Studies on Hemoglobin

1. Antigenic Properties of Human, Canine and Rabbit Hemoglobin Solutions

By E. A. RACHMILEWITZ, G. IZAK AND D. NELKEN

DURING the past decade the presence of hitherto unrecognized hemoglobin fractions has been demonstrated in both normal human adult hemoglobin and in cord blood hemoglobin. With the development of improved technics, the presence of various abnormal hemoglobin fractions has been recognized as characteristic of certain hemolytic diseases. While much information has become available concerning the electrophoretic mobility of the different hemoglobins, little is known about their antigenic properties. Chernoff was the first to obtain specific antibodies against adult and fetal hemoglobin, and he devised a quantitative precipitin test to distinguish between the two antigens. Similar work has been reported by Aksoy, Goodman and Campbell, and recently by McCormick and Walker who used the same method and have reported specific immunologic differences between cord blood hemoglobin and fetal hemoglobin obtained from thalassemic patients. In recent years, Heller et al. and others demonstrated that certain hemoglobin fractions of adult human and cord blood, are potent antigens, whereas others do not possess antigenic specificity. As immunologic differences permit the differentiation and identification of the various hemoglobin molecules with a great degree of accuracy, it seemed worthwhile to study the antigenic properties of hemoglobin solutions from human and animal sources.

The present report deals with certain observations on the antigenic properties of human adult and cord blood, and dog and rabbit hemoglobin. The in vitro and in vivo effect on the red blood cells of the antisera produced will be dealt with in a separate communication.

Material and Methods

Antigen Preparations

Hemoglobin solutions were prepared according to the method of Singer et al. The final hemoglobin concentration was adjusted to 4 Gm. per cent and the solution kept in a frozen state in aliquots of 5 ml. until used. Samples were prepared in this way from the red cells of normal adult humans, cord blood, healthy dogs and healthy adult rabbits. Washed cell suspensions from the same donor (type O) were prepared afresh for each injection, and the suspensions were adjusted to contain 4 Gm. per cent hemoglobin.

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566
Red cell stroma was prepared according to the method of Danon.\textsuperscript{21} The stroma was then broken up by a sonic oscillator (10 kc Raytheon oscillator) for 5 minutes, and the final suspension was made up to contain 4 Gm. per cent total protein. This final suspension still contained a trace (0.18 Gm. per cent) of hemoglobin.

Human plasma was diluted to a concentration of 4 Gm. per cent total protein and stored in a frozen state until used.

Freund's adjuvant was prepared according to the original description of Freund.\textsuperscript{22}

Globin and hemin solutions were prepared from normal adult human hemoglobin according to the methods of Kassenaar et al.\textsuperscript{23}

**Immunization Procedures**

Thirty-five female rabbits weighing approximately 2.5 Kg. were used in these experiments. Three rabbits were kept in each cage and were fed a fresh vegetable diet. The animals were injected with a mixture of 5 ml. antigen and 5 ml. adjuvant twice weekly for 5 consecutive weeks. Each injection dose was divided into two equal parts, one being injected into the deep subcutaneous tissue in the inguinal area, and the other into the abdominal subcutaneous tissue. Ten days following the last injection, the animals were bled by cardiac puncture and the serum collected and frozen until used. Five animals were immunized against human adult hemoglobin, three against cord blood hemoglobin, two against dog hemoglobin, five against rabbit hemoglobin, three against human intact red cells, three against human red cell stroma, three against human plasma; five animals were injected with Freund's adjuvant alone, while two rabbits received saline only. Four animals died during the immunization procedure. The cause of death was not determined.

The following tests were performed with the immune sera obtained: capillary precipitin test,\textsuperscript{24} agar diffusion test,\textsuperscript{25} agar plate test,\textsuperscript{26} agar electrophoresis, immunoelectrophoresis and complement fixation test. The details of these latter two methods follow.

