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Heterozygosity of the Sheep: Polymorphism of 'Malic Enzyme', Isocitrate Dehydrogenase ($NADP^+$), Catalase and Esterase

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Abstract

In contrast to other reports, it is found that the sheep has approximately as much enzyme variation as man.

Most of the genetically interpretable enzyme variation in heart, liver, kidney and muscle from 52 sheep (Merinos or Merino crosses) is in the NADP-dependent dehydrogenases [two 'malic enzymes' and the supernatant isocitrate dehydrogenase ($NADP^+$)] and in the esterases. Ten different loci for NAD-dependent dehydrogenases are electrophoretically monomorphic, as are five different NADH diaphorases from heart muscle and 15 different major proteins from skeletal muscle.

It is highly statistically significant that NADP-dependent dehydrogenases and esterases are polymorphic but representatives of several other major classes of enzymes are not. The physiological significance of this polymorphism may be related to the role of these enzymes in growth and detoxication, sheep having been selected by man for faster growth, of wool or of carcass, and for grazing a wide variety of plants.

Introduction

The domestic sheep *Ovis aries* is polymorphic for several blood proteins and for erythrocyte potassium levels (see reviews by Tucker 1971; Agar *et al.* 1972). However, an apparent contradiction is the scarcity of enzyme polymorphism reported for sheep compared with certain other domesticates or with man (see reviews by Brewer and Sing 1969; McDermid *et al.* 1975).

For 15 different enzymes in erythrocytes, several with multiple zones and with evidence of determination by two or more loci, sheep were polymorphic for only one—an NADH diaphorase (Brewer and Sing 1969). In contrast, these same workers report that man is polymorphic for six of these same loci and has rare variants at six other of these loci. One locus, carbonic dehydratase, not reported as polymorphic in sheep by Brewer and Sing (1969), is known to have a low frequency variant which occurs in a few breeds at gene frequencies above the usual 1 or 5% cut-off points defining 'polymorphism' *v.* 'monomorphism' (Tucker 1971; McDermid *et al.* 1975).

The present study has as a main objective an attempt to answer the question: is the sheep deficient in genetic polymorphism of the usual electrophoretically surveyed enzymes in comparison with man and other mammals? An important secondary objective involves comparison of certain organs, which have high levels of a number of enzymes, with erythrocytes, which are relatively deficient in many electrophoretically useful enzymes although they have high activities of a few enzymes necessary for keeping haemoglobin in a functional state (Agar and Smith 1973). This comparison forms an important control in studies measuring protein polymorphism. Frequently in heterozygotes a variant enzyme has less activity than the normal enzyme and if,

as in studies on erythrocytes, even the normal enzyme is present in small amounts, there is a danger that surveys confined to only erythrocyte enzymes will miss some variation.

Materials and Methods

Sheep

Most experiments were carried out on blood and organs from 52 slaughtered sheep from the Roseworthy Agricultural College, South Australia. The sheep were either from the commercial Merino flock, or were crossbreds involving Merinos crossed with Border Leicesters, Poll Dorsets or Suffolks.

Organ Extracts

From the 52 sheep slaughtered, samples were taken of blood, heart, liver, kidney and skeletal muscle (the latter taken from the upper part of the leg). In addition, samples of brain, pancreas and small intestine were taken from a few sheep. To ascertain the prevalence of certain enzyme variants in some sheep slaughtered in South Australia, organs were also sampled from 36 sheep at a local abattoir and from three Corriedales kept by the authors at Normanville, S.A.

As soon as possible after slaughter, organs were placed in plastic bags and the bags submerged in crushed ice. Within 6 h, 1-g samples were removed, blotted clean of excess blood, and minced finely with a single-edged razor blade. Heart tissue was further triturated by grinding with glass beads in a mortar. The mince was stored frozen, having been brought to the laboratory at Normanville on dry ice.

In preliminary experiments a variety of extractants was tried. Best results were generally obtained with a 0·02 M lithium glycyl-glycine solution, pH 8, to which 1% digitonin was added. This solution was also useful in haemolysing erythrocytes. In general 1 ml of extractant was added to 1 g of mince, except for heart and muscle where the ratio was 2 : 1.

