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IMMUNOLOGICAL CONDITIONS OF TRANSFERRIN TYPES IN CATTLE

By

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Among the newer methods for fractionating proteins which have come into use of late years, starch-gel electrophoresis (*Smithies* 1955, a, b.) and immunoelectrophoresis (*Grabar and Williams* 1953) have arosed interest in medical circles as possible aids in solving the question of paternity. With the help of starch-gel electrophoresis, haptoglobin groups may be determined on serum (*Smithies* 1955, a, b, *Smithies and Walker* 1955, 1956, *Galatius-Jensen* 1957, 1960), and also β-globulin types (*Smithies* 1957, 1958 and *Harries* et al. 1958). Furthermore with immuno-electrophoresis it is possible to determine Gc-types (*Hirschfeld* 1959, 1962, *Hirschfeld and Beckman* 1960).

The reason for the good results obtained by the two methods is the better possibility for separation which they afford as compared with the electrophoretic methods hitherto used.

The starch gel promotes separation, because during electrophoresis the protein molecules must pass through the pores of the gel. By that process, proteins with the same electrophoretic mobility, but under other electrophoretic forms may now be separated, provided the protein molecules are of different size. The high separation obtainable by immunoelectrophoresis is due to the fact that electrophoresis is combined with antigen-antibody reaction, whereby proteins which migrate with the same velocity during electrophoresis can now be separated if they show different diffusion rates in agar gel and differ in respect to antigen.

In parentage control in cattle it is of interest to supplement blood typing with tests based on other principles and here electrophoresis by the starch gel has proved useful. With that type of

electrophoresis, polymorphism has been demonstrated in the β-globulin area in samples of serum or plasma.

It is possible to discriminate between six different phenotypes in that area as it contains 3—5 protein spectra, designated A, B, C, D and E with diminishing electrophoretic mobility from A to E. It has been demonstrated that heredity is probably determined by 3 allelic genes: β^A, β^D and β^E (*Ashton* 1958, *Smithies and Hickman* 1958, *Højgaard* 1959, *Moustgaard and Møller* 1960). The appearance and nomenclature of the six phenotypes are seen in Fig. 1 and in Table 1 (from *Højgaard, Moustgaard and Møller,* 1960) the relation between genotype, serum type and phenotype is shown. With autoradiography (*Giblett et al.* 1959) it has been demonstrated that the β-globulin variations in both man and cattle are due to the iron-carrying protein substance transferrin.

Table 1. Serum-β-globulintypes in cattle and the relation between genotype and phenotype. (*Højgaard, Moustgaard and Møller,* 1960).

Genotype	Serum type	Phenotype (β-globulin band)
β^A/β^A	AA	ABC
β^D/β^D	DD	BCD
β^E/β^E	EE	CDE
β^A/β^D	AD	ABCD
β^A/β^E	AE	ABCDE
β^D/β^E	DE	BCDE

As serum types exist, it is of interest to clarify whether cattle sera of different types possess the same antigen structure and whether the various transferrin spectra are immunologically related. Investigations (*Ashton* 1959) seem to indicate that the ability of the embryo to survive depends on whether the embryo is of the same genotype as the mother since a larger number of calves of the mother's type, than of the father's have been found, although an equal number of types of each parent should be expected. This is not the case however, when the mother possesses the E-gen, for an antagonism seems to exist between the E-gene of the mother and the E-gene of the calf. In the same publication (*Ashton*) mentions other possibilities for an uneven distribution of the materials. To clarify the antigen relation, the various types were subjected to an immunoelectrophoretic investigation in which both starch and agar were used as carrying media.

