

## Serum Protein Binding of Myoglobin, Hemoglobin and Hematin

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**R**ECENTLY much attention has been focused on the protein binding of hemoglobin. The hemoglobin binding proteins, collectively called haptoglobin, have been thoroughly studied by several workers.<sup>1-5</sup> Identification of these substances has helped to clarify the concept of "renal threshold" for hemoglobin excretion.<sup>5,6,9</sup>

Subsequently, in an investigation of the difference in "renal threshold" of myoglobin and hemoglobin, Javid et al.<sup>10</sup> reported that there was no binding of myoglobin by haptoglobin and in fact could not demonstrate binding of myoglobin by protein. However, Lathem<sup>11</sup> later presented evidence of protein binding of myoglobin. This protein-pigment complex was found to have electrophoretic characteristics similar to but not identical with protein bound hemoglobin. In addition, various workers<sup>5,8,12,13</sup> have described hemoglobin and hematin binding by a globulin which does not appear to be a haptoglobin. This protein apparently binds the heme portion of the hemoglobin molecule in contrast to haptoglobin which binds the globin fraction.<sup>7</sup>

This study was devised to investigate further the binding of heme compounds, especially myoglobin, by human serum proteins.

### MATERIALS AND METHODS

Hemoglobin was obtained by distilled water lysis of red blood cells with removal of stroma by centrifugation.

Alkaline hematin was prepared by the addition of 5 drops of 1 N. NaOH to 0.5 ml. of 2.5 Gm. per 100 ml. hemoglobin solution.

Myoglobin was prepared by the method of Ginger et al.<sup>14</sup> from human muscle obtained at autopsy. This material was found to be spectrophotometrically characteristic of metmyoglobin and migrated as a single band on paper and starch-gel electrophoresis.

Each of these pigments was then added separately in varying concentrations to normal sera of haptoglobin types 1-1, 2-1, 2-2, 0-0,\* and to anhaptoglobinemic sera from patients with hemolytic anemia. Serum hemochromogen concentrations were determined by the method of Crosby and Furth.<sup>15</sup> Electrophoresis was then performed on Whatman #3 filter paper using a pH 8.6 barbital buffer and a pH 7.0 phosphate buffer.<sup>16</sup> Starch-gel electrophoresis was performed according to the technique of Smithies.<sup>17</sup>

The paper strips were stained with benzidine or with bromphenol blue. Pigment binding was quantitated by using the benzidine stained strips run at pH 7 and a Spinco automatic recording densitometer.<sup>18</sup> Hemoglobin binding capacities were determined by the method of Laurell and Nyman<sup>8</sup> and by the method described by Lathem.<sup>19</sup> The latter method is

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\*We are indebted to Dr. B. S. Blumberg, National Institutes of Health, Bethesda, Md., for providing the type O-O serum. The term "type O-O" is misleading as an haptoglobin-O gene probably does not exist. Giblett and Steinberg believe that this anhaptoglobinemic state is related to the presence of a modified Hp2 gene.<sup>20</sup>

Fig. 1.—Diagrams of paper electrophoresis of normal and anaptoglobulinemic sera at pH 8.6 and 7 are shown. The point of application is indicated by the vertical limb of the arrow which is pointed in the direction of migration. The cathodal aspect of the 8.6 strips is not shown. The pigment-free serum is stained with bromphenol blue. The remaining sera to which the indicated pigments have been added are stained with benzidine. Free hemoglobin is usually seen in such sera and is shown by dotted blocks.

