BOVINE TRANSFERRINS: SIALIC ACID AND THE COMPLEX PHENOTYPE

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THE electrophoretic polymorphism of bovine transferrin was first described by ASHTON (1957) and SMITHIES and HICKMAN (1958). Nine alleles are now known, all apparently at a single locus (ASHTON 1958, 1959; KRISTJANSSON and HICKMAN 1965; ASHTON and LAMPKIN 1965). Some of the alleles are characteristic of particular breeds, but others (Tf^A , Tr^{D1} , Tf^{D2} , Tf^E) are widespread. Nomenclature and references are given by ASHTON *et al.* (1967).

The phenotypes associated with some of the common genotypes are shown in Figure 1. The complexity of the patterns produced by single alleles has led to much speculation on the molecular composition of bovine transferrin, particularly since it differs in this respect from human transferrin, where each allele is represented by a single protein product (GIBLETT 1962). There is obvious resemblance to lactate dehydrogenase, where the pattern is produced by different combinations of two kinds of subunits into a tetramer (see VESELL 1965, for a recent review). On the other hand, attempts to demonstrate subunits in human transferrin have not been successful.

FIGURE 1.—Acrylamide gel electrophoresis of some common bovine serum transferrin phenotypes. Preparation of samples and electrophoresis were as given in the text. Samples were inserted at the bottom. The protein near the origin is a portion of the γ -globulin which remains in the rivanol supernatant. Mobility of the D1 and D2 patterns is very similar and cannot be distinguished on this gel. The AD is actually AD1, the D is heterozygous D1D2, and the DE is D2E.

Gel filtration and ultracentrifuge studies of bovine transferrin give a single symmetrical peak, indicating that all transferrin bands are of approximately equal size, with a molecular weight of approximately 103,000 (HINES 1965).

In addition to the polypeptide portion, transferrin has a small amount of carbohydrate. This has not been extensively studied in bovine transferrin, but in human transferrin, each molecule of molecular weight ca. 90,000 is reported to have four residues of sialic acid, four residues of galactose, eight residues of mannose, and eight residues of N-acetylglucosamine (JAMIESON 1965).

Treatment by neuraminidase splits off the terminal sialic acid residues, decreasing the electrophoretic mobility by steps corresponding to the number of residues lost (PARKER and BEARN 1962). Such treatment of bovine transferrin altered the pattern by two charge units but did not appear to change it. This led to the suggestion that bovine transferrin has only two sialic acid residues available to the enzyme and that these are not involved in producing the complex electrophoretic pattern.

The suggested differences between bovine and human transferrins would have considerable evolutionary significance and led us to examine in somewhat more detail the structural relationships among the bovine bands produced by a single allele.

METHODS

Samples: The cattle blood samples used in the experiment were obtained from the Austin Livestock Community, Austin, Texas. The blood was collected immediately after the cattle were killed. EDTA was used as an anticoagulant. The cells were centrifuged down and discarded. The plasma was kept at -20°C until used.

The transferrin phenotypes of the samples were determined by acrylamide gel electrophoresis. The samples were pretreated in the following way to remove most of the proteins other than transferrin (SUTTON and KARP 1965): For one drop of plasma, one drop of 0.15% ferric chloride solution was added; then four drops of 0.6% solution of rivanol (2-ethoxy-6, 9-diaminoacridine lactate) was added. The mixture was well shaken by hand and the precipitate then centrifuged down. The clear yellow supernatant was used for electrophoresis. Only plasma with a homozygous Tf A pattern was used.

Acrylamide gel electrophoresis: Acrylamide gels (7%) were prepared with 0.08 M tris, 0.23 M borate buffer, pH 7.5. Electrophoresis was carried out with a field strength of 20 v/cm for 4.5 hours in conventional apparatus. The gel was stained for protein with amido black.

Isolation of the individual bands: The procedure of SUTTON and KARP (1965) was used for isolating pure transferrin, substituting ultrafiltration for the DEAE-Sephadex step as a means of concentrating the rivanol supernatant after it has passed through the starch pad. In the final purification step, starch block electrophoresis, the individual bands are visible and can be eluted separately. As is usually the case with starch block electrophoresis, the slower bands may be slightly contaminated with faster moving bands.

From 100 ml of plasma, about 40 mg of each of the two strong bands and about 25 mg of the next strongest band were obtained. The fastest band, very faint at best on gels, could not be seen on the starch block and was not studied. For convenience (and with some hindsight), we have designated the fastest band isolated as A_4 , the next A_3 , etc. We therefore are reporting on bands A_2 , A_3 , and A_4 .

Ultracentrifuge studies: Homogeneity determination on bovine transferrin by ultracentrifugation was carried out in a single sector cell at 59,780 rpm with a Spinco Model E ultracentrifuge. About 5 mg of purified transferrin A was dissolved in 1 ml of 0.1 M phosphate buffer, pH 8.0. The picture was taken after 36 minutes at 59,780 rpm.

Treatment with neuraminidase: The neuraminidase used in the experiments was an extract from *Clostridium perfringens* type V (Sigma Chemical Company). The enzyme activity was 1854 units/ml. The pure transferrins were incubated at 37°C with about 270 units of neuraminidase per mg protein in 0.5 ml of acetate buffer, pH 6.5 (BLUMBERG and WARREN 1961). Samples were removed at time intervals and stored frozen. The maximum incubation was 72 hours. Further incubation resulted in no change of final pattern.