**Immunoelectrophoresis:** Two per cent chemically pure agar* solution was prepared in citrate buffer, pH 6.4, with an ionic strength of 0.025, and poured into an agar block which measured 7 x 4 x 0.3 cm. The material to be tested was applied across the width of the block in the midline by means of Whatman No. 3 filter paper slices (0.4 x 0.2 cm.). The same paper was used to bridge the distance between the block and the buffer. The buffer solution in the containers was of a pH identical with that of the block, but its ionic strength was 3 times as high as that of the buffer in the block. The voltage applied was 35 V/cm and the amp. 3.6 ma/cm. By this method a distinct separation of the various components could be obtained within 2 hours. The hemoglobin under these conditions migrated towards the negative pole, and four distinct fractions could be identified when normal adult hemoglobin was subjected to electrophoresis. The non-hemoglobin protein, on the other hand, moved to the positive pole and when human washed red cell hemolysate was subjected to electrophoresis, between three and four non-hemoglobin protein fractions could be distinguished at the completion of the run. After electrophoresis had been completed, a trough was cut along the longitudinal axis of the block in the midline, the antiserum placed therein and the block incubated for 24–48 hours at 4 C. From this stage the procedure followed was as described by Grabar.\textsuperscript{27}

**Complement fixation.** All sera was inactivated at 56 C. for 30 minutes. One-tenth ml. amounts of doubling dilution of the antiserum was used as the antibody. The antigen consisted of 0.1 ml. of a 1/100 dilution of the standard hemoglobin solution. Two full units of guinea pig complement in 0.1 ml. were always used. The tubes were shaken and incubated at 6 C. for 2 hours and at 37 C. for 30 minutes before the addition of the hemolytic system. Ambocepter and a 2 per cent erythrocyte suspension were added in 0.1 ml. quantities. After the addition of the hemolytic system, the tubes were again incubated at 37 C. for exactly 30 minutes before reading the results.

* Bacto agar, Difco.
Table 1.—Titers Obtained in the Complement Fixation Tests between the Various Antigens and Antisera

<table>
<thead>
<tr>
<th></th>
<th>Anti-Adult Human Hb* Serum</th>
<th>Anti-Cord Blood Hb Serum</th>
<th>Anti-Dog Hb Serum</th>
<th>Anti-Rabbit Hb Serum</th>
<th>Anti-Human Red Cell Stroma Serum</th>
<th>Anti-Human Plasma Serum</th>
<th>Anti-Freund's Adjuvant Serum</th>
<th>Saline in Selected Animals Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human adult Hb (dilated 1:100 with saline)</td>
<td>1:1500-1:25000</td>
<td>1:380</td>
<td>0-1:80</td>
<td>0</td>
<td>1:80-1:1280</td>
<td>1:320</td>
<td>0-1:80</td>
<td>0</td>
</tr>
<tr>
<td>Cord blood Hb (dilated 1:100 with saline)</td>
<td>1:400-1:1280</td>
<td>1:2600-1:4600</td>
<td>1:80-1:1280</td>
<td>1:80-1:1280</td>
<td>1:320-1:1280</td>
<td>0-1:320</td>
<td>0-1:80</td>
<td>0</td>
</tr>
<tr>
<td>Dog Hb (dilated 1:100 with saline)</td>
<td>0-1:180</td>
<td>0-1:40</td>
<td>1:800-1:1280</td>
<td>1:80-1:1280</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rabbit Hb (dilated 1:100 with saline)</td>
<td>0-1:200</td>
<td>0-1:160</td>
<td>0-1:20</td>
<td>1:512-1:1600</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Human red cell stroma</td>
<td>1:40-1:320</td>
<td>1:40-1:320</td>
<td>0</td>
<td>0</td>
<td>1:80-1:160</td>
<td>1:80-1:160</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Human plasma</td>
<td>±</td>
<td>0-1:80</td>
<td>0</td>
<td>0</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>1:400-1:1600</td>
</tr>
<tr>
<td>Freund's adjuvant</td>
<td>±</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0-1:40</td>
</tr>
<tr>
<td>Saline</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Hb = hemoglobin.
†RBC = red blood cells.
See also text.
The protein content of the immune sera was determined according to the method of Lowry et al.28

RESULTS

Quantitative Measurement of the Antibodies Produced

The results obtained with the precipitin test indicated that some of the antisera produced a precipitate with their corresponding antigens in dilutions as high as 1:10,000; however, the accuracy and reproducibility of these tests, whether done in test tubes or in the capillary tubes, proved rather doubtful in our hands. On the other hand, both accurate and highly reproducible results were obtained with the complement fixation test. It can be seen from table 1 that high antibody titers against the corresponding antigens were obtained within each group of rabbits immunized with these antigens.

