Variants of Ovine Alkaline Serum Phosphatases and Their Association with the R-O Blood Groups. (29056)

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When the serum of various mammalian species is subjected to electrophoresis in starch gels, it is possible, as shown by Lawrence *et al*(1), to demonstrate as many as 11 systems of enzymes by staining the gels with enzyme dyes. Utilizing the method for staining phosphatases, Gahne(2,3) detected intraspecific differences in cattle and showed that the fastest moving band (A zone) is inherited as a dominant trait. Similarly, Arfors *et al*(4) demonstrated polymorphism in human serum-phosphatase and were able to show that the phenotype which they designated as "type 2" is negatively correlated with the A agglutinogen of human red cells. It is known that A of man is closely related serologically to J of cattle and R of sheep(5,6). Therefore, it was not unexpected that the A phosphatase of cattle would be negatively correlated with J. Indeed, such a correlation was demonstrated by Rendel and Gahne(7) but the magnitude was less than that between human A and phosphatase "2." The present report is concerned with the description of phosphatase phenotypes in sheep and their distribution with respect to the 3 blood groups R, O (or r), and i of the R-O system of sheep(8,9,10).

Materials and methods. Blood samples were obtained from 177 sheep. Two of the samples (both from Group i females) were obtained by Professor B. A. Rasmussen of University of Illinois. The remainder were from animals in the breeding flocks of the University of California. In all, there were 105 sheep of Group R (9 ♂♂, 96 ♀♀), 67 of Group O (3 ♂♂, 64 ♀♀), and 5 of the rare group i (all ♀♀). When first bled, the ewes were either in early pregnancy or had not yet conceived.

Serum samples were subjected to electrophoresis, essentially after the methods of Gahne(3). The gels measured 130 × 215 × 6 mm permitting the running of 13 to 15 samples per gel. The samples were inserted on filter paper (9 × 6 mm) 2 cm from the cathode side on the long axis of the gels. They were run at 165 V and approximately 25 mA for 25 minutes, at which time the inserts were removed. Thereafter, 300 V was applied for 3 and ¾ hours.

Each gel was sliced and one-half was incubated for 2 hours in a staining solution containing sodium α-naphthyl phosphate and garnet GBC salt as active ingredients(1). However, for adequate staining of sheep phosphatases it was necessary to increase the pH of the sodium acetate buffer to at least 5.4. After staining the gels were treated in methanol/water/glacial acetic acid (5:5:1) to make them more amenable for reading and handling.

Results. On properly stained gels, the phosphatases appeared as dark blue bands or zones on a sorrel background, whereas the albumins, transferrins, and slow α-globulins stained yellow.

Fig. 1 illustrates the gamut of variation observed in this study of ovine phosphatases and their positions with respect to known serum proteins. Most of the phosphatase activity was found in a region approximately 1.7 cm in length between the transferrin and slow α-globulin zones. All individuals had a phosphatase band (labelled A in Fig. 1) of varying intensity just behind the transferrins. Atten-

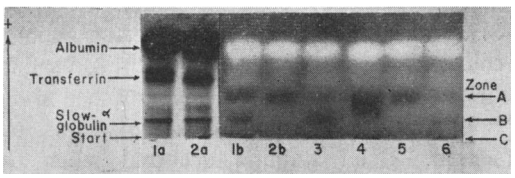

FIG. 1. Phosphatase zymograms of six sheep. The phosphatase zones are marked A, B, and C at the right. Animals No. 1, 3, 4, and 6 are of blood group O, and 2 and 5 of Group R. To the left are two samples stained with amido black for comparison.

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TABLE I. Classification of the Phosphatase B Band in Animals of Blood Groups R, O and i. The 4 animals of phosphatase type X are excluded.