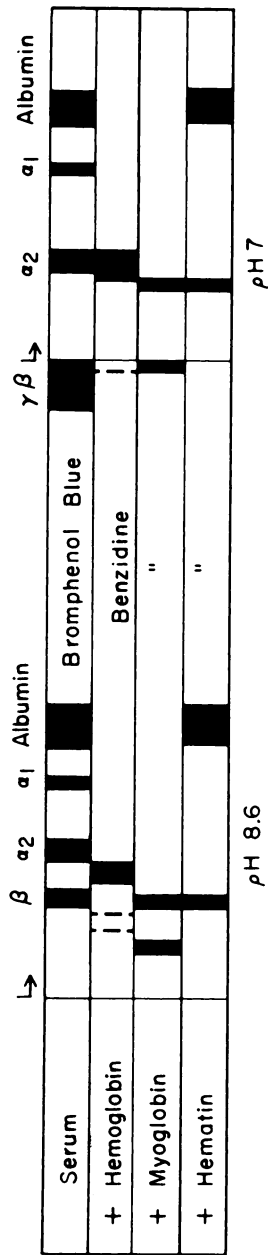
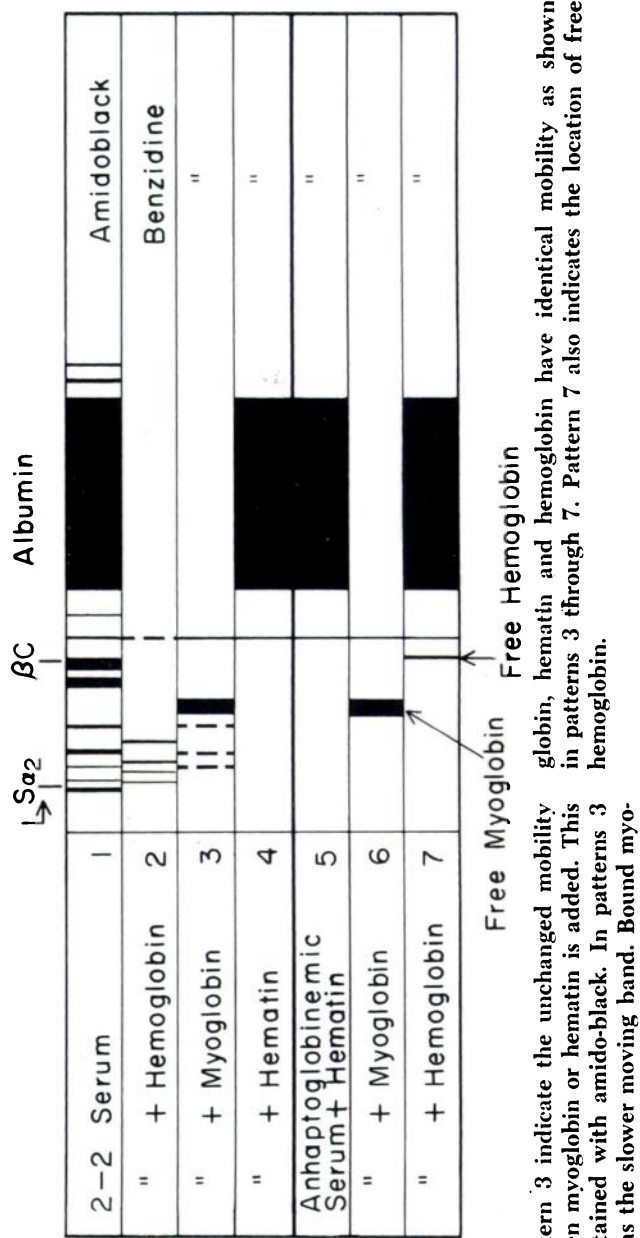


Fig. 2.—This diagram illustrates the results of starch gel electrophoresis of normal type 2-2 and anaptoglobulinemic serum with pigments added as indicated. Pattern 1 shows normal type 2-2 serum stained with amido-black. In patterns 2-7, only those bands which are benzidine positive are shown. The dotted line in pattern 2 represents the nonhaptoglobin hemoglobin binding. The solid lines indicate the haptoglobin-hemoglobin complex. The dotted lines of the haptoglobin bands when myoglobin or hematin is added. This is seen if the gel is counterstained with amido-black. In patterns 3 and 6 free myoglobin is seen as the slower moving band. Bound myo-



easier for estimation of binding capacity, but we found variation of up to 15 mg. per cent on duplicate determinations.

The starch gels were cut into duplicate halves. One part was stained initially with benzidine and counterstained with amido-black as suggested by Giblett.<sup>20</sup> This allowed accurate delineation of the heme containing site in relation to the other bands. The benzidine positive bands appeared black compared to the blue color of the other proteins. The other half of the gel was stained with amido-black only.

## RESULTS

### *Normal Serum*

All normal serum bound\* hemoglobin, myoglobin and hematin. Figure 1 illustrates the paper electrophoretic patterns at pH 8.6 and 7.

Table 1 shows the amounts bound. Although there was definite binding of myoglobin and hematin, the method did not allow distinction between binding by haptoglobin or another protein fraction. The use of starch gel electrophoresis and anhapto-globinemic sera allowed this distinction to be made.

