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THE CHARACTERIZATION OF EQUINE PREALBUMIN (Pr) PROTEINS BY ANTIGEN-ANTIBODY CROSSED ELECTROPHORESIS

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EK, NILS: *The characterization of equine prealbumin (Pr) proteins by antigen-antibody crossed electrophoresis.* Acta vet. scand. 1979, *20*, 180—190. — Selected equine Pr phenotypes from a total of 55 horses of mixed breeds were investigated. The horse sera were subjected to acid starch gel electrophoresis at pH 4.8, followed by right angle electrophoresis in agarose gels containing rabbit-produced anti-Pr protein. This technique gives peaks in the agarose gels corresponding to the Pr zones in acid gels.

The investigation revealed patterns of the Pr protein which were more complex than those seen when using ordinary acid starch gel electrophoresis. The phenotypes FF, II and LL showed a total of eight peaks, each with three main peaks in the front. Ahead of these, the Pr II and Pr LL phenotypes each had a fourth small peak. The basic fast pattern for these two phenotypes therefore consisted of four bands. The Pr WW and Pr SS showed a similar picture as regards the fast moving peaks. The Pr NN type appeared with two peaks in the front, one small and one large and with two slow moving ones. The Pr UU type had four peaks, but only in the area of the main Pr U band in acid gels.

Four heterozygous Pr phenotypes appeared as a combination of the corresponding homozygous phenotypes, the number and height of the peaks depending on positions and overlappings of these in the respective homozygotes. Thus the Pr FW phenotype showed a total of 10 peaks.

The effect of variations in pH of the starch gel buffer was studied. The Pr NN and Pr FF phenotypes were run at pH 4.8, 5.0, 5.2 and 5.4. With increasing pH, the slow moving peaks weakened and moved closer to the fast ones. At pH 5.4 only one large fast moving peak remained.

horse; Pr prealbumin; antigen-antibody crossed electrophoresis.

When horse serum is subjected to discontinuous starch gel electrophoresis at pH 4.8, numerous protein bands appear ahead of albumin. The most anodal series of these zones belongs to

one genetic system called the Pr system (*Gahne* 1966, *Brænd* 1970). Its phenotypes were explained by eight codominant alleles: Pr^F, Pr^I, Pr^L, Pr^N, Pr^S, Pr^T, Pr^U and Pr^W (*Brænd* 1970).

A similar prealbumin system has been reported in man (*Fagerhol & Brænd* 1965). This protein, a protease inhibitor, has been named Pi (*Fagerhol & Laurell* 1967) and is the same as α_1-antitrypsin (*Fagerhol & Brænd* 1966). When human serum is subjected to acid starch gel electrophoresis followed by antigen-antibody crossed electrophoresis on agarose gel, the Pi allele product appears as a pattern of eight zones (*Fagerhol* 1969). Earlier investigations of the equine Pr protein led to the conclusion that this corresponds to α_1-antitrypsin (*Ek* 1977).

The purpose of the work reported in the present paper was to study and characterize selected equine phenotypes by the technique of antigen-antibody crossed electrophoresis.

MATERIALS AND METHODS

Sera from a total of 55 horses were investigated. They were selected among samples sent to the Blood Group Laboratory, Department of Internal Medicine I, for routine parentage control. The homozygous phenotypes Pr FF, Pr II, Pr LL, Pr NN, Pr SS, Pr UU, Pr WW and the heterozygous phenotypes Pr FW, Pr IU, Pr LS and Pr NU were represented, there being at least two samples of each except for Pr WW and Pr IU of which there was one respectively. The samples were stored at —20°C.

Preparation of the Pr fraction

A sample of horse serum Pr phenotype FF (2 ml) was absorbed into a double 13 × 0.5 cm strip of filter paper about 1 mm thick, and subjected into electrophoresis on acid starch gel. The technique employed was as described by *Brænd* (1970). Immediately after the run, a strip about 1 cm broad was cut out of the gel from the Pr zone at right angles to the direction of run. A gel previously run in the same way and stained with Amido Black was used as a marker for the position of the zones.