The extracting procedure was varied in special cases. As a check on possible alteration of mobility, some samples were extracted simply in dilute electrophoretic buffers. For comparison of mitochondrial versus cytoplasmic location of certain dehydrogenases, organ minces (unfrozen) were ground with glass beads together with four volumes of 0·25 M or 0·44 M sucrose, buffered to pH 8·0 with either tris or lithium glycyl-glycine (without digitonin). These samples were then centrifuged first at low speed (500 *g*, 15 min) and then at higher speeds (10 000 *g*, 30 min; 38 000 *g*, 1 h) to yield a supernatant (cytosol) fraction and particulate fractions, including mitochondria which were subsequently extracted with 1% digitonin together with freezing and thawing. To check for maximal possible sharpness of electrophoretic resolution of pyridine–adenine dinucleotide-linked enzymes, some organ extracts were made with 1 mM NAD or NADP added. For most enzymes in this category the improvement was only slight, but the resolution of glucose-6-phosphate dehydrogenase* and phosphogluconate dehydrogenase (decarboxylating) by NADP and the resolution of glutamate dehydrogenase by NAD was clearly improved.

Addition of 1·5 mM 2-mercaptoethanol improved the sharpness and altered the satellite zone pattern for the more slowly moving (mitochondrial) isocitrate dehydrogenase ($NADP^+$)—but also often caused some deterioration in the resolution of the faster moving (supernatant) isozyme of the same specificity. In attempts to vary the resolution of regulatory enzymes in the glycolytic pathway, i.e. hexokinase, 6-phosphofructokinase and pyruvate kinase, organ extracts were tried with and without 1·5 mM 2-mercaptoethanol, 1 mM reduced glutathione, 1 mM ATP, 1 mM $MgSO_4$ or with the EDTA-containing diluted electrophoretic buffer specified below. Activities were altered to some extent but no important improvement was noted over the usual extractant. It is likely that our failure to find 6-phosphofructokinase after electrophoresis of muscle or to find only irregular amounts of true hexokinase after electrophoresis of liver could be overcome by improvements in extracting procedures for these sensitive enzymes, although we were able to consistently obtain 6-phosphofructokinase in kidney extracts and hexokinase in heart muscle by our present methods. As is to be expected, certain organs yielded higher activity and better resolution of some enzymes; these particular combinations were used in the survey of the 52 sheep from Roseworthy Agricultural College and are detailed in Table 1.

* For EC numbers of enzymes, see Table 1.

Electrophoresis

All enzymes were surveyed initially in the following five different electrophoretic systems; however, a system was used in surveying most or all of the 52 sheep only when reasonably sharp resolution was found (see Table 1) (by reasonably sharp resolution it is meant that the zone is less than 4 mm wide when the staining begins). The five electrophoretic systems used (and the abbreviations used in Table 1) are as follows.

(1) *A–G (acrylamide)*: acrylamide polymer gradient electrophoresis, Gradipore (Margolis and Kenrick 1968) using the following modification of the pH 8·7 buffer of Aronsson and Grönwall (1957). A stock solution was made up of 121 g tris, 15·6 g EDTA and 9·2 g boric acid in 1 litre. The stock solution was diluted 1 part stock solution into 20 parts final solution for electrophoresis. Best results were obtained when the commercially prepared gels were pre-electrophoresed in this buffer for 1–2 h at 100 V before the samples were inserted. Samples containing 5 μl of centrifuged organ extract or haemolysate were used. Initial electrophoresis was at 100 V for 10 min; the sample spacers were then removed, the surface of the gel washed gently with distilled water followed by the electrophoretic buffer, and electrophoresis then started at 250 V and approximately 50 mA. When heating was a problem, the circulating buffer was run through a plastic bag surrounded by ice. Although the length of the run was varied for certain purposes, for most research using gradient electrophoresis for detection of polymorphism the higher voltage run was for 2 or 3 h.

(2) *A–G (starch)*: The same buffer as in (1), but using a starch gel.

(3) *F–W*: starch gel using a modification of the discontinuous system of Ferguson and Wallace (1961). For the gel buffer there is a 1 : 20 dilution of the following stock solution: 2·4 g lithium hydroxide (monohydrate), 23·6 g boric acid, 31·5 g citric acid (monohydrate), and 111·6 g tris, all dissolved into 1 litre of solution. For the bridge solution there is a 1 : 5 dilution of the following stock solution: 6 g lithium hydroxide (monohydrate) and 59 g boric acid in 1 litre of solution. In contrast to the diluted buffers the concentrates will keep well for months at room temperature.

