MULTIPLE ELECTROPHORETIC FORMS OF CARBONIC ANHYDRASE IN RED CELLS OF DOMESTIC CATTLE (*BOS TAURUS*) AND AMERICAN BUFFALO (*BISON BISON*)¹

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BY combining the methodology of starch-gel electrophoresis (SMITHIES 1955) with histochemical staining, it is possible, as shown by HUNTER and MARKERT (1957), to demonstrate numerous zones of enzymatic activity directly in the supporting medium. MARKERT and MØLLER (1959) introduced the term "isozyme" which they used to denote multiple molecular forms of the same enzyme, a cogent example being the multiple molecular forms of lactate dehydrogenase. Using this approach, which has been referred to as the "zymogram display", numerous isozyme polymorphisms have since been reported as reviewed by SHAW (1965). Among such reports was one from this laboratory (GRUNDER, SARTORE and STORMONT 1965) concerned with the description of a system of aliesterases demonstrable in hemolysates of rabbit red cells and apparently under the control of a pair of codominant autosomal alleles. Somewhat analogous systems of red cell esterases have also been described in studies of mice (PELZER 1965), *Peromyscus* (RANDERSON 1965), and primates (TASHIAN 1965).

Concomitantly with our studies of rabbit red cell esterases we sought to detect electrophoretic variation in the red cell esterases of domestic cattle and American buffalo, and were successful. However, in line with certain observations made by TASHIAN and colleagues (see TASHIAN 1965), we found that these particular isozymes are more appropriately classified as carbonic anhydrases (CA) even though they do exhibit esterase activity. The results of those studies are the subject of this report.

MATERIALS AND METHODS

Blood samples: The cattle samples for the family studies (Table 1) were obtained from both dairy and beef animals in the University herds at Davis, and from a privately owned herd of Guernseys. Those for the population surveys (Table 2) were limited solely to bulls used in artificial breeding and they were shipped to the Serology Laboratory from all parts of the United States (a few from Canada) for recording permanent blood-typing records of identification. Samples from 94 Longhorn cattle (45 males and 49 females) were obtained from animals in the herd maintained at the Wichita Mountains Wildlife Refuge in Cache, Oklahoma.

The bison samples were obtained from 23 males and 28 females in a herd of approximately 150 maintained at the Theodore Roosevelt National Wildlife Refuge in North Dakota.

All blood samples were collected in citrated-saline (2% sodium citrate and 0.5% NaCl).

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Hemolysates were prepared by laking washed, packed red cells with two volumes of distilled water.

Electrophoresis and staining: Hemolysates were subjected to horizontal starch-gel electrophoresis. The gels were prepared as described by KRISTJANSSON (1963). Optimum resolution of the CA zones was obtained using an electrolyte buffer of pH 8.7 (0.10 M NaOH and 0.30 M boric acid) and a gel buffer (pH 7.3) containing 0.0042 M citric acid and 0.0144 M Tris (hydroxymethyl) aminomethane. Each gel was prepared using approximately 28 g of hydrolyzed starch (Connaught Medical Research Laboratories, Toronto, Canada) and 250 ml of gel buffer. The hemolysates were adsorbed onto 9×6 mm paper strips (Beckman Inst. No. 319329) and inserted in an incision made 4 to 6 cm from the cathode end of the gels. Up to 12 samples were run in each gel. They were run at a gradient of 6 V/cm for 30 min, at which time the paper inserts were removed. Thereafter, 11 V/cm were applied until the borate boundary had migrated 9 to 11 cm beyond the insertion line (the origin). To prevent heat denaturation and loss of enzyme activity, the runs were performed at 4°C.

Each gel was sliced horizontally into three slices, two of which were incubated either at room temperature or at 37°C in staining solution containing a synthetic naphthol ester as substrate, fast blue BB salt as the dye coupler and phosphate buffer in the amounts described by GRUNDER *et al.* (1965). The stained gels were rinsed in water and fixed in a 50% solution of methanol.

The third slice was stained with a protein dye (either nigrosin or buffalo black) and destained in a 5:5:1 solution of methanol/water/glacial acetic acid.

Identification of the kinds of esterases: The substrates used to detect and identify the different kinds of esterases were α -naphthyl acetate, β -naphthyl acetate, α -naphthyl butyrate, α -naphthyl propionate, α -naphthyl caprylate, naphthol AS-D acetate, and β -carbonaphthoxy choline iodide. However, routinely only α -naphthyl acetate and α -naphthyl butyrate were used. Inhibition tests were performed in the manner described by GRUNDER *et al.* using 10^{-4} M eserine sulphate, 10^{-4} M tetraethyl pyrophosphate (TEPP), 10^{-4} M diisopropyl-fluorophosphate (DFP), 10^{-4} M ethylenediamine tetraacetic acid (EDTA) and 10^{-4} M acetazolamide as inhibitors. The criteria set forth largely by AUGUSTINSSON (1961) were used in the attempts to classify the kinds of esterases.

