

IS HEMOGLOBIN ANTIGENIC?*

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A review of the literature on the properties of hemoglobin as a possible antigen shows that the results obtained by various workers differ.

Thus in work reported by Leblanc,¹ a precipitin for beef hemoglobin was obtained by immunizing a dog with a preparation obtained by saturating laked, washed, red cells with ammonium sulphate after removal of globulins and stromata. This preparation was by no means pure since no attempt was made to free the hemoglobin from possible contamination by other proteins which may have been carried down by the crystals. Levene² was unable to produce either a lysin or an agglutinin for red cells by immunization with twice crystallized hemoglobin. Ford and Halsey,³ working with various fractions of the red cell, were unable to demonstrate antibodies—precipitins, lytic or agglutinating substances in rabbits or anaphylaxis in guinea-pigs injected with dog or hen hemoglobin recrystallized 4–5 times from 25% alcohol. These authors used special precautions to exclude protein contamination and their results appear to be more reliable than other workers in this field. Schittenhelm and Weichardt⁴ and Dittrich⁵ carried out experiments with both hemoglobin and the protein fraction of that molecule, globin. The former report two anaphylaxis experiments on guinea-pigs, using horse hemoglobin prepared by precipitation with ammonium sulphate according to the method of Schulz⁶ and recrystallized three times. One sensitized animal died on receiving an injection of 100 mg. 14 days after the initial injection while the second animal showed merely a rise of 2 C. in body temperature, which however was but little more than the normal variability. Hemoglobin (100 mg. injected intravenously into guinea-pigs) was nontoxic; no influence on the blood pressure of rabbits was noted. Contrasted with these results are their experiments carried out with globin. This substance, like protamin and thymus histone, was found to be toxic.⁶

Extensive anaphylaxis experiments with dog and beef hemoglobin have been reported by Bradley and Sansum.⁷ They found that not only is hemoglobin

Received for publication April 28, 1919.

* Aided in part by a grant from the George Williams Hooper Foundation for Medical Research.

¹ *La Cellule*, 1901, 18, p. 337.

² *Jour. Med. Research*, 1904, 12, p. 191.

³ *Jour. Med. Research*, 1904, 11, p. 403.

⁴ *Ztschr. f. Immunitätsforsch. u. exper. Therap.*, 1912, 14, p. 609.

⁵ *Arch. f. exper. Path. u. Pharm.*, 1892, 29, p. 247.

⁶ *Ztschr. f. physiol. Chem.*, 1898, 24, p. 449.

⁷ Schittenhelm, A., and Weichardt, W.: *München. med. Wehnschr.*, 1912, 59, p. 1089. Cf. also, Thompson, W. H.: *Ztschr. f. physiol. Chem.*, 1900, 29, p. 1; Gay, F. P., and Robertson, T. B.: *Jour. Exper. Med.*, 1912, 16, p. 479; Taylor, A. E.: *Jour. Biol. Chem.*, 1908, 5, p. 311; McCruden, F. H.: *Amer. Chem. Soc. Abstr.*, 1912, 6, p. 1181.

⁸ *Jour. Biol. Chem.*, 1914, 18, p. 497.

antigenic with regard to its ability to produce anaphylaxis in sensitized guinea-pigs, but it is also specific for the species from which it was obtained. The hemoglobin was prepared from laked, washed, red cells to which toluol had been added and the crystals washed with a little water and dried. The preparation used for the second injection was less pure. A possible criticism of these experiments is that if the hemoglobin used by Bradley and Sansum for sensitization was slightly contaminated with foreign protein and since the preparation used for the second injection was less pure, the anaphylaxis obtained by them may have been due, not to the hemoglobin, but to the contaminating protein. Rosenau and Anderson⁸ found that a millionth of a c c of horse serum was sufficient to sensitize guinea-pigs and Wells⁹ states that one twenty millionth of a gm. of pure egg albumin will sensitize sufficiently to produce typical anaphylactic symptoms, while one millionth of a gm. will sensitize fatally. Bradley and Sansum while admitting that globin is toxic and nonspecific† (since it is nonantigenic) and that the relatively simple hematin portion of the hemoglobin molecule may be the same in all hemoglobins, believe that the specific differences found by them in various hemoglobins must be found in the protein or globin fraction which, however, are lost in the ordinary acid cleavage. To accept this idea we would have to assume that the union of nonantigenic globin with the nonprotein substance hematin, as combined in hemoglobin, produces an antigenic compound. Doubt is cast on this work by the fact that the animals sensitized to the purest dog hemoglobin used reacted also to dog serum.