Specificity of the Antibodies Produced

Attempts were made to employ the precipitin test to determine the specificity of the antibodies which, however, were unsuccessful for reasons given in the previous paragraph. On the other hand, the complement fixation test was successfully employed in the quantitative estimation of the specificity of the different sera. Anti-human adult hemoglobin serum gave a positive complement fixation test in dilutions up to 1:1280 with cord blood hemoglobin, up to 1:100 with dog hemoglobin, up to 1:200 with rabbit hemoglobin, and up to 1:320 with human red cell stroma. With its own antigen, however, the titer was as high as 1:25,000. Similarly, anti-cord blood hemoglobin serum gave positive results but of low titers against adult human hemoglobin, human red cell stroma, dog hemoglobin, rabbit hemoglobin and human plasma, but high titers against its own antigen. Complement fixation tests performed with each of the antisera against all the various antigens revealed that, with the exception of the control serum, all the sera contained antibodies against several antigens although their antibody titers were highest against the antigens employed in their production, as shown in the examples cited above. The detailed results are summarized in table 1. Experiments were performed in which the antisera were incubated with various antigens in an attempt to absorb their "non-specific" or "specific" antibody content. Thus, if the anti-adult human hemoglobin serum was incubated with a dilute solution of cord blood hemoglobin prior to the complement fixation test, this serum lost almost completely its ability to combine with cord blood hemoglobin, while its original antibody titer against adult human hemoglobin decreased considerably. Similar results were obtained when any of the other immune sera were absorbed with the appropriate antigens. It should be noted that absorption of anti-human adult or cord-blood hemoglobin sera with dog hemoglobin did not eliminate the reaction between these sera and rabbit hemoglobin. Some of these findings are summarized in table 2. Using the absorption procedure, we were able to obtain sera which preserved a substantial "specific" antibody titer, as shown by the complement fixation tests. Such sera will henceforth be referred to as "specific" sera, and they were utilized in some
Table 2.—Results of Complement Fixation Tests Performed between Various Antigens and Immune Sera after the Latter Had Been Incubated with “Non-Specific” Antigens

<table>
<thead>
<tr>
<th>Antihuman Adult Hemoglobin Serum</th>
<th>Before adsorption</th>
<th>Adsorbed on cord blood Hb*</th>
<th>Adsorbed on dog Hb</th>
<th>Adsorbed on rabbit Hb</th>
<th>Adsorbed on human red cell stroma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human adult Hb*</td>
<td>1:25000</td>
<td>1:14000</td>
<td>1:25000</td>
<td>1:20000</td>
<td>1:12500</td>
</tr>
<tr>
<td>(diluted 1:100 with saline)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cord blood Hb</td>
<td>1:1280</td>
<td>1:320</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(diluted 1:100 with saline)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dog Hb</td>
<td>1:80</td>
<td>0</td>
<td>1:40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(diluted 1:100 with saline)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit Hb</td>
<td>1:160</td>
<td>1:80</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(diluted 1:100 with saline)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human red cell stroma</td>
<td>1:320</td>
<td></td>
<td></td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Anti-Cord Blood Hemoglobin Serum</th>
<th>Before adsorption</th>
<th>Adsorbed on human adult Hb</th>
<th>Adsorbed on dog Hb</th>
<th>Adsorbed on rabbit Hb</th>
<th>Adsorbed on human red cell stroma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human adult Hb</td>
<td>1:380</td>
<td>1:100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(diluted 1:100 with saline)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cord blood Hb</td>
<td>1:4600</td>
<td>1:2300</td>
<td>1:4000</td>
<td>1:3600</td>
<td>1:3600</td>
</tr>
<tr>
<td>(diluted 1:100 with saline)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dog Hb</td>
<td>1:40</td>
<td>0</td>
<td>1:60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(diluted 1:100 with saline)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit Hb</td>
<td>1:160</td>
<td>1:80</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(diluted 1:100 with saline)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human red cell stroma</td>
<td>1:320</td>
<td></td>
<td></td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

of the immuno-electrophoretic procedures to be described later in this paper.

It may be seen from table 3 that anti-human adult hemoglobin serum gave a positive complement fixation test with globin, and a lower titer with hemin. The simultaneous presence in the antigen of globin and hemin in the proportions in which they occur in the hemoglobin molecule did not result in a higher titer than when globin alone was used as the antigen, and the titer was far below that seen with adult human hemoglobin.