The strip was cut into pieces of about 1 × 1 mm, and suspended in 10 ml phosphate buffer 0.05 M, pH 7.4. The suspension was then homogenized in a tube. The final volume was about 20 ml.

Preparation of antiserum

Antiserum against the Pr protein was prepared by immunizing a rabbit with the suspension made from the starch gel. On each occasion the rabbit was given 20 ml of the suspension, 5 ml being injected subcutaneously at four different sites. Two immunizations were carried out at an interval of 10 days followed by six immunizations at weekly intervals. One week after the last immunization, 30 ml of blood was drawn from the ear vein.

The antiserum was tested by immunoelectrophoresis against horse serum. The immunoelectrophoretic method used was as previously described (*Ek* 1974).

Antigen-antibody crossed electrophoresis

The technique used was essentially as described by *Fagerhol & Laurell* (1967). A 50 µl sample of horse serum was absorbed into a 1.0 × 0.5 cm strip of filter paper about 1 mm thick and subjected to electrophoresis on acid starch gel based on the technique of *Brænd* (1970). The acidity of the gels was, however, varied so as to study the effect of pH on the precipitation patterns and their peaks. When electrophoresis was completed, the starch gel was sliced horizontally, and a 2 mm broad strip then cut out from the bottom part of the sliced gel (2 mm thick) along the migration path of the proteins. This gel strip was inserted into a slot cut in an agarose gel (1 mm thick) containing 5 % of the anti-Pr protein serum previously produced. The seams between the edges of the starch and agarose gels were sealed with liquid agarose of 45°C. The agarose gel was subjected to electrophoresis (20 V/cm) with conventional barbital buffer. The separated proteins then migrated from the starch gel into the agarose with the migration direction perpendicular to that of the previous run.

On completion, a smooth filter paper was moistened with physiological saline and applied directly to the entire gel film. This was overlaid with three to five dry absorbent paper towels and covered with a plastic plate bearing and distributing a weight of 2 kg over a 8 × 8 cm area. The plate was kept under this pressure for 30 min. After careful removal of the absorbent pads the compressed and now thinner gel was washed in a saline bath overnight, dried in a stream of air and stained with Amido Black.

Photographic technique

Photographs were taken on Ilford FP 4 films type 120 with illumination from below.

RESULTS

The antiserum against Pr protein gave only a single precipitation line on immunoelectrophoresis against horse serum (Fig. 1).

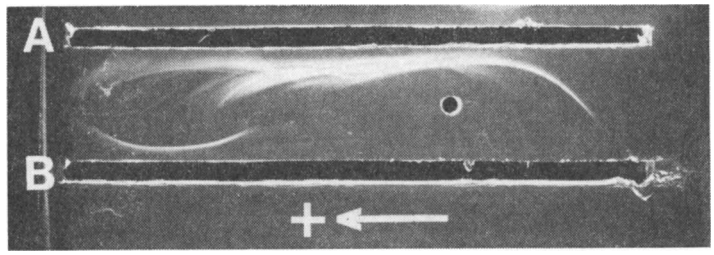

Figure 1. Photograph showing immunoelectrophoresis of horse serum against anti-Pr protein serum and anti-horse serum on agarose gel with barbital buffer (pH 8.6).
Trough A: Anti-horse serum
Trough B: Anti-Pr protein serum
Well : Horse serum

Peak patterns of Pr phenotypes are shown in Fig. 2. A typical Pr FF pattern appeared with a total of eight peaks, which were numbered 1 to 8. There were three major peaks in the front, corresponding to the three-band pattern described by *Brænd* (1970). The additional five peaks were situated between the application line and the three major peaks. The relationship between the heights of peaks Nos. 1, 2 and 3 varied, as did the relationship between the heights of the five slowest moving peaks. The most typical finding, however, with respect to the peaks of slower mobility was two peaks, somewhat smaller than 1, 2 and 3, about midway between the application line and the main peaks.