RESULTS

As indicated in a photograph of three gel slices in Figure 1, three major regions of esterase activity were evident in the runs performed on cattle hemolysates. These are labelled I, II and III in decreasing order of mobility of the zones involved.

Up to five zones could be seen in the esterases of region I. Those zones hydrolyzed α -naphthyl acetate (Figure 1-B) and β -naphthyl acetate. They were inhibited only by EDTA but the degree of inhibition was slight at 10^{-4} M. They were not stainable with the protein dyes. Therefore, the isozymes of region I may be classified as A esterases. No qualitative differences in the rate of migration of the zones in region I were evident. However, there were marked differences in the degree of staining from sample to sample and we attribute this variation largely to the way in which the samples were handled from the time of collection to their use in the various tests.

Region II exhibited two zones with esterase activity and these were observed in three phenotypes labelled F, FS and S in Figure 1. Like the zones of region I, those of region II hydrolyzed α -naphthyl acetate (Figure 1-B) and β -naphthyl acetate, and were partially inhibited by EDTA. However, unlike the zones of region I, they were readily stained with the protein dyes (Figure 1-A) and were

FIGURE 1.—Starch gel showing the carbonic anhydrase zones CA-F and CA-S, and the three CA phenotypes F, FS and S. These zones and the phenotypes are seen best in slice A which is stained with nigrosin, a protein dye. They are also seen in slice B which was developed in a solution containing α -naphthyl acetate as substrate. Also evident in slice B are the multiple zones of arylesterases (A esterases) in region I, and the leading zone of aliesterase (B esterase) in region III. Slice C, which was developed in a staining solution containing α -naphthyl butyrate as substrate, shows the full extent of staining of the zone or zones in region III. Other markers in the figure are BB for the borate boundary and "origin" for the point of insertion of the hemolysates at the beginning of the run. Hb is the symbol for hemoglobin.

inhibited by acetazolamide, a specific inhibitor of carbonic anhydrase (CA). Therefore, they were classified as carbonic anhydrase (CA) and so labelled in Figure 1.

Region III, which partially overlapped region II, was characterized by a leading band that hydrolyzed α -naphthyl acetate (Figure 1-B), α -naphthyl butyrate (Figure 1-C) and α -naphthyl propionate, followed immediately by a broad zone that hydrolyzed α -naphthyl butyrate (Figure 1-C) and α -naphthyl propionate but not α -naphthyl acetate (Figure 1-B) or any of the other substrates. This zone (or zones) was inhibited by TEPP and DFP but not by the other inhibitors, and was not stainable with the protein dyes. Accordingly, it may be classified as an aliesterase or B esterase. No genetic variation was detected.

In the gel slices stained with the protein dyes, numerous faintly staining zones could be seen in the entire region beyond zone CA-F to the borate boundary. None of those zones exhibited esterase activity and there was no intraspecific variation except in the intensity of staining.

With the discontinuous system of buffers used in these studies, hemoglobin migrated first cathodally and then anodally just beyond the origin as shown in

Figure 1. The leading boundary appeared invariably as a wavy line. There was no suggestion of esterase activity in the region occupied by the Hb.

Storage of hemolysates for as long as three years at -20°C had no effect on the zymogram patterns described here. Although repetitive freezing and thawing of the samples had no apparent effect on the enzymatic activity of the CA zones, there was progressive decrease in the activity of the esterase isozymes in regions I and III, particularly those of region III. The remarkable stability of the CA isozymes is indicated by the fact that heating the hemolysates for 20 min at 56°C had no noticeable effect on their staining capacity.

When the bison samples were electrophoresed and the gel slices were treated in the manner described for the cattle studies, the overall results were much the same with the exception of the position of the A esterase zones in region I and the number of CA zones and phenotypes. As may be seen in the photo of the gel slice in Figure 2, which contains five bison and one cattle samples, the zones of A esterase in the bison hemolysates migrated considerably faster than those of cattle, thereby providing a marker which clearly distinguished the two species. How-

FIGURE 2.—A gel slice, developed in the α -naphthyl acetate substrate, which shows the results of a run on five bison hemolysates of CA phenotypes 1-1, 1-2, 1-3, 2-3 and 2-2, and one cattle hemolysate of phenotype FS. Note that the zone CA-S of cattle migrates at about the same rate, if not precisely the same rate, as CA-3 of bison, whereas CA-F of cattle migrates slower than CA-1 and CA-2 of bison. This slice also shows that the bison zones of A esterase in region I are shifted considerably anodally with respect to the cattle A esterase zones. The leading zone of the B esterase of region 3 is also visible and, as may be seen in this Figure, bison and cattle are indistinguishable with respect to that zone.

ever, as in the cattle studies, there was no evidence for intraspecific variation in the isozymes of region I.