MATERIAL

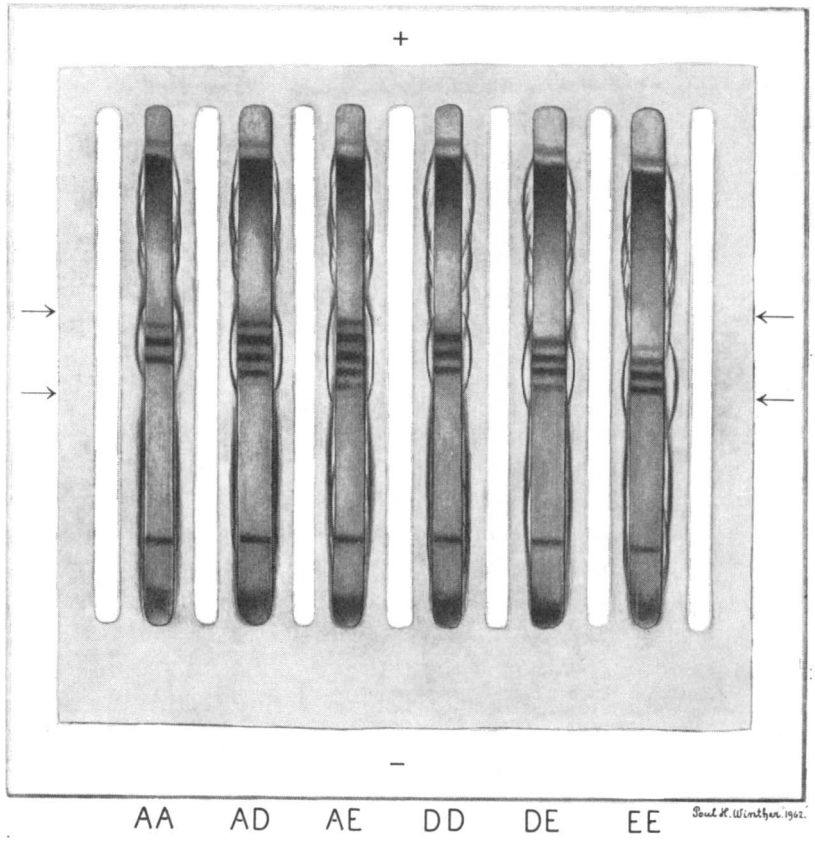
Antigen: Circa 230 plasma samples received at the department for a serum type determination.

Antibody: Anti-cattle serum, diluted 1:2, produced in rabbits by immunization with pooled cattle serum. Antigen and antibody were both stored at —20°C.

METHODS AND RESULTS

Immunoelectrophoresis is normally made in agar gel, which, unlike starch gel, is a medium well-adapted for precipitation. Starch, however possesses a good, electrophoretic separation ability, and it was therefore reasonable to combine starch-gel electrophoresis with antigen-antibody reaction in agar gel as demonstrated by *Poulik* 1956, 1959, *Havez and Biserte,* 1959 and *Allison* 1959. The author has followed the above principle in the present publication. Plasma samples of the six different transferrin types were first subjected to electrophoresis in starch gel according to the method described by *Højgaard* 1959. In electrophoresis was used the discontinnous "Tris"-buffer system described by *Poulik* 1957.

Electrophoresis requires 3—4 hours with a gradient of 6 volts per cm. After the samples which have been sucked up on pieces of filter paper are introduced into the gel, their location is carefully noted so that the migrations can be easily found again, for the filter paper is removed after half an hour. At the close of electrophoresis, and after the removal of the upper half of the gel, the migration routes of the single samples are cut out of the gel. The migration routes are bisected longitudinally and one half of each route is stained with amido black, the other half is placed on a glass plate (13×13) cm. with 1.3 cm. between each strip. A one percent agar solution is poured out to stiffen between the strips level with the surface of the strips. Between each pair of strips is arranged a 0.3 cm. wide antibody reserve, of the same length (9 cm.) as the strips and 0.5 cm. apart from each of them. After covering the plate with antibodies for diffusion, the plate is stored until the following day when the precipitation curves will have developed. A longer diffusion time, 4—5 days, causes no characteristic changes. The starch strips are now removed and the plate is washed for ca. 48 hours in saline (0.92 %), and for ca. 24 hours in distilled water after which it is dried and stained with amido black. The colored reference may now be



F i g. 1. The six different types which may be determined by starch gel electrophoresis of cattle serum. At the level of each group of transferrin bands the characteristic arch of precipitation is seen, following the cource of the bands.