Chemical studies: Protein concentration was estimated by the procedure of LOWRY *et al.* (1951), using the Folin-Ciocalteu reagent. Sialic acid was determined by the thiobarbituric acid method of WARREN (1959), following acid hydrolysis (0.1 N H₂SO₄ at 80°C for one hour).

RESULTS

The ultracentrifuge pattern of the mixture of pure bovine transferrin A components is shown in Figure 2. It exhibits a single symmetrical peak, indicating that the component proteins are of approximately equal size.

The effect of the neuraminidase on individual band mobility is shown in Figure 3. Each band shows a stepwise reduction in the mobility. The A₂ band (position 2) shows two additional slower bands; A₃ shows three; and A₄ has four. The mobilities of the new bands appear to be identical to the next slowest, naturally occurring band. This suggests that there are two sialic acids per molecule in the A₂ position, three in the A₃ position, and so on. The suggestion was confirmed by the chemical analysis of the sialic acid content of individual bands, as shown in Table 1.

DISCUSSION

Transferrins epitomize the range of electrophoretic patterns encountered among the various isozyme systems. The complex pattern of bovine homozygotes is comparable to that of lactate dehydrogenase from a number of species, where it depends on the combinations of two kinds of subunits into a tetramer. Our failure to dissociate bovine transferrin into subunits, while negative evidence, led us to consider other possibilities. The finding that the carbohydrate portion is variable explains fully the complex patterns on gel electrophoresis, although it does not rule out subunits or other variations in the polypeptide chain. The question of whether there may be contaminating proteins which may account for our results can only be answered with negative evidence. We see no evidence of

FIGURE 2.—Ultracentrifuge pattern of purified bovine transferrin type A. Conditions are given in the text.

FIGURE 3.—Acrylamide gel showing the effects of neuraminidase on purified bovine transferrin A and on isolated components of transferrin A.

contaminating proteins on electrophoresis or on ultracentrifugation. Most important perhaps is the failure to note any protein components which do not change with the various genotypes. This sets the upper limit of contamination but would not rule out a substance with very high sialic acid content present in a small amount.

These studies led to two suggestions of genetic interest: (1) The loci directing

TABLE 1

Sialic acid assays of individual bovine transferrin A components

Transferrin band	Protein concentration mg/ml	Sialic acid micromoles/ml	Equivalents of sialic acid per 100,000 mol wt
A ₂	3.24	0.0720	2.22
A ₃	3.42	0.1005	2.94
A ₄	2.28	0.0960	4.21

Results are the average of four assays. A molecular weight of 100,000 has been assumed in calculating the number of residues per molecule.

synthesis of the polypeptide portion of transferrins in mammals produce similar products, in spite of considerable variability in transferrin patterns among species. (2) Inherited differences in the ability to attach nonprotein moieties to proteins may contribute to variability both within and among species.

Studies underway in this and other laboratories should provide evidence on the first suggestion. In support of it is our failure to find peptide differences on fingerprints of isolated bovine transferrins A₂ and A₃ (unpublished), a finding compatible with the idea of a single kind of polypeptide chain for each allele. Much more evidence will be needed on the primary structure of transferrins from several species before confidence can be placed in this idea.

The second suggestion seems obvious but is perhaps useful to emphasize in view of the current great interest in electrophoretic variations of enzymes. Too often, patterns are interpreted on the basis of inadequate chemical or genetic evidence, and there is a tendency to forget that proteins consist of more than polypeptide chains.

The bovine transferrin pattern is similar to that observed by PARKER and BEARN (1962) in cord blood of human beings. In that case, treatment with neuraminidase abolished the multiple bands, producing a slow moving band corresponding to zero sialic acid residues. They proposed that the system for attaching the carbohydrate side chains matures after birth. By analogy, the bovine system would not reach the stage of "maturity" characteristic of man and some other mammals.

In both the studies of PARKER and BEARN and our own on the effects of neuraminidase on bovine transferrin, enzyme treatment failed to shift all of the bands to the zero sialic acid form. Possibly more active preparations of neuraminidase would have accomplished this, but bovine transferrin seems to be intrinsically more resistant than human transferrin to attack by neuraminidase. The use of isolated bands in the present study clearly shows the potential of all bands to go to the zero sialic acid state.

In most electrophoretic runs of bovine transferrin, there is a very faint band faster than the A₄ band. Such a band is often seen in runs with human transferrin also. Although the protein in this band has not been isolated and studied, it seems unlikely to be related to sialic acid differences, since in human transferrin all the molecules have at least four residues, but this band remains very faint. More likely, it is due to changes in other parts of the molecule and may be partially denatured material produced in handling.

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SUMMARY

Bovine transferrins produce a complex pattern on gel electrophoresis. Individual bands from plasma giving a homozygous Tf A pattern were isolated and subjected to neuraminidase digestion and to sialic acid analysis. The results

indicate that the complexity of the homozygous transferrin patterns can be explained in terms of the number of sialic acid residues per molecule, with no need to invoke heterogeneity of the polypeptide portion of the molecule.

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