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Inability of Haptoglobin to Bind Myoglobin

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THE EXISTENCE of a hemoglobin-reactive substance in the sera of normal subjects was first described in 1939 by Polonovski and Jayle,[1] who found enhancement of the peroxidative activity of hemoglobin (Hgb) on addition of serum. This substance they named haptoglobin (Hp). Subsequent studies showed that this factor selectively binds Hgb, and it is now established that virtually the entire Hgb-binding capacity of serum is due to its hp content. Normally the Hp in 100 ml. of serum can bind about 125 mg. Hgb[2]. The renal handling of Hgb has recently been shown to depend to a large extent upon its binding with Hp. Thus Hgb does not appear in the urine until the Hgb-binding capacity of blood is exceeded.[2-4] This accounts for the pigment being present simultaneously in the plasma and in the urine of patients with hemoglobinuria, a finding incorrectly attributed to a high renal threshold for free Hgb.[5,6]

Myoglobin (Mb) is only slightly increased in the plasma even when it spills readily in the urine. It was presumed that the renal threshold of Mb was 15 mg./100 ml. because its molecular weight is only 17,000 in contrast to 68,000 for Hgb.[6] Since the demonstration of Hgb binding by Hp it was of interest to determine whether Mb is similarly bound. So far our studies have failed to reveal any binding of Mb by Hp. Evidently the lower "renal threshold" of Mb represents its presentation to the glomerulus as free Mb, with a molecular weight of 17,000, in contrast to the Hgb-Hp complex with an estimated molecular weight of 310,000.[4]

Materials and Methods

Mb was obtained by saline or water extraction, in the cold, of human pectoralis, rectus, ilio-psoas and deltoid muscles obtained at postmortem. Initially, purification was attempted by dialyzing the crude extract against saline to remove the soluble impurities and then injecting the material intravenously into rabbits. A rapid diuresis ensued and the specimen obtained by catheterization contained high concentrations of human Mb, with no demonstrable Hgb, and presumably no rabbit Mb, since the rabbit has been reported to have none.[7] The report of Liang[8] that rabbit serum does not bind Hgb caused us to purify the aqueous muscle extract by a method recommended to us by Perkoff[9] modified from that of previous workers.[10-12] The method is as follows: 10 to 20 Gm. muscle was ground in a meat grinder, mixed with distilled water, and allowed to stand overnight at 10° C. The mixture was then centrifuged and the supernate was adjusted to pH 7 with 2 per cent NH_4OH. Saturated basic lead acetate, in the amount of ¼ the initial volume, was added to this solution, presumably precipitating all proteins save Hgb and Mb. Then, 4.72 Gm. phosphate powder mixture (2.88 Gm. K_2HPO_4

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to 1.84 Gm. KH_2PO_4) were added to each 10 ml. of the supernate and the mixture was shaken vigorously. The Hgb was precipitated, and a clear reddish brown supernate containing Mb practically free of Hgb and other proteins was obtained. The Mb moved electrophoretically as a single distinct band when stained with benzidine or with a protein stain (naphthalene black, B 200). When the Mb solution was examined in a Beckman DU Spectrophotometer, no discernible Hgb band could be seen.[13] The solution was dialyzed overnight against distilled water, and the Mb solution thus obtained was used for electrophoresis.

Samples of serum were obtained from normal adults, refrigerated and used within three days of collection. The sera, when electrophoresed alone, showed no benzidine staining bands, implying lack of significant hemolysis. AA Hgb was obtained by the usual method of lysing washed normal red cells with distilled water, and extracting with toluene. All Hgb samples were Seitz-filtered and/or centrifuged at 3000 rpm for 10 minutes to remove remaining red cell stroma. Identical results were obtained whether the hemoglobin solutions were centrifuged and presumably contained some stroma or were the stroma-free Seitz-filtered preparations. Concentrations of Hgb and of Mb were determined colorimetrically by the cyanomethemoglobin method in a Coleman Junior Spectrophotometer, at 540 mμ. Samples of serum containing Hgb or Mb were prepared by diluting solutions of Hgb and of Mb, of known concentration, with serum to obtain the desired final concentrations of the two pigments. All such mixtures were incubated at 37°C. for 15 minutes before being subjected to electrophoresis. In estimating the binding capacity of the serum for Hgb, the effect of diluting the serum with Hgb solution, though slight, was taken into consideration.