The Pr II phenotype also had three major peaks in the front numbered 2, 3 and 4. In addition there was a fourth one, No. 1 which was usually covered by peak No. 2 and which needed a more acidic gel for clear separation from peak No. 2. It should be noted that peak No. 1 of phenotype Pr FF is not the same as

peak No. 1 of the Pr II phenotype. The basic pattern of the fastest Pr II zones was thus a four-peak pattern. Of slow moving peaks there were two distinct ones, Nos. 6 and 8, and two, Nos. 5 and 7, which were barely recognizable.

The Pr LL phenotype showed two patterns. One pattern had a similar appearance to that of Pr II with peaks Nos. 2, 3 and 4 being the highest and with peak No. 1 usually hidden by peak No. 2. Two slower peaks, 5 and 6, appeared regularly. The second

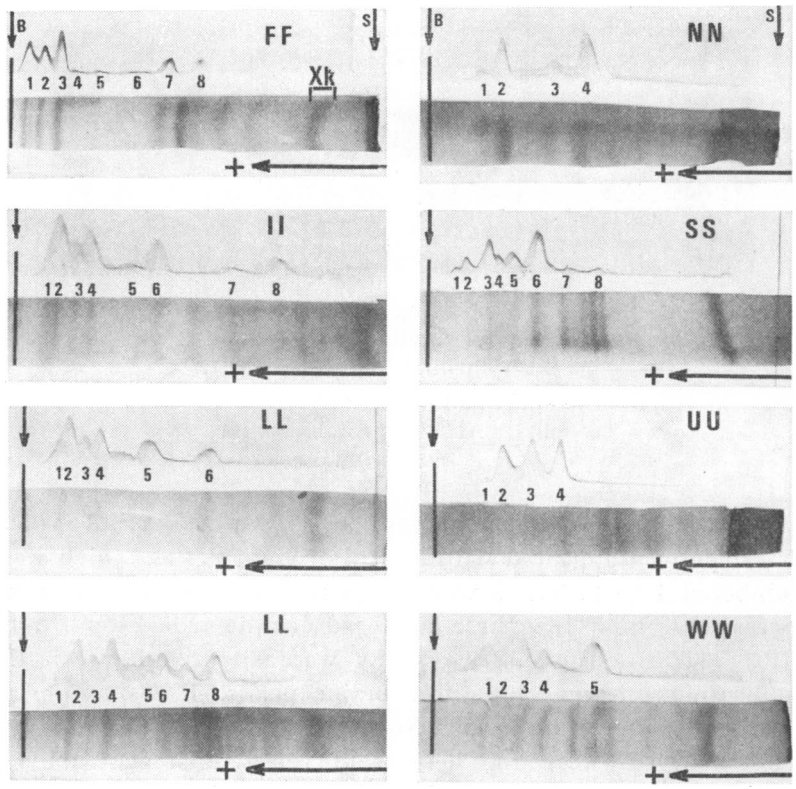

Figure 2. Photographs of stained agarose gels after antigen-antibody crossed electrophoresis of horse sera from eight Pr homozygotes. The antiserum in the agarose gels was anti-Pr protein (1:20). Initial separation was performed by starch gel electrophoresis with pH at 4.8 in the gel buffer. The lower part of each photograph is of the stained gel after acid starch gel electrophoresis. The upper part is of the stained agarose gel after right angle electrophoresis. S marks the insertion of starting point and B the position of the borate boundary for acid starch gel electrophoresis. The peaks in each homozygote are numbered according to migration rate, No. 1 being the most anodal.

Pr LL phenotype also showed three major peaks, Nos. 2, 3 and 4, but in contrast to the first in which peak No. 2 was the strongest, in this case peak 2 and 4 were about equal. Furthermore, there were four slower peaks, numbered 5, 6, 7 and 8.

The Pr NN showed two peaks in the front, one small (No. 1) and one large (No. 2). There were two slower moving peaks, a small one (No. 3) and one (No. 4) about the same height as No. 2. A fifth peak was sometimes seen.

There was some variation among the Pr SS patterns. Four horses showed the same peak pattern as presented in Fig. 2, whereas two showed an additional band with a peak almost as high as peak No. 3.