The gels were made using 12 g hydrolysed starch (Connaught Laboratories) to 100 ml of diluted gel buffer. Samples were inserted on pieces of Whatman No. 3 filter paper and the inserts were removed from the gel after 10–20 min of electrophoresis at 120 V, the current being usually between 20 and 25 mA. The gels were then electrophoresed at a voltage as high as possible without overheating—at room temperatures varying over the range 10–35°C the voltages were 300 V at the lower temperatures and 200 V at the higher temperatures. A fan was used to facilitate cooling of the gels. Electrophoresis was completed in 2–3 h, the line of discontinuity having travelled approximately the length of the gel tray (12·5 cm).

(4) *pH P*: starch gel using potassium phosphate buffers of pH 7, 6·7 or 6·0, diluted from stock concentrates to a final ionic strength of 0·02 or 0·01. The diluted solution was used in both the gel and the bridges. The phosphate buffers yielded excellent resolution of several of the dehydrogenases.

(5) *S–K*: starch gel using Shaw and Koen's (1968) pH 7 tris–citrate buffer. The stock solution contains 27 g tris and 18·1 g citric acid (monohydrate) per litre. It was diluted 35 ml into 1 litre final volume for both gel and bridge buffers. The system was especially useful for the NAD-dependent malate dehydrogenases which resolve poorly in the Ferguson–Wallace buffer.

For pyridine–adenine dinucleotide-linked enzymes we checked resolution with and without coenzyme in the gel (and, in the discontinuous system, the cathodal bridge as well): 5 mg per 100 ml of NAD (0·08 mM) or of NADP (0·07 mM) for starch gels, and for the recirculating buffer in Gradipore electrophoresis 5 mg of coenzyme per litre was used. Addition of the appropriate coenzyme sharpened the zones of glucose-6-phosphate dehydrogenase, phosphogluconate dehydrogenase (decarboxylating) and, on some occasions, isocitrate dehydrogenase ($NADP^+$), but had only marginal effects on 'malic enzymes'. Slight but significant improvement of resolution occurred for several of the NAD-dependent dehydrogenases, especially for glutamate dehydrogenase, which yielded a sharp zone, approximately 2 mm wide, in liver and kidney extracts; this is much better resolution than we have obtained for this enzyme in many other species.

Enzyme Localization after Electrophoresis

Most of the enzymes were localized by conventional methods (Manwell and Baker 1970, 1977*a*, 1977*b*; Manwell 1977) but a few changes are worth noting.

Regardless of differences in the pH optima of various dehydrogenases, the best staining involves a compromise between pH optima and stability of the staining mixture. For each sliced gel, in staining for dehydrogenases, 10 ml of potassium phosphate buffer, pH 7·0 and ionic strength 0·03,

was used; to this was added 10 mg substrate, 4 mg NAD (or 2 mg NADP where appropriate), 1 mg phenazine methosulphate (PMS) and 10 mg thiazolyl blue [MTT = 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide]. In addition, for dehydrogenases requiring divalent ions 0·2 ml of 1 M $MgCl_2$ or 0·05 ml of 0·1 M $MnCl_2$ was added. To minimize background colour development when enzyme activity was low, the gel slice was first pre-incubated for 20 min in 50 ml of the diluted pH 7 buffer if the gel was at a pH of 8 or higher. For UDPglucose dehydrogenase only 2 mg of this substrate was necessary for adequate staining.

As a further check on specificity of dehydrogenases, some gels were examined under long wavelength u.v. light after addition of only 10 mg of substrate and 3 mg of coenzyme in buffers of different pH and with appropriate ions. For example, by addition of carbonate and pyruvic acid in the presence of NADH it was possible to show that the 'malic enzyme' was decarboxylating, as its electrophoretic position was identical to that detected with the reaction in the 'normal' direction (from malate to pyruvate plus carbon dioxide) and did not overlap isozymes of lactate dehydrogenase, which react, albeit slowly, with NADPH and pyruvate. Dehydrogenases examined by changes in coenzyme fluorescence after electrophoresis coincided with those stained by the usual techniques except in one case: when UDPG was added with NAD after electrophoresis of liver extracts, a second, more slowly moving zone which showed only faintly if at all with tetrazolium staining, was revealed; because of the breadth of this zone it was not useful in surveying for genetic variation, but the possibility that the 'classical' methods of staining for enzymes after electrophoresis might inhibit certain isozymes needs to be considered.