Figure 2 shows a diagram of the results of the starch-gel electrophoresis. It is seen that when hemoglobin is added to serum, the haptoglobin bands are slowed in mobility and become benzidine positive, indicating union with hemoglobin. On the other hand, following the addition of myoglobin or hematin, no alteration in mobility of the haptoglobin bands occurred nor did they stain with benzidine. The bound band of both the myoglobin and the hematin is seen just ahead of the beta-C band. This was a constant finding, unrelated to the haptoglobin type, thus indicating binding by a nonhapto-globin protein. When a quantity of hemoglobin was added equal to, but not exceeding, the binding capacity of normal serum, a benzidine-positive complex was seen in the gel in addition to the haptoglobin bands (fig. 2). This band was identical in mobility to bound myoglobin and hematin and migrated just ahead of free hemoglobin.

### *Anhapto-globinemic Sera*

In order to determine if binding of these pigments occurred in the absence of haptoglobin, one type 0-0 serum and six sera from patients with hemolytic anemia were tested.

As shown in table 1, the type 0-0 serum bound all three pigments, as did three of the hemolytic anemia sera. Two of the hemolytic anemia sera bound no pigment, while the sixth bound hemoglobin and myoglobin but was not tested for hematin binding.

The electrophoretic patterns are shown in figures 1 and 2. From the paper patterns one could not be certain that haptoglobin was absent. The starch gel patterns, however, clearly demonstrated that none was present. It was

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\*Binding as used here refers to binding by globulin only. Methemalbumin was seen when hemoglobin was added in excess of the globulin binding capacity, with concentrations of myoglobin exceeding 100 mg. per 100 ml. and at any concentration of hematin. We were unable to saturate the albumin binding of hematin with additions up to 200 mg. per cent.

Table 1.—Binding Data of Heme Pigments.

| Serum Source and Type                   | Hemoglobin Binding Capacity mg. % | Myoglobin Added mg. | Myoglobin Bound mg. %  | Hematin Added mg. | Hematin Bound mg. %    |
|---|-----------------------------------|---------------------|------------------------|-------------------|------------------------|
| Normal (2-1)                            | 100                               | 35                  | 5                      | 53                | 29                     |
|   |                                   | 55                  | 14                     | 157               | 38                     |
|   |                                   | 73                  | 22                     |                   |                        |
|   |                                   | 87                  | 18                     |                   |                        |
|   |                                   | 112                 | 23                     |                   |                        |
| Normal (2-2)                            | 65                                | 26                  | 2.5                    |                   |                        |
|   |                                   | 48                  | 6.0                    |                   |                        |
|   |                                   | 87                  | 10.0                   |                   |                        |
| Normal (2-2)                            | 85                                | 44                  | 5                      | 83                | 16                     |
|   |                                   | 144                 | 8                      | 155               | 16                     |
|   |                                   | 152                 | 5                      |                   |                        |
| Normal (2-1)                            | 140                               | 63                  | 1                      | 86                | 16                     |
|   |                                   | 68                  | 3                      | 135               | 16                     |
| Normal (2-2)                            | 30                                | 140                 | 12                     |                   |                        |
| Iron Deficient (2-2)                    | 60                                | 40                  | 12                     | 27                | 4                      |
|   |                                   | 50                  | 5                      | 46                | 9                      |
|   |                                   |                     |                        | 105               | 14                     |
|   |                                   |                     |                        | 158               | 20                     |
|   |                                   |                     |                        | 212               | 11                     |
| Local Infection                         |                                   | 22                  | 2                      |                   |                        |
|   |                                   | 50                  | 4                      |                   |                        |
|   |                                   | 72                  | 5                      |                   |                        |
|   |                                   | 90                  | 4                      |                   |                        |
| 0-0                                     | Approximately 10                  | 100                 | Visible Not Measurable | 100               | Visible Not Measurable |
| Autoimmune Hemolytic Anemia             | 14                                | 130                 | 13                     | 186               | 28                     |
| Hemolytic Transfusion Reaction (anti-c) | < 2 mg. % Visible Not Measurable  | 100                 | 0                      | 100               | 0                      |
| Same as above During Recovery           | 6                                 | 90                  | 6                      | 90                | 16                     |
| Thalassemia Sickle Disease              | 13                                | 104                 | 8                      | -                 | -                      |
| Hereditary Spherocytosis                | Visible Not Measurable            | 60                  | Visible Not Measurable | 85                | Visible Not Measurable |
| Sickle Cell Anemia                      | 0                                 | 100                 | 0                      | 100               | 0                      |
| Paroxysmal Nocturnal Hemoglobinuria     | 0                                 | 115                 | 0                      | 100               | 0                      |

also evident in the starch gel that all three of these pigment-protein complexes migrated identically with each other and with the nonhaptoglobin protein pigment complexes seen with normal serum.