Starch gel electrophoresis was carried out according to the method of Smithies[14] for 10 to 16 hours at 4 volts/centimeter and 10°C. The strips were cut into duplicate halves and one half was stained for proteins,[14] the other for Hgb and Mb by the benzidine stain.[2] The starch used was Starch Hydrolyzed.*

When mobilities of bands in different samples were to be compared, two samples were introduced side by side in the same strip of gel. Mobilities of the bands were taken to be indentical if they formed a continuous band across the strip, a criterion which Smithies[14] found to be more reliable than the comparison of absolute mobilities in different strips.

Results

Our studies confirm the observations on the binding of Hgb by Hp as reported by Allison and Rees,[2] Laurell and Nyman[4] and Lanthem and Worley.[3] Hgb was shown to migrate on starch gel more rapidly than Mb, as it does on paper.[15] Hgb, in concentrations below 125 mg. per cent, was bound to Hp, but in concentrations of 150 mg. per cent or higher a band having the mobility of free Hgb also appeared. Mb, on the other hand, moved as a single band in serum at concentrations of 25, 50, 125, and 200 mg. per cent, always having the same mobility as Mb alone (fig. 1). Moreover, the presence of Mb in serum caused no change in mobility of the Hp bands. At the concentrations of Mb employed, no benzidine-staining material was noted in the albumin region, whereas methemalbumin bands were consistently present when 200 mg. per cent or more of Hgb was used. Similar results were obtained with Mb prepared by chemical fractionation or rabbit injection technics.

Discussion

Although Mb was first claimed to be distinct from Hgb by von Kölliker,[16] it remained for Camus and Pagniez[17] in 1902 to show that Mb was excreted

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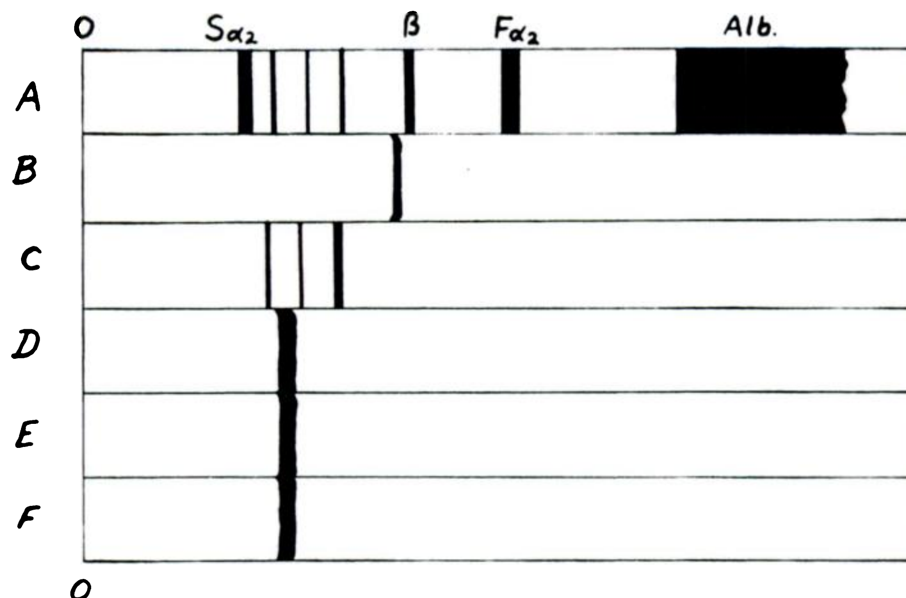

FIG. 1.—Diagram of starch gel strips stained with naphthalene black (A, B and D) and with benzidine (C, E and F). Only anodic segments of the strips are shown; samples were introduced at 0.