The Pr UU phenotype appeared with four peaks only, none being in a markedly slower position. The No. 1 peak was, however, close to No. 2.

The Pr WW peak pattern consisted of five major peaks, the slowest one being as high as the fastest of those in the front.

The patterns of four heterozygous phenotypes are shown in Fig. 3. The heterozygous phenotypes appeared as combinations of the corresponding homozygous peak patterns. In some cases there was a large number of peaks whereas in others the peaks

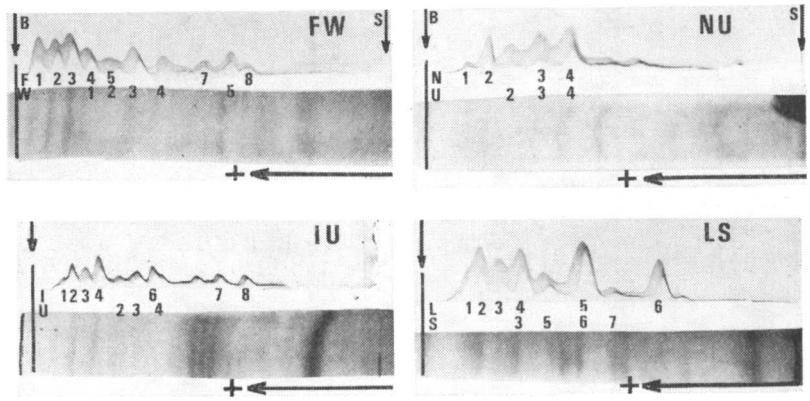

Figure 3. Photographs of stained agarose gels after antigen-antibody crossed electrophoresis of horse sera from four Pr heterozygotes. The conditions were as given in Fig. 2. The peak pattern as demonstrated (Fig. 2) for the Pr FF phenotype also appears in the Pr FW phenotype. The same is the case for Pr WW phenotype. The peaks representing the products of the Pr^F and Pr^W alleles are numbered as in the respective homozygotes.

overlapped. The Pr FW showed a total of 10 peaks and the Pr IU nine whereas the Pr LS and NU showed fewer peaks.

The effect of variations in pH of the starch gel buffer is shown in Fig. 4. The peak patterns of the Pr NN and Pr FF phenotypes shown in the photographs marked I are the same