The patient with the transfusion reaction shown in table 1 is of interest in that return of nonhaptoglobin binding was seen during recovery. Later specimens were not available to determine the return and type of haptoglobin.

#### DISCUSSION

Hemoglobin binding by plasma proteins was first described by Palonovski and Jayle who proposed the name haptoglobin.<sup>1,2</sup> Numerous studies of hapto-

globin have followed, and these have been reviewed by Nyman.<sup>8</sup> Reich earlier had reported globulin binding of hematin.<sup>12</sup> In addition to haptoglobin, another hemoglobin binding protein was described by Neale.<sup>13</sup> This protein, referred to as "heme binding beta protein" by Nyman was found to differ from haptoglobin in several ways.<sup>8</sup> It was thought to be a beta rather than an alpha 2 globulin. Its affinity for hemoglobin was much lower than that of haptoglobin. This protein also was found to bind the heme portion of the hemoglobin molecule while haptoglobin bound the globin portion. In fact, Neale found that this substance bound hematin as well as hemoglobin and further noted that, like haptoglobin, it was absent in some cases of hemolytic anemia.<sup>13</sup>

Protein binding of myoglobin has been studied by Javid et al. and by Lathem.<sup>10,11</sup> The former, using human serum, human myoglobin and starch-gel electrophoresis, found no evidence of binding. Lathem, on the other hand, using dog serum, dog myoglobin and paper electrophoresis was able to demonstrate binding. He found, as did we, that the bound myoglobin had similar, but not identical, paper electrophoretic characteristics to those of protein bound hemoglobin. By the addition of both pigments to the same serum sample, he obtained results which suggested that myoglobin and hemoglobin competed for some of the same binding sites. Our findings substantiate this and indicate that the common site is not haptoglobin, as shown by the location of the bound complex on starch gel and by the demonstration of binding in an haptoglobinemic sera. Further evidence indicates that the same protein binds hemoglobin, myoglobin and hematin. (1) The bound complexes have identical mobility in starch gel, (2) comparable binding ability for each pigment, and (3) absence of myoglobin and hematin binding in cases of hemolytic anemia where no hemoglobin binding occurs.

We were not able to show definitely whether this heme binding globulin is an alpha 2 or beta globulin. The starch-gel procedures suggest that it is an alpha 2 globulin.

When hemoglobin binding capacity is determined by paper electrophoresis, this nonhaptoglobin binding contributes only slightly (about 10 mg. per cent) to the total binding capacity of normal serum, but it may represent most or all of the binding in sera from patients with hemolytic disease. With abnormal hemolysis it appears that the sequence of events is first depletion of haptoglobin and then, if severe enough, depletion of the heme binding globulin. Neale has shown that during recovery the reverse order obtains; i.e., return of heme binding globulin, then return of haptoglobin.<sup>12</sup>

#### SUMMARY

Using paper and starch-gel electrophoresis we have demonstrated binding of hemoglobin, myoglobin and hematin by human serum protein which is not a haptoglobin. This protein was present in all sera tested except that from two patients with hemolytic anemia and was also present in serum genetically deficient in haptoglobin.

The binding capacity of this protein is low and contributes only slightly to

the total hemoglobin binding capacity of normal serum. However, it may represent most or all of the binding in sera from patients with hemolytic disease.

#### SUMMARIO IN INTERLINGUA

Per medio de electrophorese a papiro e a gel de amylo nos ha demonstrate le ligation de hemoglobina, myoglobina, e hematina per un proteina de sero human que non esseva un haptoglobina. Iste proteina esseva presente in omne le seros testate, excepte in illos de duo patientes con anemia hemolytic. Illo non mancava in seros geneticamente deficiente in haptoglobina.

Le capacitate ligatori de iste proteina es basse. Illo contribue solmente pouco al total capacitate hemoglobino-ligatori de sero normal. Tamen, il es possibile que illo representa le plus grande parte o mesmo le totalitate del ligation presente in seros ab patientes con morbo hemolytic.