Thomsen¹⁰ prepared horse hemoglobin by crystallizing twice from 25% alcohol and was able to demonstrate specific anaphylaxis in guinea-pigs sensitized with this hemoglobin. He believes that anaphylaxis induced by red cells is largely due to the hemoglobin. Our own observations¹¹ in this field lead us to believe that a protein other than hemoglobin is the antigen concerned in the production of specific immune bodies for the red cell.

In considering the properties of hemoglobin as a possible antigen two questions arise, namely: (a) does combination of globin which is nonantigenic, with hematin, which is not a protein and is also nonantigenic,¹² as found in hemoglobin, result in the latter substance acting as an antigen? (b) Is hemoglobin, owing to the toxic constituent globin, likewise toxic? That globin is nonantigenic and toxic appears to be well established, although experiments have been reported by Browning and Wilson¹³ to the effect that globin is not only antigenic, but shows marked species specificity. The later experiments of Gay and Robertson¹⁴ and Schmidt¹⁵ contradict this and confirm the fact

⁸ Bull. Hyg. Lab., U. S. P. H. S., 1906, No. 29.

⁹ Jour. Infect. Dis., 1908, 5, p. 449.

† This may not be strictly true. Thus Kossel, Ztschr. f. physiol. Chem., 1913, 88, p. 163, states that the content of amino acids varies in different protamins, and is specific for the species from which the preparations are derived.

¹⁰ Ztschr. f. Immunitätsforsch. u. exper. Therap., 1909, 3, p. 539.

¹¹ Bennett, C. B., and Schmidt, C. L. A.: Jour. Immunol., Vol. 4. To appear shortly.

¹² Gay, F. P.: Personal communication.

¹³ Jour. Path. and Bacteriol., 1909, 14, p. 174.

¹⁴ Jour. Exper. Med., 1913, 17, p. 535.

¹⁵ Univ. of Cal. Pub. Path., 1916, 2, p. 157.

that globin is nonantigenic and likewise toxic. Combination of globin with hematin appears to markedly, if not entirely, decrease the toxic action of this protein as a review of the early literature on hemoglobin by Kuntzen and Krummacher¹⁶ shows. These workers injected horse hemoglobin crystallized three times from 25% alcohol in doses of 0.5-1.3 gm. per kg. into guinea-pigs and rabbits without toxic effects. Barratt and Yorke¹⁷ likewise confirm these results in a general way, although they used only laked red cells free from stromata.

Our experiments were initiated in the belief that the positive results obtained by other workers can be ascribed to impurities in the preparations of hemoglobin and for this reason our preparations were crystallized repeatedly in order to include as little protein contamination as possible. Crystalline proteins exhibit marked ability to carry down the menstruum from which they are deposited, the observations of Schulz and Zsigmondy¹⁸ showing that egg albumin must be recrystallized 3-6 times to insure a preparation of reasonable purity. We have made use of the well-known fact that reduced hemoglobin is more soluble than the oxyhemoglobin for the purpose of recrystallizing hemoglobin. For our experiments three preparations were employed, each crystallized by a somewhat different method. These we shall designate as A, B and C.

For preparation A we proceeded as follows: Oxalated dog blood was freed from serum and washed six times with equal volumes of physiologic salt solution, the red cells being thrown down by centrifuging and the supernatant fluid removed each time. The corpuscles were then laked with 3 volumes of water and the insoluble stromata removed by centrifuging. To this solution sufficient alcohol was added to make a concentration of 25% and the mixture cooled by the use of ice and salt, causing crystallization of the hemoglobin. The crystals were thrown down by centrifuging, the supernatant fluid decanted, and to the crystals 3-4 volumes of distilled water added. The oxyhemoglobin crystals were brought into solution by passing in CO₂, the reduced form being much more soluble. This solution was centrifuged to remove insoluble material, oxygen passed through it for an hour, and placed in a freezing mixture as previously, again causing crystals of oxyhemoglobin to deposit, which in turn were separated from the mother liquor by centrifuging. This method of crystallization was repeated 5 times. Before the 5th crystallization the hemoglobin solution was passed through a porcelain filter to remove any possible suspended material. A portion of these crystals were air dried. These did not completely redissolve, due probably to some denaturization. Another portion which was subsequently used as antigen in the fixation tests was kept frozen until used.

¹⁶ Ztschr. f. Biol., 1900, 40, p. 228.

¹⁷ Ztschr. f. Immunitätsforsch. u. exper. Therap., 1912, 12, p. 333; Brit. Med. Jour., 1914, 1, p. 235. Cf. also Sellards and Minot, Jour. Med. Research, 1917, 37, p. 161.