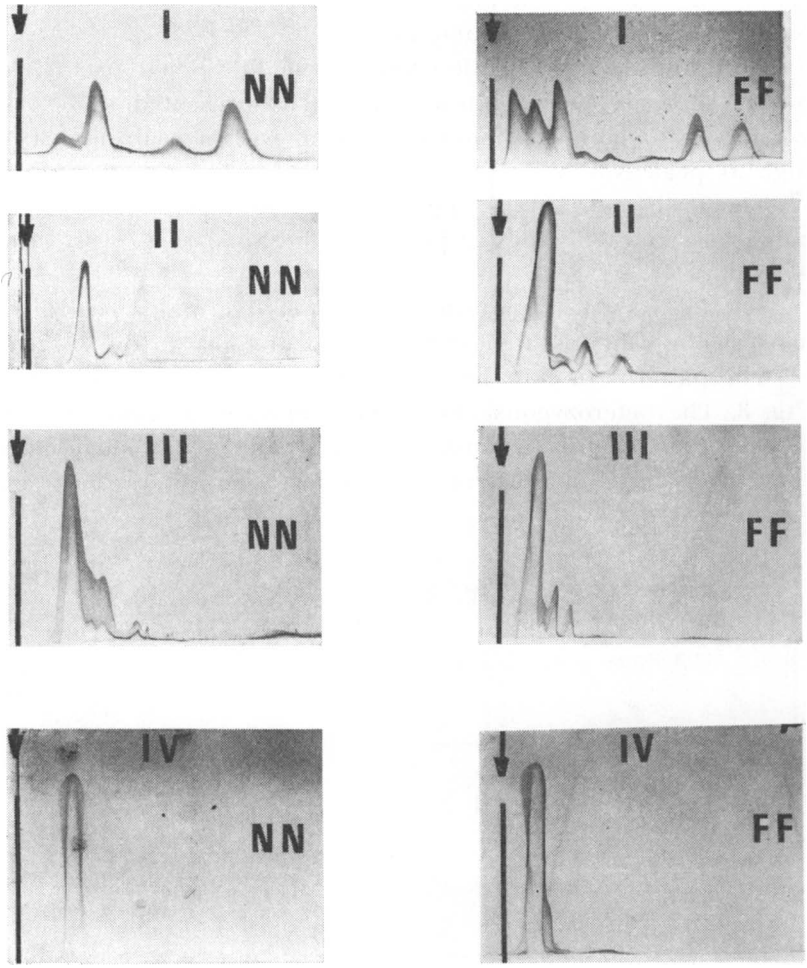

Figure 4. Photographs of stained agarose gels after antigen-antibody crossed electrophoresis of horse sera which had been run in starch gels at different pH.

I: Starch gel buffer with pH 4.8
II: „ „ „ „ „ 5.0
III: „ „ „ „ „ 5.2
IV: „ „ „ „ „ 5.4

as those already described and presented in Fig. 2, the pH of the starch gel buffer being 4.8. When runs were made at pH 5.0, there were marked differences in peak pattern (photographs marked II) as compared with runs at pH 4.8. The fastest peaks in both phenotypes increased in relative height, whereas the slowest peaks disappeared from their positions. Other slow peaks could instead be seen closer to the fast peaks. In the case of the Pr NN pattern this slow peak was strong, in contrast to the Pr FF pattern in which the two slow peaks were considerably weaker than the fast one in the front. At pH 5.2 (photographs marked III) the differences from the picture obtained at pH 4.8 were even more pronounced. The slower peaks moved still closer to the fastest one and became even weaker in comparison. At pH 5.4 (protographs IV) the slower peaks were reduced so much that only one large fast peak could be seen.

DISCUSSION

The technique of antigen-antibody crossed electrophoresis reveals more complex patterns of Pr zones than could earlier be observed (*Brænd* 1970). In this connection it should be mentioned, however, that the existence of additional slower moving zones had always been suspected (*Brænd* 1978, personal communication), but these could not be demonstrated using the original technique.

These complex patterns with up to eight peaks correspond to the patterns described by *Brænd* (1970) in so far as the zones which migrate in the front are concerned. Thus the three-band patterns of the Pr FF, the Pr II and the ordinary Pr LL as detected by acid gel electrophoresis, appear with three corresponding peaks. In addition the Pr II and the Pr LL phenotype each has a fourth peak among the fast ones, so that the basic pattern of the faster bands consists of four zones, of which zone No. 3 was not included among those originally described (*Brænd* 1970). A similar picture could be seen for the Pr WW phenotype, whereas the appearance of peaks for the Pr NN, Pr SS and Pr UU phenotypes falls into another category. Nevertheless there where similarities to the one faint and one strong band as originally described also as regards their faster zones and corresponding peaks.

The second Pr LL phenotype differs clearly from the ordinary Pr LL phenotype. This difference may correspond to that

seen for deviating phenotypes having the Pr L band which was reported by *Brænd* (1970). This has also been noticed over the years (*Brænd* 1978, personal communication), but has so far not been confirmed by genetic studies.

The differences between the Pr phenotypes with regard to number and height of the slower peaks are remarkable. In the human Pi system, *Fagerhol* (1969) and *Fagerhol & Laurell* (1967) found eight protein zones when sera were subjected to electrophoresis on starch gels at pH 4.9, but although there were differences in the rate of migration, the basic pattern of eight zones was consistent. The author does not know the reason for such a difference between humans and horses in this respect.

An attempt to quantitate the peaks was made, but because of the many variable factors which influence final separation, this was not pursued. The results presented in Fig. 2 suggest, however, an explanation for the differing strength of specific zones which is regularly seen with the ordinary technique. The most typical example in this connection is the Pr U band and its appearance. This band is usually faint with the ordinary technique, in fact so weak that it is often difficult to see, particularly in heterozygotes. As can be seen from Fig. 2 the fastest strong band (No. 2) of the Pr UU phenotype comprises only about one third of the total amount of protein. A similar picture exists for the Pr SS phenotype and the zone (No. 3) used for determination with the acid gel technique, since the Pr S band also often appears weak.