Further clarification of the antigenic properties of hemoglobins was attempted by the application of Oudin’s agar diffusion method, and later by a micro-modification of this method according to Viazov et al. When the various antisera were tested against their respective antigens, two or more precipitate bands developed in some columns. An example of such a pattern
Table 3.—Titers Obtained in the Complement Fixation Test with Anti-Adult Hemoglobin Serum against Globin and Hemin

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody Titer</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Globin</td>
<td>1:320</td>
<td>Globin concentration 100 mg.%</td>
</tr>
<tr>
<td>Hemin</td>
<td>1:160</td>
<td>Hemin concentration 100 mg.%</td>
</tr>
<tr>
<td>Globin + hemin</td>
<td>1:320</td>
<td>Adjusted to contain physiologic proportions of globin and hemin. Final concentration 100 mg.%</td>
</tr>
<tr>
<td>Adult human hemoglobin</td>
<td>1:25000</td>
<td></td>
</tr>
<tr>
<td>Freund's adjuvant</td>
<td>1:80</td>
<td></td>
</tr>
</tbody>
</table>

is provided in figure 1 which illustrates the development of three distinct precipitate rings over a period of 72 hours when adult human hemoglobin solution was placed on one side of the column and the antiserum on the other. The anti-cord blood hemoglobin serum also produced three precipitin bands against its antigen, while the anti-dog hemoglobin serum and the anti-rabbit hemoglobin serum each produced one precipitin ring against their respective antigens. As in the case of the complement fixation test, here too the antisera were tested against the various antigens. The anti-adult hemoglobin serum produced a precipitin ring with cord blood hemoglobin, while the antiserum prepared against this latter hemoglobin gave positive results with normal adult hemoglobin solution (fig. 1). The results obtained with the remaining antisera will not be discussed in detail, as they were very similar to those obtained with the agar plate method to be described below.

The agar plate method, as described by Ouchterlony, offered additional information concerning the antigenic properties of the hemoglobins under
Fig. 2.—Illustrates some of the results obtained with the agar plate method when sera were placed in the central holes and the different antigens around. The similarity of the precipitate patterns obtained between anti-cord blood hemoglobin serum and cord blood hemoglobin on the one hand, and the same serum and the hemoglobin solution of a thalassemic patient on the other, is striking. Both anti-rabbit and anti-dog hemoglobin sera produced precipitates with cord blood hemoglobin, whereas no precipitate developed between anti-cord blood hemoglobin serum and either rabbit or dog hemoglobin solutions. S = antiserum, H = human hemoglobin, F = cord blood hemoglobin, D = dog hemoglobin, Sal = saline, R = rabbit hemoglobin, STR = red cell stroma, PL = human plasma, FA = Freund’s adjuvant, THA = hemoglobin of thalassemic patient.

study. The precipitates which developed between the antisera and the various antigens are illustrated in figure 2. It can be seen that the serum obtained from a rabbit immunized against human hemoglobin solution produced two distinct lines of precipitate with its own antigen, one of them heavy and the other fainter, a faint and a heavy line of precipitate against cord blood hemoglobin, two lines of precipitate against human plasma, and one line against human red cell stroma. No precipitate developed between this antiserum and the dog and rabbit hemoglobins. It should be pointed out that the formation of a precipitate between the anti-adult hemoglobin serum and cord blood hemoglobin, as well as the fainter line of precipitate between this serum and its own antigen, could be prevented by mixing a dilute solution of cord blood hemoglobin with the agar before the application of the respective antisera and antigens. Similarly, the development of the other precipitate lines, such as between anti-human adult hemoglobin serum and plasma, could also be prevented by mixing the appropriate antigen solutions with the agar before the performance of the test.22 Antiserum against cord blood hemoglobin produced three lines of precipitate with its own antigen, one of which (the middle line in figure 2) could be prevented by prior absorption of this serum in adult human hemoglobin. This serum also produced distinct lines of precipitate against adult human hemoglobin, human plasma and human red cell stroma. Two lines of precipitate formed between this serum and hemoglobin
Fig. 3.—Schematic drawing of agar electrophoretic patterns of three different hemoglobin solutions. The point of application is on the right-hand side of the figure. Adult human hemoglobin (upper block) separated into four distinct bands. The slowest moving fraction corresponds to hemoglobin A₁, the fastest moving band is fetal hemoglobin. The nature of the other two fractions has not been clarified as yet. Note the increase in the fetal fractions in both the cord blood hemoglobin (middle block) and the hemoglobin of a thalassemic patient (lower block).

prepared from a patient with thalassemia. The development of these latter precipitates could be prevented by mixing the various antigens with the agar before it had solidified. Most of the antisera tested produced precipitate lines not only with the antigen against which they had been prepared but with other antigens as well. In all instances, however, the "non-specific" precipitate formation could be prevented by absorption of the tested antiserum with the respective "non-specific" antigen prior to the application of the former to the agar plate.