#### REFERENCES

- Polonovski, M. and Jayle, M. F.: Peroxydas animals, leur specificite. *Bull. Soc.Chim.Biol.* 21:66-91, 1939.
- Polonovski, M. and Jayle, M. F.: Sur la preparation d'une nouvelle fraction des proteines plasmatiques, l'haptoglobine. *C.R.Acad.Sc.* 211:517, 1940.
- Smithies, O.: Zone electrophoresis in starch gels: Group variations in the serum proteins of normal human adults. *Biochem.J.* 61:629-641, 1955.
- Smithies, O. and Walker, N. P.: Notation for serum protein groups and the genes controlling their inheritance. *Nature* 178:694-695, 1956.
- Allison, A. C. and Rees, W.: The binding of hemoglobin by plasma proteins (haptoglobins). *Brit.M.J.* 2: 1137-1143, 1957.
- Laurell, C. B. and Nyman, M.: Studies on the serum haptoglobin level in hemoglobinemia and its influence on renal excretion of hemoglobin. *Blood* 12:493-506, 1957.
- Bearn, A. G. and Franklin, E. C.: Comparative studies on the physical characteristics of the heritable haptoglobin groups of human serum. *J. Exper.Med.* 109:55-68, 1959.
- Nyman, M.: Serum haptoglobin. Methodological and clinical studies. *Scand. J.Clin.& Lab.Invest. Suppl. No. 39, Vol. 11*, 1959.
- Lathem, W.: The renal excretion of hemoglobin: Regulatory mechanisms and the differential excretion of free and protein-bound hemoglobin. *J. Clin.Invest.* 38:652-658, 1959.
- Javid, J., Fischer, D. S. and Spaet, T. H.: Inability of haptoglobin to bind myoglobin. *Blood* 14:683-687, 1959.
- Lathem, W.: The binding of myoglobin by plasma protein. *J.Exper.Med.* 111: 65-75, 1960.
- Reich, M.: The electrophoretic mobility of haemoglobin and its derivatives and the effect of their addition to the serum in various animal species. *Austral.J.Exper.Biol.* 34:343-348, 1956.
- Neale, F. C., Aber, G. M. and Northam, B. E.: The demonstration of intravascular haemolysis by means of serum paper electrophoresis and a modification of Schumm's reaction. *J. Clin.Path.* 11:206-219, 1958.
- Ginger, I. D., Wilson, G. D. and Schweigert, B. S.: Biochemistry of myoglobin. Quantitative determination in beef and pork muscle. *Agricultural and Food Chemistry* 2:1037-1040, 1954.
- Crosby, W. H. and Furth, F. W.: A modification of the benzidine method for measurement of hemoglobin in plasma and urine. *Blood* 11:380, 1956.
- Durrum, E. L.: A microelectrophoretic and microionophoretic technique. *J.*

- Am.Chem.Soc. 72:2943-2948, 1950.
17. Smithies, O.: An improved procedure for starch-gel electrophoresis: Further variations in the serum proteins of normal individuals. *Biochem.J.* 71: 585-587, 1959.
18. Lathem, W. and Worley, W. E.: The distribution of extracorporeal hemo- globin in circulating plasma. *J. Clin. Invest.* 38:474-483, 1959.
19. Lathem, W. and Jensen, W. N.: Plasma hemoglobin-binding capacity in sickle cell disease. *Blood* 14:1047-1056, 1959.
20. Giblett, E., Personal communication.

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SEPARATION OF THE VALYL-LEUCYL AND VALYL-GLUTAMYL-POLYPEPTIDE CHAINS OF HORSE GLOBIN BY FRACTIONAL PRECIPITATION AND COLUMN CHROMATOGRAPHY. *S. Wilson and D. B. Smith.* From National Research Council, Ottawa, Canada. *Canad.J.Biochem.Physiol.* 37:405, 1959.

Horse globin was fractionated into two components by stepwise addition of acid acetone to an acidified globin solution and by gradient elution with urea from an amberlite (carboxylic) ion exchange resin at low pH. Both methods yield preparations similar to those obtained by electrophoretic technics. One of the components has the N-terminal sequence valyl-leucyl and the other valyl-glutamyl using the isothiocyanate and DNP methods. A short note on the nomenclature of the polypeptide chains of hemoglobin is included.—*A. I. C.*

STUDIES IN ANAEMIA OF INFECTION. PART III. MECHANISM OF HYPOFERREMIA: INFLUENCE OF BODY TEMPERATURE ON PLASMA IRON. *S. Gupta, S. Kumar and V. S. Mangalik.* From Lucknow University, Lucknow, India. *Indian J.Med.Res.* 47:266, 1959.

Some correlation was observed between the degree of pyrexia and the extent of hypoferremia in patients with infection, but low plasma level was also seen in a few afebrile cases. Similar changes were also seen in experimental infection. Physical hypothermia did not produce any change in plasma iron in rabbits.—*J. B. C.*