DISCRETE CHARACTERIZATION OF THE SUB-FRACTIONS OF PORCINE HAPTOGLOBIN (BAND 5)

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ABSTRACT. — The electrophoretic pattern for porcine haptoglobin (band 5 or Hp5) as devloped with pig hemoglobin and observed after starch gel electrophoresis, consists of two bands of nearly equal width and intensity; while the pattern developed with chicken hemoglobin consists of three bands (Hp5-F, Hp5-M and Hp5-S), of which Hp5-F and Hp5-M are equally narrow and intense, and Hp5-S is approximately four times as wide and intense as the others.

A variety of electrophoretic patterns of Hp5-pig hemoglobin complexes has been detected with the use of chicken hemoglobin. These uniformly varying patterns are dependent on the quantity of pig hemoglobin complexed with Hp5 prior to the addition of chicken hemoglobin.

In pig serum after starch gel electrophoresis, three benzidine staining bands (Hp1, Hp2 and Hp3) were initially called haptoglobins (Kristjansson, 1961), but further work has shown that they are hemebinding proteins and have subsequently been named hemopexins (Imlah, 1965; Graetzer *et al.*, 1965; Schröffel, 1966; Baker, 1967; Grunder, 1968). Similarly, a fourth band Hp0 (Hesselholt, 1963), and a fifth band Hp4 (Baker, 1967), belong to the hemopexin system in pigs.

If pig serum contained no pig hemoglobin, a sixth band named Hp5 (Kristjansson, 1961), on combination with chicken hemoglobin separated into three fractions on starch gels after electrophoresis and benzidine staining (Hesselholt, 1963; Mittal, 1966). Evidence has been reported that only the benzidine staining globins in this band 5 (Hp5) region are the 'true' haptoglobins (Hesselholt, 1963; Imlah, 1965; Grunder, 1968).

This report describes discrete characterization of the sub-fractions of the 'true' porcine haptoglobins (Hp5). In addition, evidence is presented showing variation in the electrophoretic mobility of these sub-fractions when combined with various amounts of porcine hemoglobin.

MATERIALS AND METHODS

Chicken hemoglobin solution (CHB) prepared from the fresh blood of a White Leghorn hen containing approximately 7% (w/v) hemoglobin, and pig hemoglobin solution (PHB) prepared from the fresh blood of a Yorkshire pig containing approximately 11.5% (w/v) hemoglobin were used (Hesselholt, 1963). Nonlysed pig serum was prepared from the blood of an eight-month-old Duroc boar, and was used in 0.25 ml. aliquots.

Ten serial dilutions of PHB were prepared with distilled water and coded W (undiluted), W^1 (1 in 2), W^2 (1 in

4), ..., W^9 (1 in 512) respectively. CHB was used in the dilution of 1 in 2.

Experiment 1. Eleven 0.25 ml. aliquots of pig serum were placed in tubes 1 to 11. One drop (22 drops = 1 ml.) of a dilution of PHB (W to W^9 respectively) was added to a corresponding tube (1 to 10 respectively) containing pig serum. No PHB was added to tube 11 (serum control). A 0.25 ml. volume of distilled water was placed in tube 12, and one drop of PHB (W) was added to it (PHB control). The 12 tubes were incubated at 37°C. for 30 min. with periodic agitation, and stored at —20°C., until thawed and examined by stark gel electrophoresis.

Experiment 2. To first 10 aliquots prepared as in experiment 1 (addition of PHB and incubation), a drop of CHB was added to each tube (1 to 10), and they were again incubated. Six controls were included in the test: (11) serum + PHB; (12) serum + CHB; (13) serum; (14) distilled water + PHB + CHB; (15) distilled water + PHB; and (16) distilled water + CHB. The volume of serum or water was 0.25 ml., and one drop of respective hemoglobin (PHB undiluted; CHB dilution 1 in 2) was added in each case. Controls were incubated at the same time as the test samples, and stored at —20°C.

Samples in both experiments were examined by starch gel electrophoresis at 4°C., followed by benzidine staining (Smithies, 1959; Kristjansson, 1961; Hesselholt, 1963; Mittal, 1965).