placed on the plate whereby a picture is obtained as shown by the drawing, Fig. 1. Staining the strips, which have functioned as antigen reservoir during diffusion, shows that practically no protein remains in the reservoir. On the figure, and close to the transferrin strips is a distinct, well-developed curve which follows the course of the strips in anode-cathode direction. Close to the three strips of type AA, for instance, is a short curve, but near the five strips of the AE type a very long curve appears. Occasionally, and in connection with the transferrin curve, a very faint precipitation curve appears. In some cases this curve seems to start from, and in other cases to cross, the transferrin

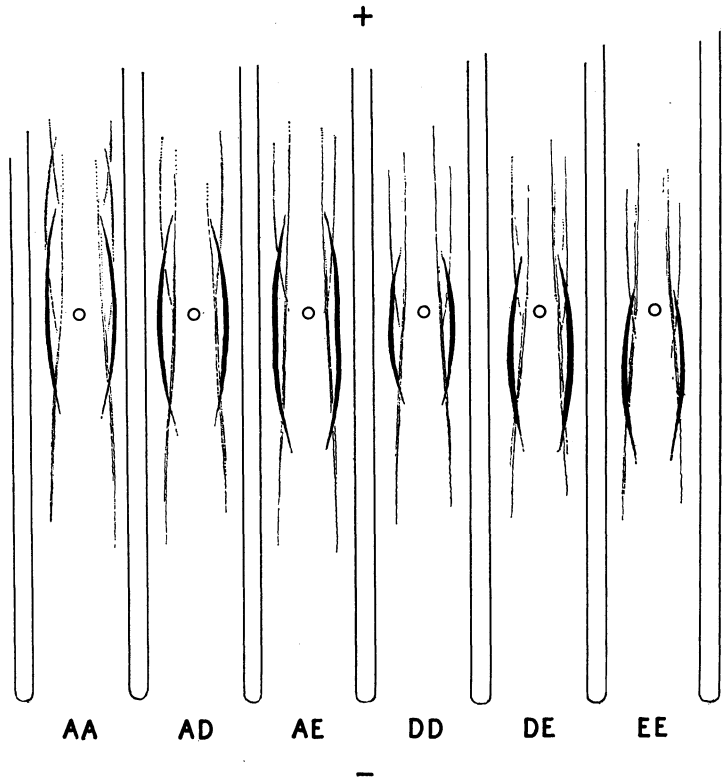


Fig. 2. Normal immunoelectrophoresis of the same six samples as shown in Figure 1. Schematically.

curve. Whether the curve has any connection with the transferrins is uncertain.

If the same plasma samples are investigated by an ordinary immunoelectrophoresis, i. e. both electrophoresis and antigen-antibody reaction taking place in the agar gel, a characteristic variation in the extent of one definite curve, located in the β-region is observed. This curve varies in the same way as the curve near the transferrin strips after diffusion from the starch gel. Therefore it is presumable that the same protein fraction is the basis of those curves, which is shown schematically, Fig. 2. This immunoelectrophoresis is carried out with 1—2 μl. plasma in 1 % agar on 13×13 cm. glass plates, using veronal buffer with lactate (*Hirschfeld* 1960), and a gradient of 7—8 v/cm.

For further study of the problem, the separate strips in the starch gel are cut out of the non-stained starch strips, leaving