¹⁸ Beitr. z. chem. Phys. u. Path., 1902, 3, p. 137; cf. also, Robertson, T. B.: The Physical Chemistry of the Proteins, New York, 1918, p. 316.

For immunizing rabbits the mother liquor from the last crystallization was used. To this, phenol to make a concentration of 0.25% was added as a preservative and the solution kept in the ice-chest. In another preparation the initial addition of alcohol was omitted and the hemoglobin crystallized from the aqueous solution. The yield of oxyhemoglobin crystals by this method is small, due to its solubility even at low temperatures and unavoidable losses in centrifuging, which could not be done at low temperatures. In the next preparation use was made of alcohol to decrease the solubility of the oxyhemoglobin which gave a relatively better yield.

For the preparation of specimen B we proceeded as follows: To the hemoglobin solution obtained as in the preceding preparation by hemolysis of washed red cells (dog) and removal of the insoluble stromata, sufficient alcohol was added to make a concentration of about 15%. The solution was then saturated with oxygen and placed in a freezing mixture and the crystals allowed to separate. They were then centrifuged off, 2 volumes of water added and CO₂ passed in to obtain a solution. Any insoluble substance remaining was centrifuged off. To the solution sufficient alcohol was added to make a concentration of 15%, the solution saturated with oxygen and crystallized as before. The hemoglobin was crystallized 6 times in the above manner except that in the last crystallization no alcohol was used. Addition of alcohol to the solution increases the yield of hemoglobin crystals. It is likewise necessary to completely saturate the solution by passage of oxygen for several hours to obtain maximum yields. Part of the hemoglobin was dissolved, phenol added to make a concentration of 0.25%, kept on ice and used for the injections. The remainder was kept frozen until used as antigen for the fixation experiments. The two preparations described above at the end of a month's time still gave the usual tests for hemoglobin.

Our third preparation C was made essentially in accordance with the directions given by Schulz.⁵ To the solution of red corpuscles freed from stromata and prepared as in the previous methods, and kept cold, sufficient ammonium sulphate was added to bring the solution to half saturation and the globulins filtered off. On raising the temperature to 40 C. and usually by the addition of a little more ammonium sulphate, hemoglobin crystals formed and could be filtered off. These crystals differed markedly from those obtained by the previous methods and instead of being red, were brown, irrespective of whether oxygen or carbon dioxide was passed into the solution. In all probability this preparation was methemoglobin as stated by Schittenhelm and Weichardt.⁴ The crystals were dissolved in water and recrystallized, this being repeated three times. Removal of the ammonium sulphate by successive addition of weak alcohol and finally washing with absolute alcohol and ether, caused the hemoglobin to become insoluble; even on addition of traces of alkali, it was difficult to obtain a satisfactory solution. To overcome this another portion of the crystals was allowed to air dry. As noted by Schulz, in this procedure the ammonium sulphate largely separates from the dark brown mass and the surface appears gray and fluffy, somewhat in appearance to hoar frost. By successively removing this top layer the whole mass of hemoglobin was brought into this condition. By this procedure a large part of the ammonium sulphate separated from the hemoglobin crystals and by rapidly draining with successive additions of ice water, could be largely removed. Before air drying this procedure would have been impossible due to the rapid solution of the hemoglobin. The preparation was then air dried. It, however, still contained a small amount of ammonium sulphate. For the antigen in the fixation experiments two preparations of hemoglobin were used: one prepared by the above

method but crystallized 6 times, and the other preparation B crystallized 5 times. Since the Schulz method of preparation in our hands appeared less satisfactory than our other methods, only two rabbits were injected with this preparation.