Another slice of the same gel as that shown in Figure 2 was stained with nigrosin and it is shown in the photo of Figure 3. In it the CA zones can be more clearly seen than in the photo of Figure 2. Altogether, three zones of Ca (labelled CA-1, CA-2 and CA-3 in the figures) were seen in the bison runs and these were observed in five phenotypes labeled 1-1, 1-2, 1-3, 2-3 and 2-2. Between samples 1-3 and 2-3, in Figures 2 and 3, is a cattle sample of phenotype FS. Note that zones CA-1 and CA-2 of bison migrated ahead of CA-F of cattle whereas zone CA-3 of bison would appear to occupy approximately the same position as CA-S of cattle. Although there is the suggestion in Figure 2 and 3 that CA-3 migrates a bit faster than CA-S of cattle, this has not been unequivocally confirmed.

With respect to the aliesterase(s) of region III, as shown by the position of the leading boundary in Figure 2, the cattle and bison samples were indistinguishable.

FIGURE 3.—This is one of the slices of the same gel as that shown in Figure 2. However, this particular slice was stained with nigrosin which permits a somewhat clearer resolution of the CA zones. In addition to the CA zones, a multiplicity of weakly staining protein zones may be seen between CA-2 and the borate boundary (BB). All cattle and bison appeared to be alike with respect to those additional protein-staining zones.

TABLE 1

Inheritance of the carbonic anhydrase phenotypes of cattle

Matings	Number of matings	Number of progeny of phenotypes		
		F	FS	S
F × F	1	1	0	0
F × FS	3	2	1	0
F × S	6	0	6	0
FS × FS	5	0	4	1
FS × S	31	0	10	21
S × S	82	0	0	82

The same was true for the multiple zones that were stained with the protein dyes (Figure 3) excepting, of course, the CA zones.

Data on the inheritance of the three CA phenotypes of cattle are summarized in Table 1. Those data are consistent with the interpretation that the two zones of cattle CA are controlled by a pair of codominant autosomal alleles CA^F and CA^S . There was, however, a significant distortion in the 1:1 ratio of FS and S offspring in the FS × S matings.

Data on the distribution of the CA phenotypes in 2,917 bulls, representing eight breeds, are shown in Table 2. We also tested 94 cattle of the Longhorn breed but these were all from one large herd and were of both sexes, and therefore are not included in Table 2. The distribution of types was 8F, 36FS and 50S with CA^F being estimated at 0.28. Assuming HARDY-WEINBERG equilibrium, it can be shown that the observed distribution of types by breeds is in good agreement with expectation.

No family data were available in connection with the bison study. Nevertheless, comparison of the observed distribution of the five phenotypes with expected distribution (Table 3) leaves little doubt that those phenotypes are controlled by a series of three codominant autosomal alleles, CA^1 , CA^2 and CA^3 .

DISCUSSION

Previous comparisons of American buffalo and domestic cattle with respect to

TABLE 2

Distribution of carbonic anhydrase phenotypes in 2,917 males representing eight breeds of cattle

Breeds	Number sampled	Phenotypes			Frequency of CA^F
		F	FS	S	
Aberdeen Angus	114	1	1	112	0.01
Ayrshire	86	1	22	63	0.14
Brown Swiss	95	1	11	83	0.07
Guernsey	352	0	31	321	0.04
Hereford	408	21	154	233	0.24
Holstein-Friesian	1102	48	341	713	0.20
Jersey	395	71	182	142	0.41
Polled Hereford	365	6	66	293	0.11

TABLE 3

Observed and expected numbers of CA phenotypes 1-1, 1-2, 1-3, 2-2, 2-3 and 3-3 in a sample of 51 bison

	1-1	1-2	Phenotypes*		2-3	3-3
			1-3	2-2		
Observed numbers	7	20	2	19	3	0
Expected numbers	6	22	2	18	3	0

* Frequencies of alleles CA^1 , CA^2 , and CA^3 are 0.35, 0.60 and 0.05, respectively.