Results and Discussion

Jayle and Moretti (1962) reported on the important role of hemoglobin in the haptoglobin metabolism of the human. Also much evidence has been presented on the characteristics of the human haptoglobins, as well as the variations in the electrophoretic mobilities of their complexes with different amounts of hemoglobin (Laurell, 1959; Smithies *et al.*, 1962; Nance and Smithies, 1963). This study focused on porcine haptoglobin and the electrophoretic mobilities of its many intermediate complexes formed with different amounts of the pig hemoglobin. These complexes have been further analysed using a heterologous hemoglobin (chicken), which characteristically separates pig haptoglobin into three sub-fractions.

In the first experiment, a constant volume of various dilutions of PHB was added to a constant volume of pig serum (constant amount of pig haptoglobin). The complexes formed between the constant amount of Hp5 and the higher amounts of pig hemoglobin (lowest five dilutions of PHB, samples 1 to 5) appear (Figure 1) as a single densely staining band (D), while the complexes between the same amount of Hp5 and lower amounts of pig hemoglobin (higher dilutions of PHB, samples 6 to 10) appear as two closely spaced and less intense bands (C and D). Serum control presented two extremely faint bands, perhaps due to extremely minute amounts of endogenous PHB in pig serum.

When tested by itself (PHB control, 12 in Figure 1), PHB separated into two bands referred to as free hemoglobin 1 (fast moving) and free hemoglobin 2 (slow moving) by Kristjansson (1961), here symbolized as pig Hb 1 and pig Hb 2 respectively (A and B bands in Figure 1; the two bands are not clear in Figure 1, they can be seen in Figure 2, samples 1, 2, 3, 11, 14 and 15). The pig Hb 2 band is wider than pig Hb 1, and both migrated faster than pig Hp5 fractions (C and D, Figure 1).

In the second experiment, again a constant volume of the various dilutions of PHB was added to a constant volume of pig serum first. Thus variable amounts of pig hemoglobin were allowed to combine with a constant amount of pig haptoglobin. Then a constant volume of a 1 in 2 dilution

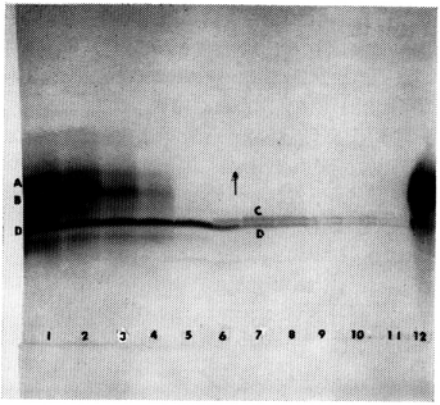

FIGURE 1. — Starch gel electrophoresis of boar serum. Benzidine stain. Arrow indicates the direction of migration of proteins in the gel. 1 to 10-serum aliquots to which serial dilutions of pig hemoglobin (W, W^1.....W^9 respectively)were added. Volumes of serum and pig hemoglobin solution (PHB) were equivalent for all samples. 11 - Serum control (no hemoglobin added); 12 - PHB control (no serum included); A - Band of pig Hb1 (also Figure 2); B - Band of pig Hb2 (also Figure 2); C - Band of pig Hp5 (fast moving); D - Band of pig Hp5 (slow moving).

of CHB (thus a constant amount) was added to each of the above mixtures.

Pig Hp5 combined with CHB alone (12 in Figure 2), separated into three fractions or bands designated Hp5-F, Hp5-M and Hp5-S (D, E and F bands respectively in Figure 2) in decreasing order of migration rate in starch gel. The Hp5-F and Hp5-M bands were narrow, and the Hp5-S band was twice as wide and intense as the others. Band C of experiment 1 (Figure 1) did not appear in the starch gel of experiment 2.

In the samples containing variable amounts of PHB, a serial change was observed in the patterns of Hp5 bands corresponding to the decreasing amounts of PHB (1 to 10, Figure 2). Hp5 was apparently saturated with PHB in samples 1 to 5 since only one band Hp5-F appeared with W to W^4, but this band decreased in width and intensity in W^5 to W^9 (6 to 10, Figure 2) as the other sub-fractions Hp5-M and Hp5-S began to appear. Hp5-M appeared in W^5, was of maximum intensity in W^6 and maintained width and intensity comparable to Hp5-F in W^7 to W^9 (8 to 10, Figure 2). Hp5-S appeared in W^6, gradually increased, and was widest and most intense in W^8 and W^9. Sample 5 (Figure 2) indicates the threshold dilution (W^4, PHB dilution 1 in 16) below which an amount of Hp5 is apparently left *unbound*, and thus is available for binding with CHB added later to develop the three bands characteristic of pig Hp5 bound with CHB (12 in Figure 2).