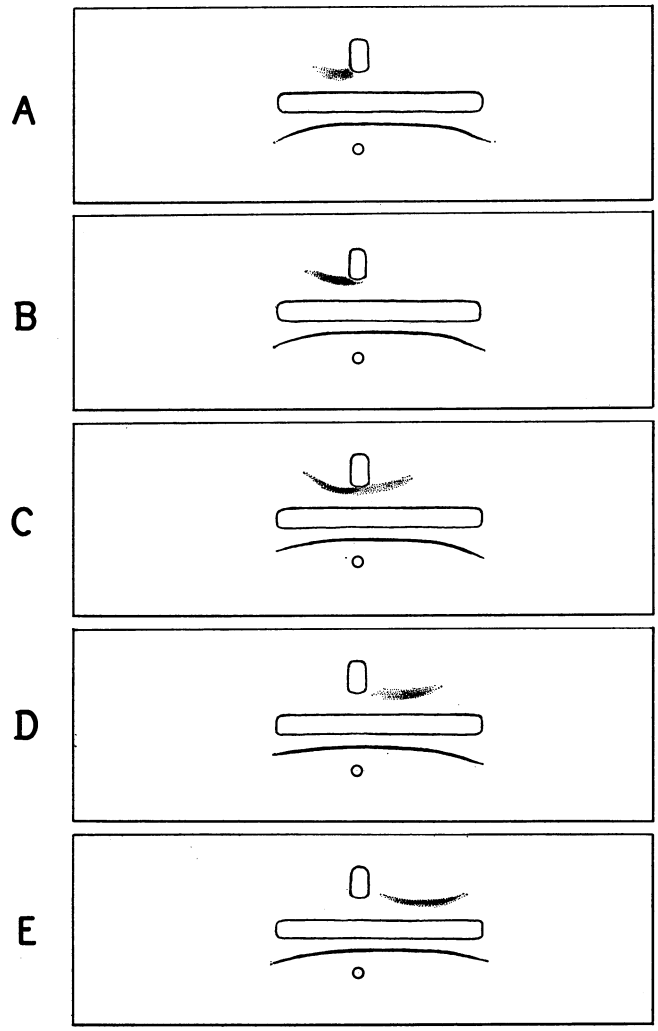


Fig. 3. The precipitation curve of the separate transferrin strips in relation to an AE-reference test. The starch block is located in the square and the reference sample in the circular cut. The antibody groove between these two cuts. The anode lies to the left. Schematically shown.

the stained strips as comparison. This causes certain technical difficulties due to shrinkage of the control strip during staining and washing. The separate, small bits cut out, are placed, each on its own slide, and covered with agar gel. After the gel has stiffened, the antibody reservoir and an antigen cavity which is then filled with a control sample of the type AE, are both esta-

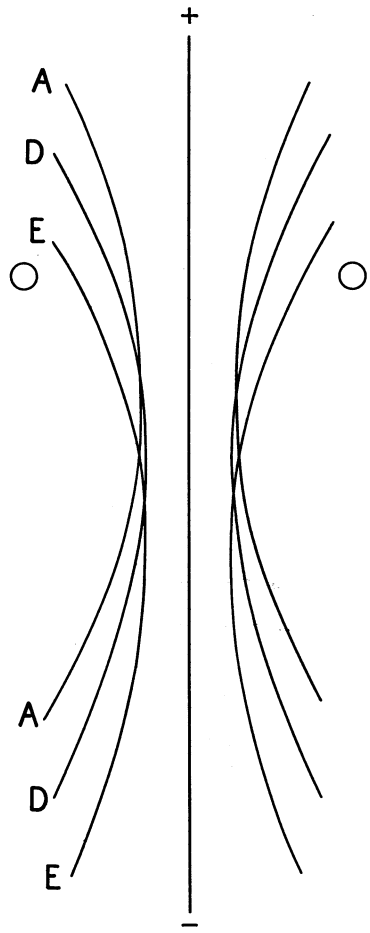


Fig. 4. The drawing used for determining transferrin types in immunoelectrophoresis.

blished. The slide is subjected to electrophoresis for two hours with 8 v/cm. and using the same type of apparatus and buffer as described by *Hirschfeld* 1960, in a modification of the method described by *Scheidegger,* 1955. At the close of electrophoresis the antibody is added and the slides then treated as was the large plate, already described. When diffusion is interrupted, the lines of precipitation appear as shown schematically in Fig. 3.

On the slide with the A-strip there is a small curve at the anode section of the long, curved AE comparison strip. The B-strip lies at almost the same place, but is more distinct. The C-strips shows a fraction at the same place as the A and B-strips,

but protrudes cathodically for the hole, where the bit of starch lay. Nearer the cathode is the curve of the D-strip, and even nearer is the curve caused by the E-strip. The bit of starch with the (presumed) E-strip proved to be nearly emptied of protein, but the slice immediately following contained the E-strip which was not properly hit in the first cut due to the above-mentioned shrinkage of the comparison strip.