A. Normal human serum. The three bands appearing between $S\alpha_2$ and β are Hp bands. B. Human AA Hgb. alone. C. Human AA Hgb. in serum (100 mg. per cent). Three Hgb-Hp bands are seen with the benzidine stain, moving more slowly than the Hp bands in A. D. Human Mb alone. E. Human Mb in serum, 125 mg. per cent. F. Human Mb alone.

selectively by the kidney. They demonstrated that the amount of Mb recovered in the urine was proportional to the amount injected intravenously, and that the urine was deeply colored while the plasma was only faintly tinged. They went on to show that the injection of similar concentrations of Hgb did not result in hemoglobinuria. Intravenous injection of a mixture of Mb and Hgb resulted in myoglobinuria alone. Some of these findings were confirmed by Whipple and his co-workers,[18] who also showed the "renal threshold" for Mb to be about 15 mg. per Kg. body weight.[19,20] It was also demonstrated[21] that the "renal threshold" for Hgb was 155 mg. per Kg. body weight, but that this threshold could be lowered as much as 60 per cent by daily injections of Hgb. Yuile et al.[22] corroborated this and found the "renal threshold" to be 17 mg./100 ml. for Mb. They assumed that both Hgb and Mb filtered through the glomeruli at a constant rate, but that the tubular reabsorption reached a maximum and remained constant at higher plasma concentrations. It was also concluded that although Mb and Hgb were cleared from the plasma by the same mechanism, Mb was cleared about 25 times as rapidly.[6] Sellard and Minot[23] had previously demonstrated that the "threshold" of Hgb was lower in patients with pernicious anemia, but they could not offer a better explanation than that of Whipple or Yuile.

Recent studies have made it clear that Hgb binds with Hp in the normal

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subject up to concentrations of 125 to 135 mg. Hgb per 100 ml. of serum.[2,4] Plasma Hp is decreased or absent in patients with march, nocturnal, and paroxysmal hemoglobinurias,[2] and following major episodes of hemolysis. Laurell and Lyman[4] showed the Hgb-Hp complex, once formed, to disappear from the plasma at a rate of 13 mg./100 ml./hour until very low plasma levels were attained; this parallels the fall in the Hgb-binding capacity of plasma. Once hemolysis ceases, the Hp level returns to normal within about a week.

Evidently even free Hgb is not handled entirely by complete filtration. The old observation that repeated Hgb injections over a period of several days (presumably eliminating the plasma Hp completely) lowers, but does not abolish, the "renal threshold" for Hgb[21] indicates other factors. A recent study by Lanthem[24] suggests that the excretory rate of free Hgb is largely determined by glomerular permeability, and that there is a small, but measurable, tubular reabsorption. It remains valid, however, that the "renal threshold" of plasma Hgb is primarily determined by its binding with Hp.

To date there are no published reports on the binding of Mb by Hp. Our in vitro studies suggest that there is no demonstrable binding of Mb by Hp of normal human serum, since the electrophoretic mobility of Mb is unchanged after incubation of various concentrations in serum known to contain good Hp levels as evidenced by the binding with Hgb. It is therefore reasonable to assume that the "low renal threshold" for Mb merely represents the availability of Mb, in the free form, to the glomeruli at low plasma Mb levels.

Summary

1. The old and the current concepts of the renal handling of extracorpuscular plasma Hgb are briefly reviewed.

2. Studies are presented on the starch gel electrophoretic behavior of Mb alone and Mb in serum.

3. It is suggested that Mb is not bound by Hp, known to bind Hgb, and that this constitutes the reason for the "low renal threshold" of Mb as compared to that of Hgb.

Summario in Interlingua

1. Es presentate un breve revista de ancian e currente conceptiones del action renal super hemoglobina extracorpuscular del plasma.

2. Es presentate studios relative al comportamento electrophoretic (a gel de amylo) de myoglobina sol e de myoglobina in sero.

3. Es suggerite que myoglobina non es ligate per haptoglobina, le qual es cognoscitemente capace a ligar hemoglobina, e que isto es le ration del existentia de un "basse limine renal" de myoglobina in comparation con illo de hemoglobina.

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