biochemical polymorphisms have concerned blood groups, hemoglobin types and transferrin types. Although the blood group comparisons (OWEN, STORMONT and IRWIN 1958; STORMONT, MILLER and SUZUKI 1961) were restricted almost wholly to the use of blood-typing antibodies (anti-A, anti-B, etc.) that had been developed and selected specifically for use in cattle blood typing, a considerable number of those reagents cross reacted specifically with the red cells of bison, as shown by antibody-absorption tests. Indeed, it was evident from the studies of STORMONT *et al.* (1961) that each of the 9 blood-group systems then recognized in cattle has its homologue in bison. However, the extent of genetic variability, as indicated by the number of alleles at each of the 9 loci, was much less than in cattle. For example, only one allele was indicated at each of the loci *F-V* and *L* of bison, and the complex *B* locus, which is represented by several hundred alleles in domestic cattle, was only represented by two alleles in bison. Although several electrophoretic variants of hemoglobin and transferrin are recognized in domestic cattle, BRAEND and STORMONT (1963) observed no electrophoretic variation in a survey of hemoglobin and transferrin types in 113 American buffalo.

Therefore, it came as somewhat of a surprise to find one locus, namely, the *CA* locus of the present study, which may support more alleles in American bison than it does in domestic cattle (*Bos taurus*).

We have already noted the ease with which American buffalo can be distinguished from domestic cattle simply by comparing the electrophoretic positions of the A esterase isozymes in region I of the gels (Figure 2). Other electrophoretic markers which can also be used to distinguish these two species of the subfamily Bovinae are their hemoglobins (BRAEND and STORMONT 1963) and transferrins (STORMONT 1964). Of interest from an evolutionary point of view will be the establishment of the number of mutations required to bring about the different electrophoretic mobilities of the CA isozymes of cattle and bison. TASHIAN (1966) points out that the human CA variant designated CA Ic differs from the normal form (CA Ia) by virtue of the substitution of arginine for glycine in a tryptic dipeptide, gly-arg. We presume, therefore, that similar amino acid substitutions will eventually be shown to account for the differences in electrophoretic mobility of the CA isozymes described in the present report.

Finally attention is called once again to the family and population data (Tables 1 and 2) on CA-F and CA-S of domestic cattle. In our opinion, those data, and the fact that the CA isozymes are readily stainable with protein dyes, indicate

that the CA types may be used reliably as an adjunct to blood types in resolving problems about questionable parentage arising in the breeding of cattle.

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SUMMARY

Hemolysates of the red cells of several thousand domestic cattle (*Bos taurus*) and 51 American buffalo (*Bison bison*) were electrophoresed in starch gels. The gels were examined for specific zones of esterase activity using a variety of synthetic naphthol esters as substrates, and also for protein staining zones. Three specifically different regions of esterase zones were described.—The most anodal region, designated I, exhibited multiple zones of esterase which were classified as A or arylesterase. No intraspecific variation of a genetic nature was observed with respect to the zones of A esterase. However, the two species could be readily distinguished on the basis of the electrophoretic positions of the A zones, those of bison migrating considerably faster than those of cattle, under the conditions described.—The zones in region II were classified as carbonic anhydrases (CA) largely on the basis of their specific inhibition by acetazolamide. Unlike the zones of esterase in regions I and III, those of region II was readily stainable with protein dyes. Each of the cattle CA zones, designated CA-F (for fast) and CA-S (for slow), could be present or absent in any individual and they were observed in three phenotypes designated F, FS and S. Both family and population data indicated that the cattle CA zones are controlled by a pair of codominant autosomal alleles, CA^F and CA^S . Allele CA^F , the least frequent of the two in a survey of nine breeds, ranged in frequency from a low of 0.01 in Aberdeen Angus to a high of 0.41 in Jerseys. Similarly, three zones of CA, designated CA-1, CA-2 and CA-3, were observed in bison. These occurred in five of six expected phenotypes based on control vested in three codominant autosomal alleles, CA^1 , CA^2 and CA^3 . The observed numbers in each phenotype agreed closely with expected numbers, assuming HARDY-WEINBERG equilibrium. The bison zones CA-1 and CA-2 migrated ahead of CA-F of cattle, whereas CA-3 appeared to migrate at about the same rate as CA-S of cattle.—Region III, which partially overlapped region II, was characterized by one broad zone of esterase when using α -naphthyl butyrate as substrate. However, when using α -naphthyl acetate as substrate only the leading boundary of this zone became stained, thereby suggesting that two B esterases might be involved. No intraspecific nor interspecific variation was detected in the esterase(s) of region III.—The data are discussed in the light of earlier comparisons of these two species with respect to blood groups and other biochemical markers, and it is noted that the CA polymorphism is so far the most extensive polymorphism detected in bison.

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