Glucuronate reductase

To 5 ml of the potassium phosphate buffer, pH 7·0 and ionic strength 0·03, was added 5 mg D-glucuronic acid (sodium salt, Sigma Chemical Co.) and 1·5 mg NADPH. A sheet of Whatman No. 1 filter paper, cut to match the size of the gel, was soaked in the above solution and placed on top of the sliced gel so as to avoid trapping any air bubbles. Within 30 min to 1 h, activity of glucuronate reductase was visible as a dark non-fluorescing region, 2–4 mm wide, against a background of bluish-yellow fluorescence of the reduced pyridine–adenine dinucleotide phosphate when the gel was illuminated with long wavelength u.v. light in a darkened room. The substrate for the enzyme in the other direction, L-gulonate, was not available to us and, thus, the reaction has not been checked both ways.

Catalase

The iodine–starch method, as modified by Numachi (1971), gave the best results with sheep catalase. Organ extracts required extensive dilution and best results were obtained with erythrocyte samples diluted in the range 1 part in 30 to 1 part in 50 with distilled water.

NADH diaphorase

Enzymes with this activity were stained with an alteration of the method of Kaplan and Beutler (1967). For each gel slice, to 20 ml of 1 : 4 diluted Aronsson–Grönwall buffer (see p. 129) was added first 5 mg digitonin, then 1 mg dichlorophenol indophenol (DCIP) (Sigma Chemical Co.) and finally 2 mg NADH and 10 mg MTT tetrazolium. The solution was then filtered through tissue paper. The blue–purple zones of NADH diaphorase detected with this technique are the same as those visualized by the decolorization of DCIP in the presence of NADH as recommended by Brewer *et al.* (1967) in their studies on the polymorphism of sheep erythrocyte NADH diaphorase, but the coupling of the reaction to MTT tetrazolium allows sharper resolution and a more permanent record. On the other hand, the MTT tetrazolium staining technique does not work well at pH values below 8, whereas the decolorization method does. The MTT technique worked well for us using digitonin and NADH (from either Sigma Chemical Co. or Calbiochem) with MTT (from either Sigma of Koch–Light), although MTT from the latter source required trituration in a mortar and pestle to dissolve it quickly with minimal exposure to light.

Checks for Artefacts in Staining

In the conventional staining methods for phosphoglucomutase, glucosephosphate isomerase, hexokinase and 6-phosphofructokinase, the detection method basically involves coupling the reaction in the gel to added enzyme(s) so that the reaction products ultimately involve a dehydrogenase which in turn is coupled to a tetrazolium dye. It is usual to incorporate these additional enzymes and the

staining reagents into an agar overlay, which is poured over the sliced gel. However, we found it equally satisfactory to soak the reagents into a filter paper which is laid over the sliced gel, providing that no air bubbles are trapped and that the overlay does not shift position during the incubation. The overlay is covered with a piece of transparent plastic.

The filter paper overlay has one important advantage over the agar overlay: when zones of colour appear, it is possible to check easily whether colour is developing first in the gel, which means an artefact, or first in the overlay, which means the reaction is dependent upon the added enzymes. For example, in staining for hexokinase it was found that some coloured zones appeared first in the gel; further investigation revealed that these were glucose dehydrogenases, which will use either NAD or NADP, and H_4 lactate dehydrogenase, which will use NADP although at a much slower rate than NAD and which was using L-lactate as a contaminant in the starch. Similarly, in staining for 6-phosphofructokinase, faint zones of H_4 lactate dehydrogenase occasionally showed up, together with, in liver samples, an unidentified dehydrogenase (but not alcohol dehydrogenase). Other workers (e.g. Shaw and Koen 1968) have reported that alcohol dehydrogenase frequently shows up in staining for NAD-linked enzymes because of traces of ethanol as a contaminant in certain reagents. This was not a problem in the present study because sheep alcohol dehydrogenase, present in liver and kidney extracts, migrates cathodally as a sharp zone in the Ferguson–Wallace discontinuous system; only L-iditol dehydrogenase, which migrates more slowly cathodally, was anywhere near the alcohol dehydrogenase zone.