Staining time	R = 104 animals Strength of B band						O = 64 animals Strength of B band						i = 5 animals Strength of B band					
	++	+	±	tr	?	−	++	+	±	tr	?	−	++	+	±	tr	?	−
1 hr	0	0	1	4	3	96	7	35	13	3	1	5	0	0	1	0	0	4
2 hr	0	0	4	3	13	84	28	23	7	3	2	1	0	1	1	0	1	2

++ = very strong B band.
+ = strong B band.
± = weak but clearly visible B band.
tr = trace of a B band.
? = a slight indication of a B band.
− = no B band.

tion should be focused specifically on the phosphatase region labelled B in Fig. 1 because the most marked and easily classified variation occurred there. The vast majority of the sheep could be classified into 2 groups with respect to staining in that region, namely, those whose serum showed marked staining and those whose serum exhibited little or no staining. The Figure shows 2 sera (Nos. 1b and 3) with typical B bands, one serum with a relatively weak B band (No. 6), 2 sera (2b and 5) which lacked B bands, and one deviating type (No. 4) called "X."

The rare X type, observed in only 4 sheep, was characterized by strong phosphatase activity in the whole region between the transferrin and slow-α zones. The trailing edge of this wide phosphatase zone extended as far or almost as far toward the cathode as the normal B band.

In some individuals a third band (C) was observed very close to the point of insertion. The intensity of C varied somewhat and its classification was therefore not entirely reproducible. Only animals of type X and those with a B band exhibited a clearly visible C band.

The phosphatases described here are of the alkaline type. The pH of the gels was about 8.5. When the acetate buffer of the staining solution was standardized at pH 4.8, its final pH after incubation of the gels reached about 6.5, and no phosphatase activity was observed. Good staining occurred when the acetate buffer was at pH 5.6. The final pH of the staining solution then became about 8.1. Good activity was also obtained when the gels were incubated in gel buffer (pH 8.8) containing Blue RR salt and sodium α-naphthyl phosphate.

It required about 2 hours' incubation in the staining solution for the phosphatase bands to develop fully. No appreciable changes took place when the gels were incubated overnight. However, the picture became often somewhat blurred; and some samples which completely lacked a B zone at the 1 or 2 hour readings showed on overnight staining an indication of a very faint B band.

Relationship of the B band to blood group O. The classification of the B phosphatase band in sheep of groups R, O, and i is summarized in Table I. Most samples from group R sheep lacked B activity, while almost all O individuals had B. Sheep of the rare group i occupied a somewhat intermediate position.

Two months after the initial bleeding, samples were again collected from the 6 animals of Group O with low or no B activity (trace or less after 2 hour staining) as well as from the 7 Group R sheep which were classified as ± or tr at 2 hours. (The Group i sheep with weak B activity were not available for resampling.) The first and second samples were tested in parallel. On retesting, 3 of the initial samples from these R individuals were reclassified as B negative, while all except one of the second samples were classified as negative. The one exceptional animal was classified as B?. The second samples from the 6 sheep of Group O showed the following B activity: negative (1 animal); trace (1); + (3); and X type (1). Also, 10 B-negative individuals of Group R and 7 B-positive sheep of Group O along with the 4 animals of phosphatase type X (3 of

Group O and 1 of Group R) were resampled. No changes had taken place in the phosphatase type of the 17 animals in the first two classes. However, the second sample from the single animal of blood Group R which had originally been classified as phosphatase type X exhibited phosphatase activity only in the A zone. The classification of the other 3 animals of type X remained essentially unchanged, though one of them approached the B type. Thus it would appear that phosphatase type X is not entirely stable from one bleeding to the next.

Discussion. The animals in this study belonged to a number of mating pens in a breeding experiment. It is intended to study the inheritance of the phosphatase types in the forthcoming lamb crop. However, because of the very close association between the B phosphatase activity and blood group O, it can obviously be inferred that the B band is genetically controlled. Since the O property of sheep serum is inherited as a recessive in contrast to the R property, it may also be inferred the B phosphatase band will appear as a recessive trait.

R and O of sheep are primarily soluble constituents of the plasma that are passively acquired by the cells. Sheep of Group R contain soluble R substance in their plasma but very little or no O substance. Sheep of Group O contain soluble O substance in their plasma but no R substance. Sheep of the rare Group i have no R substance and very little or no O substance in their plasma; furthermore, their erythrocytes are negative in tests with anti-R and anti-O(9,10,11).