However, the situation is different for the Pr N band. According to the peak pattern, the Pr N zone used for diagnosis of Pr phenotypes should also appear weak, since it corresponds to only half of the total Pr protein. This notwithstanding, the Pr N band is usually strong and easy to diagnose. At the present time, the reason for this difference between the appearances of the Pr U, Pr S and Pr N bands is not known, although a possible explanation might be a higher total amount of Pr protein in animals of the Pr NN phenotype than in those of Pr SS and Pr UU phenotypes.

As mentioned above the technique is difficult since there are so many variable factors which influence the final separation of Pr zones. Particularly important though is the acidity of the gels. This is shown by the results presented in Fig. 4, and it was also apparent from many of the gels used for the characterization of

the various phenotypes. When the pH of the gels is reduced, more and slower peaks appear. At the same time the relative heights of the peaks change. Such an unexplained peak is seen in the Pr IU phenotype (Fig. 3).

The reasons why each Pr allele gives rise to such a large number of Pr zones with the same antigenic determinant are not known. Possible explanations have been discussed for the human Pi system (*Fagerhol & Laurell*). The theory has been put forward that each allele is responsible for the synthesis of one molecular species causing a pattern of three major bands.

Bell & Carrell (1973) believed that the electrophoretic findings could be explained in terms of the carbohydrate moiety of the α_1-antitrypsin. They incubated α_1-antitrypsin with neuramidase and measured the release of sialic acid. It was shown that the electrophoretic mobility of α_1-antitrypsin decreased concomitantly with the release of sialic acid. Thus it is possible that the multiband pattern of this protein system is caused by variations in sialic acid bandings in the carbohydrate moiety of the molecules.

Another possible reason for the same antigenic determinant giving rise to different fractions may be structural changes arising during the acid starch gel electrophoresis. It is known that the labile α_1-antitrypsin rapidly and irreversibly loses its trypsin-binding capacity at pH levels below 5.0, indicating structural changes (*Fagerhol & Laurell*).

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SAMMENDRAG

Prealbumin (Pr) proteiner i hesteserum karakterisert ved antigen-antistoff krysselektroforese.

Det ble foretatt en undersøkelse av bestemte Pr fenotyper hos hest på et totalantall av 55 hester av forskjellige raser. Hestesera ble kjørt på stivelseselektroforese ved pH 4.8 etterfulgt av elektroforese i rett vinkel på agarose gel som inneholdt anti-Pr-protein produsert på kanin.

Undersøkelsen åpenbarte mere komplekse mønstre av Pr proteinet enn det som er sett ved ordinær elektroforese på stivelsesgel. Fenotypene FF, II og LL viste et totalantall på åtte topper hver med tre hovedtopper forrest. Foran disse hadde Pr II og Pr LL fenotypene hver en fjerde mindre topp. Det fremre hovedmønster for disse to fenotyper besto derfor av fire band.

Pr WW og Pr SS hadde et lignende mønster når det gjaldt de hurtige topper. Pr NN typen viste to topper forrest, en liten og en stor, og i tillegg to mere langsomtgående topper. Pr UU typen hadde fire topper uten å ha noen som vandret långsomt.

Fire heterozygote Pr fenotyper viste seg som en kombinasjon av de tilsvarende homozygote fenotyper, idet antall og høyder av topper avhang av posisjoner og overlappinger av toppene i de respektive homozygoter. Således viste Pr FW fenotypen et totalantall på ti topper.

Virkningene av variasjoner i pH i stivelsesgelbufferen ble undersøkt. Pr NN og Pr FF fenotype ble kjørt ved pH 4,8, 5,0, 5,2 og 5,4. Ved stigende pH ble de saktegående topper mindre og bevæget seg nærmere de hurtige. Ved pH 5,4 gjensto bare en hurtiggående topp.

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