The results described above suggested that the antigens used in these experiments were either "contaminated," so that each of the resulting antisera contained antibodies against several of the antigens, or there were common antigenic groups among the antigens used for immunization. In order to clarify this question, a method was devised of separating hemoglobin from non-hemoglobin protein, and of separating the various hemoglobin fractions from each other by agar electrophoresis. Figure 3 illustrates some of the patterns obtained by the method of agar electrophoresis employed here. In figure 3 (upper block) the pattern of a normal adult hemoglobin solution following electrophoresis is illustrated, and in it the slow-moving component corresponds to A₁, while the fastest moving component corresponds to the alkaline-resistant hemoglobin fraction. The middle block of figure 3 shows the electrophoretic pattern of a cord blood hemoglobin solution, and in the lower block that of a patient suffering from thalassemia is illustrated. In the latter two patterns the fetal fraction is markedly increased. The method seemed particu-
Fig. 4.—Illustrates the precipitates formed between antihuman hemoglobin serum and normal human whole blood hemolysate after the electrophoretic fractionation of the latter in agar. Two precipitates developed between the hemoglobin-containing portion of the block and the antisera, and two more precipitate lines appeared between the non-hemoglobin protein fractions and the serum.

Fig. 5.—Antihuman red cell serum produced one line of precipitate with the hemoglobin-containing part of the block and two more lines with the non-hemoglobin protein fractions of the hemolysate.

Figure 4 illustrates the pattern of precipitate formation between whole blood lysate and antiserum prepared against adult human hemoglobin solution. It can be seen that two heavy lines of precipitate developed between the serum and the hemoglobin component of the lysate. One of them, the shorter one, extends to where fraction A₁ is located, while the second line extends along the whole area covered by hemoglobin. Two additional faint precipitates developed between this serum and the non-hemoglobin protein fraction of the hemolysate. Three distinct lines of precipitate developed between whole blood lysate and anti-red cell serum (fig. 5). One such line was located along the A₁ fraction of the hemoglobin, while the other two lines occurred along the non-hemoglobin protein part of the hemolysate. A somewhat similar pattern was obtained when the antiserum applied to the block was prepared against human red cell stroma (fig. 6). In this case, however, no precipitate developed between the hemoglobin band and the antiserum. The antiserum prepared against human plasma produced a single heavy line.
Fig. 6.—Antihuman red cell stroma serum produced two lines of precipitate. One of these developed around the point of application of the hemolysate, while the other line extends over the area where the non-hemoglobin protein portion of the hemolyzate is located.

Fig. 7.—Shows one line of precipitate between the antihuman plasma serum and the non-hemoglobin protein part of the hemolyzate.

of precipitate with the non-hemoglobin protein fraction of the hemolysate (fig. 7). When anti-cord blood hemoglobin serum was applied between solutions of cord blood hemoglobin and of thalassemia patients' hemoglobin, a single line or precipitate developed after electrophoresis between the cord blood hemoglobin and the serum, extending along the whole length of the hemoglobin, while only a short line of precipitate developed between this serum and the fetal fraction of the hemoglobin of the thalassemia patient (fig. 8). It seems, therefore, that the antigenicity of the cord blood hemoglobin is identical with the alkaline-resistant hemoglobin of thalassemia patients.

DISCUSSION

The data presented here provide sufficient evidence to show that, under suitable conditions, antisera with high antibody titers can be produced against hemoglobin solutions prepared from human, canine and rabbit blood. It has also been demonstrated that the antibodies produced against these different hemoglobins are highly specific. The difficulties encountered in attempting to establish the antigenic specificity of these proteins are well illustrated by the need for five different methods to arrive at a sufficiently well-documented conclusion. The complement fixation provided the information that the sera
Fig. 8.—Illustrates the lines of precipitate formed between anti-cord blood serum and cord blood hemoglobin on the one hand (upper block), and a hemoglobin solution from a thalassemic patient (lower block). The precipitate line between the former hemoglobin solution and antiseraum extends along the whole length of the block, whereas between the serum and the thalassemia patient’s hemoglobin it appeared only along the area where the fetal fraction of the hemoglobin solution is located.

of rabbits given repeated injections of hemoglobin solutions contained antibodies against the respective antigens. There was, however, a considerable overlap when this method was applied to test the specificity of the various antisera. Thus the complement fixation test was positive up to a high titer when anti-human adult hemoglobin serum was tested against cord blood hemoglobin, and vice versa. This finding is not surprising in view of the fact that there is sufficient adult hemoglobin in cord blood and sufficient fetal hemoglobin in adult red cells to produce antibodies when injected repeatedly into rabbits.

