, T., *Munch. med. Woch.*, 1902, xlix, 473. (c) Landsteiner, K., *Biochem. Z.*, 1918-19, xciii, 115; 1920, civ, 280.

taining approximately 0.1 per cent of hemoglobin. 0.1 cc. of 1 per cent of sodium chloride solution was added to a control tube and 0.08 cc. of Serum 3 was put in all. The results are shown in Table II.

It is not unlikely that the inhibition observed in these tests depends upon the existence in the various hemoglobins of a common component, such as hematin, in view of the analogous inhibition shown in the case of proteins containing a common radical which had been introduced into the molecule.^{22c} However, a solution of hematin itself caused no inhibition, just as it failed to give reactions with precipitin sera.²³

TABLE II.
Inhibition of Precipitation.

Dog.	Horse.	Rabbit.	Chicken.	Control.
Tr.	0	+ -	+	++

Identity of the Precipitable Substance with Hemoglobin.

Because of the negative results of many authors, the difficulty of producing strongly reactive antibodies, and the observations of Hektoen and Schulhof, it is of great importance to settle the question as to whether the antigen in our experiments is really hemoglobin itself, or some impurity difficult to remove.

The most striking result reported by Hektoen and Schulhof was that the precipitin reaction remained unaltered in solutions in which the hemoglobin had been destroyed with acetic acid and the globin removed. However, hydrochloric acid, when not greatly diluted, destroyed the antigen.

We repeated the experiments with acetic acid and hemoglobin at room temperature in the concentrations given by the authors, removing test portions from time to time. After removal of the globin by neutralization, a series of dilutions was made and compared with dilutions of the original hemoglobin solution. Precipitin tests were made on these solutions, and rough estimations were made of how much of the original antigenic value remained, by comparing

²³ Gay, F. P., quoted by Schmidt and Bennett.¹¹

the acid-treated tubes with the hemoglobin dilutions. In this way it was found that after $\frac{1}{2}$, 2, and 22 hours, about 50, 60, and 75 per cent, respectively, of the antigen had been destroyed. Part of the hemoglobin was still spectroscopically demonstrable even after the action of 0.01 N hydrochloric acid instead of 0.1 N acetic acid for 24 hours.

In view of this fact, the following experiment was set up.

A 10.2 per cent solution of twice recrystallized oxyhemoglobin was prepared by dissolving a suspension of the crystals with the aid of the minimum amount of normal sodium hydroxide. 50 cc. of the solution were diluted to 100 cc., and although neutral to litmus, the solution was acidified with 2 cc. of normal acetic acid in order to avoid any question as to its reaction. 50 cc. of the resulting 5 per cent solution were mixed with 50 cc. of 0.05 N hydrochloric acid and allowed to stand at room temperature for 4 $\frac{1}{2}$ hours. The brown solution was then neutralized with 0.1 N ammonium hydroxide containing 5 per cent of ammonium chloride, allowed to stand overnight, and centrifuged. A determination of hemoglobin by Stadie's method²⁴ showed 0.2 of the original amount still to be present, while a similar solution, allowed to stand overnight before neutralization, still contained 0.1 of the original amount. The solutions showed the absorption bands of methemoglobin, and reacted with immune serum with approximately the degree of intensity to be expected from the analytical findings.

It thus becomes evident that, under the conditions given, hemoglobin is not completely split into hematin and globin.

The fact that the antigen is destroyed to an appreciable extent in a short time by acetic acid of a lower concentration than 0.05 N is in itself strong support for the belief that hemoglobin is the substance active in the serological tests; for other antigens such as the serum proteins are influenced in their precipitin reactions only by acid of much higher concentration.

To sum up, there are a number of grounds for ascribing the antigenic action to hemoglobin itself. In the first place, removal of the stromata by filtration and of globulins by ammonium sulfate followed by recrystallization, leaves the precipitability of the hemoglobin solution intact, as does also treatment with aluminium cream according to Marshall and Welker,²⁵ as Hektoen and Schulhof found. In

²⁴ Stadie, W. C., *J. Biol. Chem.*, 1920, xli, 237.

²⁵ Marshall, J., and Welker, W. H., *J. Am. Chem. Soc.*, 1913, xxxv, 820.

the second place, the observation that our immune sera reacted to an equal extent with solutions of purified hemoglobin and with extracts of red cells containing all the accompanying impurities points in the same direction. In the third place, the precipitin reaction permits the detection of hemoglobin in as low a concentration as 0.001 per cent, which compares favorably with the strength of common precipitins. It would be difficult to reconcile this fact, and that of the inhibition found at a concentration of 0.05 per cent, with the assumption of an active impurity. Finally, it can be shown that hemoglobin is actually carried down in the precipitate formed in hemoglobin solutions by immune serum. The important observation that the precipitate is red was originally made by Leblanc and Ide.²⁶ Since it could be objected that the hemoglobin might have been carried down by adsorption, and not have been actually involved in the precipitin reaction, tests were made under conditions which eliminate this possibility.

The following immune sera of rabbits were used.

A. Antihemoglobin Serum 2.....	20 drops
B. " " 3	20 "
C. Anti-horse serum.....	10 "
D. Anti-human "	20 "
E. Anti-donkey "	20 "

The tubes were set up as follows:

In each tube 0.2 cc. of 0.1 per cent hemoglobin solution plus

1. 1.8 cc. saline + A.
2. 1.8 " " + B.
3. 1.8 " 1:500 horse serum + C.
4. 1.8 " 1:500 human " + D.
5. 1.8 " 1:500 donkey " + E.

At the end of 1 hour the precipitates were centrifuged off, washed with saline, and again centrifuged. The differences observed were very striking. The sediment in Tubes 1 and 2 was definitely red, while that in the other tubes was pure white in spite of the fact that the antigen-antibody complex was precipitated in a hemoglobin solution of the same concentration as that in the first two tubes. One is forced to the conclusion that the red color of the precipitate in the hemoglobin-antihemoglobin system is actually due to antigen-antibody combination.

The following additional experiment was run.

1 cc. of 8 per cent hemoglobin solution was diluted with water to 30 cc. and treated with 20 cc. of 0.01 N hydrochloric acid. After 2 hours the globin and

²⁶ See Leblanc,² p. 362; Ide,³ p. 266.

hematin were precipitated by neutralization. The precipitin test showed in comparison with hemoglobin solutions of known strength that about 80 per cent of the antigen in the pale yellow-brown solution had been destroyed. 1 cc. of the solution was then treated with enough antihemoglobin serum to induce maximum precipitation, after which the precipitate was handled as in the preceding experiment. In this case, too, the sediment was definitely red, showing that the antibody was bound to a pigmented antigen and not to a colorless split product.

From the above experiments, therefore, it would seem that in order to prove definitely that the antigen or an active part of it can be split from the hemoglobin, it would be necessary to show conclusively that any active solution obtained by treatment with acid contains no hemoglobin, methemoglobin, or closely related derivative.

CONCLUSIONS.

Sera produced by immunization with crystalline oxyhemoglobin react species-specifically with hemoglobin solutions.

Evidence is presented that in this reaction the hemoglobin itself is the active substance.

Conversion of oxyhemoglobin into methemoglobin, carbon-monoxo hemoglobin, or cyanhemoglobin does not alter the response to the precipitating immune serum.

Not only the hemoglobin of homologous species, but also that of other species causes inhibition in greater or less degree of the precipitin reaction.