To test the accurracy with which the types of transferrin may be determined by immunoelectrophoresis, about 230 samples of plasma were investigated both by starch gel and immunoelectrophoresis. Immunoelectrophoresis was made on slides with two samples per slide and using the same apparatus as described above. It proved to be rather difficult to judge the curves without a standard for comparison, therefore the stained preparations, with the help of a magnifying apparatus were projected on a pattern drawing of all the types as shown in Fig. 4.

As only the variations in the one β-curve were of interest, the antibody cavity was made rather short (2.5 cm.) to save antibodies. A comparison of the results from the two different methods showed an agreement of 75—80 per cent.

Absorption of antibody with the various transferrin types can also give information on the antigen characteristics of each separate type. This absorption may take place in the gel itself, as described by *Björklund* 1952, *Dray and Young* 1959, and by *Hirschfeld* 1960. Such absorption experiments show that all types absorp all antibody components. If, for instance, an AA-sample is used as absorbent, this sample will absorb the antibody against the A, B and C-strips and also exhaust the anti-serum for antibody against the D and E-strip.

DISCUSSION

It appears from the experiments that the polyvalent rabbit anti-cattle serum posesses antibodies to all five transferrin components. Fig. 1 shows that on the level with the transferrin strips, there is formed a long even curve, not a row of small curves intersecting each other. Therefore the five transferrins must be immunologically related. This was verified by the absorption experiments and proves than an anti-serum produced from any one of the types may be used to demonstrate the presence of all the types. The immunological relationship between the different

transferrin fractions does not indicate that the uneven distribution found in *Ashton*'s material is due to an antibody formation in the mother against the foetus, due to a different transferrin type in mother and foetus.

The immunoelectrophoretic determination of the transferrin types is found to be more uncertain than starch-gel electrophoresis. This may partly be due to the coarser electrophoretic separation in the agar, and partly because quantitative changes in the transferrins may change the location of the precipitate. Furthermore, immunoelectrophoresis requires an antibody and it is more difficult to determine the types by this procedure than by starch-gel electrophoresis. As a result, immunoelectrophoresis will hardly ever be used in routine determinations of transferrin types in cattle.

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SUMMARY

Electrophoretic separation of cattle plasma in starch gel, with subsequent antigen-antibody reaction in agar gel and absorption experiments have proved that transferrins in cattle are immunologically related. It was also shown that transferrin types in cattle may be demonstrated by immunoelectrophoresis although an agreement between the two methods was only 75—80 per cent. This discrepancy may be due to poorer electrophoretic separation ability in agar gel and also to the influence of quantitative differences on the location of the precipitates.

ZUSAMMENFASSUNG

Die immunologischen Verhalten der Transferrintypen bei dem Rindvieh.

Mittels elektrophoretischer Trennung von Rinderplasma in Stärkegel und nachfoldender Antigen- Antikörper Reaktion in Agargel und Absorptionsversuche wird es nachgeweist, dass die Transferrinen des Rindviehs immunologisch verwandt sind. Es wird weiterhin gezeigt, dass die Transferrintypen immunoelektrophoretisch nachgeweist werden können, jedoch nur mit 75—80 % Übereinstimmung zwischen den beiden Methoden. Diese Nichtübereinstimmung kan von dem geringeren elektrophoretischen Separationsvermögen in Agargel und der Einwirkung quantitativer Unterschiede auf der Lage der Precipitaten herrühren.

RESUMÉ

Transferrintypernes immunologiske forhold hos kvæget.

Med elektroforetisk separation af kvægplasma i stivelsegel og påfølgende antigen-antistofreaktion i agargel og absorptionsforsøg påvises det, at transferrinerne hos kvæget er immunologisk beslægtede. Endvidere vises det, at kvægets transferrintyper kan påvises immunelektroforetisk, dog kun med en overensstemmelse mellem de to metoder på 75—80 %. Denne uoverensstemmelse kan skyldes den ringere elektroforetiske separationsevne i agargel og kvantitative forskelles indvirkning på precipitaternes beliggenhed.

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