As shown by the complement fixation test and the agar plate method, antihuman hemoglobin sera reacted with dog and rabbit hemoglobin solutions. These reactions could be eliminated by the absorption of the sera with a dilute solution of the respective antigen. It should be noted, however, that while absorption of antihuman hemoglobin serum with dog hemoglobin solution eliminated the reaction between them, the same antiserum would still react with rabbit hemoglobin, although in low titer. It seems reasonable to conclude, therefore, that the cross-reactivity observed between the various antisera and the hemoglobin solutions was not due to some other, non-hemoglobin protein such as catalase or methemoglobin reductase which may have been present in any of the antigens used in these experiments, some of which were shown to be good antigens. The above observations suggest that some common antigenic properties exist between human, dog and rabbit hemoglobins which can be demonstrated by the above methods. The explanation of this common antigenicity of hemoglobins of these species is not apparent. One could mention in this respect the finding of Hunt and Ingram showing that the structure of the polypeptide chains alpha and beta,
which are the constituents of the main hemoglobin fractions, vary little from species to species; thus it can be speculated that the antibodies prepared against them may cross-react.

The reason for the relatively low titer found in the complement fixation test between antihuman adult serum and globin solution is not clear. It is possible that the isolation of globin brought about such structural changes in the molecule as to render it "unrecognizable" by the antibody produced against the intact material.

It has been shown by Heller et al.\textsuperscript{13} that the $A_2$ and $F$ hemoglobin fractions are strongly antigenic, while the $A_1$ fraction seems to be a weak antigen. The immunoelectrophoretic patterns observed in the present study indicate that one of the precipitate lines developed between the antihuman adult hemoglobin serum and that portion of the block where the $A_1$ fraction was located. These results seem to indicate that the $A_1$ hemoglobin can be indentified immunologically. Since, however, under the conditions employed here for the electrophoretic separations of various hemoglobins, the $A_2$ hemoglobin fraction is not discernable, it cannot be excluded that this latter component migrated together with the main fraction, and that the resulting precipitate developed between the $A_2$ hemoglobin and antiserum rather than between the $A_1$ hemoglobin and antiserum. Precipitate lines which developed between the fetal fractions of both adult and cord blood hemoglobins and that of thalassemia patients on the one hand, and anti-cord blood hemoglobin serum on the other hand, indicate that all these hemoglobin fractions are immunologically indistinguishable. These findings agree with those of Heller et al.\textsuperscript{13} and are contrary to the results reported by McCormick and Walker and others.\textsuperscript{17,18}

Whether the finding that hemoglobin is a fairly good antigen has any significance in human pathology cannot be stated at present. It should be mentioned, however, that the incubation of anti-hemoglobin with mature red cells causes profound changes in these cells in vitro. Furthermore, the injection of these antisera into experimental animals is accompanied by severe hematologic changes. The results of these latter investigations will be published separately.

**Summary**

Specific antisera with high antibody titers could be prepared against adult, cord blood, dog and rabbit hemoglobin solutions. These sera were employed to study the antigenic properties of the above hemoglobins.

Common antigenic properties could be detected between hemoglobin solutions prepared from human, canine and rabbit blood.

No immunologic difference could be found between the fetal fractions of adult human blood, cord blood and thalassemia patients' hemoglobin solutions.

**Summario in Interlingua**

Specific antiseros con alte titros anticorporee poteva esser preparate contra solutiones de hemoglobina human adulte, human de cordon, canin, e de
conilio. Iste seros esseva empleate pro studiar le proprietates antigenic del mentionate hemoglobinas.

Commun proprietates antigenic poteva esser detegite pro solutiones de hemoglobina preparate ab sanguine human, canin, e de conilio.

Nulle differentia immunologic poteva esser trovate inter le fractiones fetal de solutiones de hemoglobina ab (1) normal sanguine adulte, (2) normal sanguine de cordon, e (3) sanguine de patientes con thalassemia.

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STUDIES ON HEMOGLOBIN. I


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