For the immunization experiments healthy rabbits, not previously used, and from different sources, were usually given 3 successive injections at 1- or 2-day intervals and after about 5 days this was repeated. Ten days after the last injection the rabbits were bled and fixation experiments carried out with the inactivated sera. With various pure proteins we have never failed to obtain immune bodies when animals were immunized according to the above method. Using antigen (A) three rabbits were given 6 injections, the total quantity of hemoglobin injected being: Rabbit N, 0.780 gm.; Rabbit S, 0.650 gm.; and Rabbit D, 0.680 gm. Alexin fixation experiments with the inactivated sera were carried out using the following doses: 5% sheep cells, 0.2 cc; rabbit vs. sheep cell amboceptor, 0.2 cc; 10% alexin, 0.2 cc ($1\frac{1}{4}$ units); antigen (crystallized 5 times and kept frozen), 0.15 cc (one quarter of the minimum inhibiting dose); sera from rabbits injected with hemoglobin in amounts of 0.4, 0.3, 0.2, 0.1 cc of 1:10, 1:50, etc. dilutions, and the volume brought to 1.2 cc with salt solution. Readings were taken after the usual half-hour incubation and after standing in the ice-chest overnight. Positive fixation with these sera was not obtained. Sera N and S in a dosage of 0.4 cc of 1:10 dilution caused a slight inhibition of hemolysis but insufficient to be called positive. In dilutions and doses lower than 0.4 cc of 1:10, slight inhibition of hemolysis was caused by the sera alone. Precipitin tests were likewise negative. The sera of these rabbits did not contain an agglutinin nor did they, in presence of alexin, cause lysis of a suspension of dog cells. It is apparent that repeated injection of hemoglobin preparation A did not produce immune bodies in the sera of these rabbits. Two normal guinea-pigs were sensitized with 10 and 20 mg. of hemoglobin injected subcutaneously and a month later given a second injection of 150 mg. intraperitoneally. In the observations for symptoms of anaphylaxis we have followed the scheme used by Wells and Osborne¹⁰ in their protein studies. No general symptoms of anaphylaxis were shown by the animals. Pig (a) may be said to have shown a possible slight reaction (fall of several degrees in body temperature) as a result of the second injection while the change in the second animal was within the normal variability of temperature change when proteins are injected.

Using hemoglobin preparation B, three rabbits were injected as follows: two received 7 injections of 200 mg. each and the third 5 injections of the same dosage. Fixation experiments were carried out as before using two antigens, (a) the solution which was used for the injections, (b) the preparation which had been kept frozen. Using dosages and dilutions of 0.4 cc of 1:10 and upward of inactivated sera, no inhibition of hemolysis was observed. Precipitin tests were likewise negative. These sera contained no sensitizer which in the presence of alexin could cause hemolysis of dog cells. The serum of rabbit C in a dilution of 1:10 caused agglutination of dog cells, but this was probably due to the occurrence of a normal agglutinin rather than production through immunization.

Three guinea-pigs were sensitized with 1 mg. each of this hemoglobin preparation given subcutaneously. One month later each animal received 100 mg. injected intraperitoneally. The animals showed no symptoms of anaphylaxis. In two animals the fluctuation in body temperature after injection was within

¹⁰ Jour. Infect. Dis., 1911, 8, p. 66.

the normal variability, in the third it was slightly greater. Marked reactions such as have been reported by Bradley and Sansum and which one ought to expect with a soluble substance such as hemoglobin, were not obtained.

Using hemoglobin prepared according to the Schulz method (preparation C) two rabbits were given 7 injections each, a total of 950 mg. The inactivated sera from these animals were used to carry out fixation experiments using as antigens, preparation C crystallized 6 times and B crystallized 5 times. No fixation was obtained. The precipitin tests were also negative.

As has been previously mentioned, it is regarded by most observers that hemoglobin is nontoxic in moderate doses. We have confirmed this fact. Two guinea-pigs were injected with 70 mg. each of hemoglobin B intracardially. No symptoms were exhibited by the animals. Two other pigs were injected with 200 mg. of the same preparation intraperitoneally. No symptoms and very little effect on temperature were noted. When it is recalled that Gay and Robertson¹⁴ obtained severe reactions in animals injected with 10 mg. of globin intravenously, it is apparent that the combination of globin with hematin as found in hemoglobin has the effect of rendering the protein non-toxic.

SUMMARY

Hemoglobin was prepared by three methods, one by crystallization from ammonium sulphate as described by Schulz, and the other two by precipitation as oxyhemoglobin (with and without addition of alcohol) at low temperatures and resolution by reduction. Each of the three preparations was recrystallized a number of times.

Rabbits were immunized with each of the above preparations and both fixation and precipitin tests carried out. These were negative. In the sera of these rabbits no sensitizer was found, which in the presence of alexin, cause a hemolysis of dog cells. Agglutinins were likewise not produced. Hemoglobin failed to sensitize guinea-pigs for the anaphylaxis reaction. The conclusion that hemoglobin is nonantigenic seems warranted.

Doses of hemoglobin very much larger than the amount of globin which will cause toxic symptoms were injected both intravenously and intraperitoneally into guinea-pigs and toxic symptoms were not shown. Combination of toxic globin with hematin as occurs in hemoglobin renders it nontoxic, but does not, as in the instance when globin is combined with casein,¹⁴ give, when rabbits are immunized, antibodies for itself.