As already mentioned, the R substance of sheep, A of man, and J of cattle are serologically related. For example, all of these substances cross react with natural bovine anti-J (6), and antisera produced in A (or J)-negative rabbits against human red cells of type A cross react strongly with red cells of J-positive cattle and sheep of group R(12). Each of these 3 species has a specific phosphatase activity which is negatively correlated with the presence of the R-J-A-antigens. The negative correlation is strongest in sheep and least pronounced in cattle. The sheep O substance seems to be related to the human secretor substance (H) because the reaction between anti-O and sheep O cells is completely inhibited by saliva from human secretors of the ABH substances(6). Similarly the saliva from R and O sheep inhibits the agglutination of human O red cells by *Ulex* extract, whereas saliva from Group i sheep does not(10). It is also established(13) that certain cattle possess a soluble plasma substance designated Oc which inhibits the reaction between anti-O and sheep O cells. The question arises whether or not it is the presence of the O substance which is essential for the manifestation of the B phosphatase band in sheep. Seemingly the answer may be forthcoming when phosphatase phenotypes are examined in a large number of Group i sheep. But Group i sheep are rare. In the meantime it may be fruitful to examine the phosphatase types in cattle which have been classified not only with respect to the presence or absence of J substance in their plasma but also with respect to the occurrence of the Oc substance. In contrast with sheep, cattle may manifest either or both the J and Oc properties and some lack both. Thus it should be possible to establish whether the presence of some Oc activity is necessary for the appearance of the bovine phosphatase band designated A by Gahne (2,3).

Summary. When sheep sera are subjected to electrophoresis in starch gels and the gels are stained with phosphatase dyes, it is possible, with few exceptions, to demonstrate 2 clearly demarcated zones of phosphatase activity named A and B, in decreasing rate of migration. All 177 samples exhibited activity in zone A and some in zone B. It is shown that phosphatase activity in zone B is highly correlated with the presence of the soluble blood group substance called O (or r). The nature of the association between "B" phosphatase and the O substance is not known.

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On a Method for Calculation and Analysis of Results in the McKenzie Assay for Thyrotropin (TSH).* (29057)

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In the elegant biological method reported by McKenzie(1) for assessing TSH activity, one measures in suitably prepared animals, the radioactivity of 2 samples of blood; at time zero, a first sample of blood (internal control) is withdrawn and TSH injected immediately thereafter; 2 hours later, a second sample of blood (experimental) is obtained. McKenzie reported that there is a (linear) relationship between the log of the dose of injected TSH and the difference in radioactivity counts between the 2 samples of blood. We have recently become interested in this assay in studying the neurohumoral control of the secretion of TSH(2). In this paper is proposed a new method for calculating and analyzing the results obtained in this technique which greatly improve its reliability and usefulness. Similar problems have been recently discussed regarding the ovarian ascorbic acid depletion test for LH(3,4). As in the case of the LH assay, we will introduce here for the TSH method the principle of covariance adjustment and analysis. A detailed discussion of this method of calculation, including its limitations as well as the mathematical requisites it necessitates has been published(3,4). The second principle regarding the TSH assay discussed here is transformation of the original data into metameters more amenable to classical mathematical analysis.

Materials, methods, results. 1. *Method to assess TSH activity.* This is essentially that reported by McKenzie(1) with minor modifications for practical convenience. Female SA mice (body weight 10-15 g) (Duterme Farms, Condé s/Huisne, France) are kept in temperature controlled rooms (22°C ± 2) on a low iodine diet (Nutritional Biochemicals, Cleveland, Ohio) with double (glass) distilled water as drinking fluid, for 10 days. They are then injected i.p. with 1.5 μc I^{131} carrier free, followed 5 hours later by 10 μg 1-thyroxine s.c.; 24 hours later they received a second injection of 5 μg 1-thyroxine s.c. and are used 48 hours after this last injection for bioassay of TSH. Under rapid ether anesthesia, 0.25 ml of blood is withdrawn from the jugular vein in a heparinized tuberculin syringe and the material to be tested (for TSH activity) is immediately injected by the same route in a total volume of 0.3 ml; 2 hours later, again under ether anesthesia, a second 0.25 ml blood sample is taken from the same or contralateral jugular vein. The radioactivity has been measured in a well scintillation counter followed by a manual scaler (Saphymo, Paris) and more recently in a fully automatic well-counter (Tracerlab) with pulse analyzer and autoprinter.

2. *Principle of covariance adjustment and*

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