When tested by itself, CHB separated into four fractions (CHB control, 16 in Figure 2) designated chicken Hb 1, Hb 2, Hb 3 and Hb 4 (G, H, I and J respectively in Figure 2) in decreasing order of migration in starch gel. Bands of chicken Hb 1, Hb 2 and Hb 3 were of identical width, the Hb 4 band being wider.

When PHB and CHB were mixed, they migrated separately, apparently unaffected by the presence of the other (14, Figure 2), and the fractions of each migrated at the same rates as when tested independently (compare control 14 with 15 and 16, Figure 2). Control 13 (serum control) produced no detectable band. The pattern for serum + PHB control (11 in Figure 2) appeared similar to that of sample 1 of experiment 1 (1 in Figure 1), as expected.

In conclusion, for pig Hp5, two discrete sub-fractions have been observed with the use of homologous

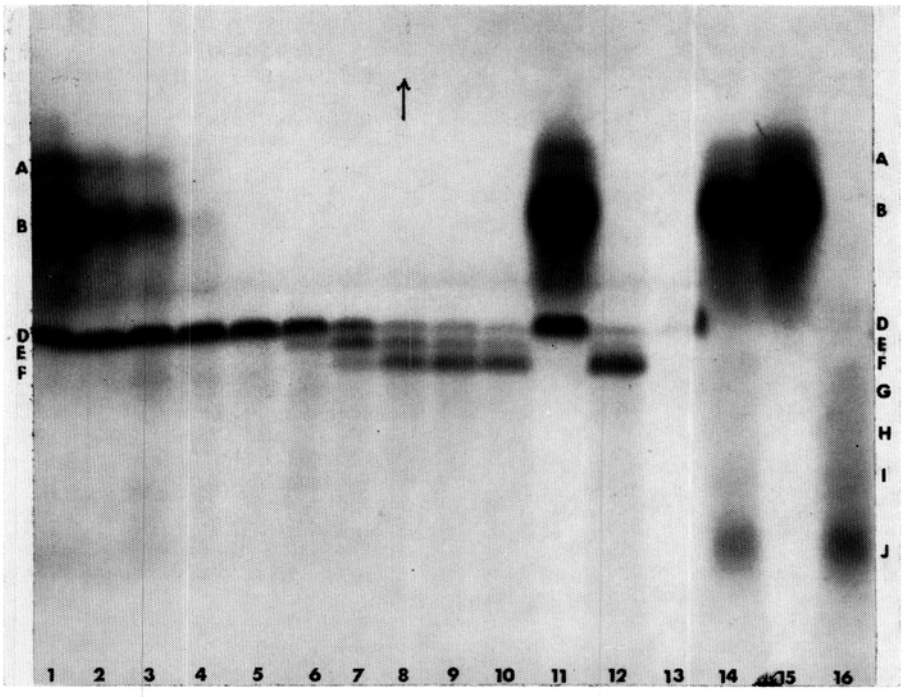

FIGURE 2. — Starch gel electrophoresis of boar serum. Benzidine stain. Arrow indicates the direction of migration of proteins in the gel. 1 to 10-serum aliquots to which serial dilutions of PHB (W,W^1.....W^9, respectively) were added first, and then chicken hemoglobin (dil 1 in 6) was added; 11 — serum + PHB control; 12 — serum + chicken hemoblobin (CHB) control; 13 — serum control; 14 — PHB + CHB control; 15 — PHB control; 16 — CHB control. A — Band of pig Hb1; B — Band of pig Hb2; D — Band of pig Hp5-F; E — Band of pig Hp5-M; F — Band of pig Hp5-S; G — Band of chicken Hb1; H — Band of chicken Hb2; I — Band of chicken Hb3; J — Band of chicken Hb4.

hemoglobin while three discrete sub-fractions have been detected with the use of heterologous (chicken) hemoglobin. The wide variety of patterns of pig Hp5-hemoglobin complexes observed in this study appeared dependent on the amount of PHB with which pig Hp5 was complexed. As the amount of PHB decreased so that it was insufficient for complex formation with all the molecules of Hp5 sub-fractions, the remaining unbound molecules formed complexes with CHB which was added later, thus gradually producing the characteristic three band pattern (7 to 10, Figure 2).

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