Scoring for Genetic Variation

We used the same procedure as in previous studies (e.g. Manwell and Baker 1975) of scoring enzymes as: *polymorphic*, when there is clear genetically interpretable variation comparable to that established as genetic for that particular protein in other species; *variable*, when there is electrophoretic variation which is not readily interpreted; and, *monomorphic* when the zone shows no significant variation in electrophoretic position. Even then an element of arbitrariness remains: '*monomorphic*' is placed in quotation marks for a few situations where real genetic variation occurs but at a low frequency, e.g. the 5 or 1% cut-off points used by several workers. The pattern of variation for 6-phosphofructokinase involves a set of several overlapping zones in some individuals, reminiscent of the heterozygote pattern in other multi-subunit proteins; however, the combination of indistinct resolution and the lack of genetic data on the variation of this enzyme results in assigning it to the *variable* rather than *polymorphic* status for the present.

Results

The survey of the organs of 52 sheep for 36 enzyme loci and 15 major muscle proteins, assumed to represent 15 loci, is shown in Table 1. The patterns of variation are similar to those observed by others (Manwell and Baker 1970; McDermid *et al.* 1975). The only unusual feature is that the major, slowly migrating 'malic enzyme' occurs in the supernatant rather than the mitochondrial fraction, and occurs mainly in heart rather than liver, although it also occurs in kidney, brain and in erythrocytes. The pattern of variation is with three hybrid zones in heterozygotes, although in some heterozygotes only two of these zones are in high concentration. Whether the slight difference in pattern among heterozygotes is the result of yet additional variation of this enzyme, i.e. of isoalleles of the S variant, is not known. A pattern of three hybrid zones has recently been observed for variation of 'malic enzyme' from other mammals (Povey *et al.* 1975). There are other confusing aspects about 'malic enzymes'. Although Povey *et al.* (1975) state that human erythrocytes lack 'malic enzyme', other workers claim to have purified a 'malic enzyme' from human erythrocytes (Snyder and Reddy 1971). We have found that sheep erythrocytes, even when freed from leucocytes by differential centrifugation or by passage through cotton wool, have the same 'malic enzyme' which shows polymorphism in heart and other organs.

Table 1. Variation of enzymes and other proteins in organs of sheep

Enzyme or other protein and coenzyme, if any	Genetic variation (and no. of sheep)	Remarks, including organs surveyed and electrophoretic systems used[A]
Alcohol dehydrogenase (EC 1.1.1.1); NAD	Monomorphic (52)	Liver, kidney; F–W, A–G (starch)
Glycerol-3-phosphate dehydrogenase (NAD^+) (EC 1.1.1.8); NAD	Monomorphic (52)	Liver; F–W, S–K, pH 6 P
L-Iditol dehydrogenase (EC 1.1.1.14); NAD	Monomorphic (52)	Liver; F–W.; seems identical with dehydrogenase activity on xylitol.
Glucuronate reductase (EC 1.1.1.19); NADP	'Monomorphic' (34) $p_F = 0{\cdot}01$ $p_S = 0{\cdot}99$	Liver; F–W, S–K; heterozygote has two major overlapping zones and satellite.
UDPglucose dehydrogenase (EC 1.1.1.22); NAD	Monomorphic (52)	Liver; F–W.
Lactate dehydrogenase (EC 1.1.1.27); primarily NAD		Heart and kidney for H_4 and H_3M; liver is variable but usually has all
H chain locus	Monomorphic (52)	five isozymes; muscle has all five;
M chain locus	Monomorphic (52)	F–W, S–K, A–G (starch and acrylamide)
Malate dehydrogenase (EC 1.1.1.37); NAD		Liver, kidney; S–K, pH 6 P; A–G (acrylamide) better for super-
Supernatant locus	Momomorphic (52)	natant than mitochondrial isozyme.
Mitochondial locus	Momomorphic (52)	
Malate dehydrogenase (decarboxylating) ($NADP^+$) = 'malic enzyme' (EC 1.1.1.40); NADP		Heart for both isozymes; major zone also in kidney, brain and erythrocytes; although Mn^{2+} said
Trace zone (heart only)	Polymorphic (52) $p_F = 0{\cdot}54$ $p_S = 0{\cdot}46$	to be ion requirement, best staining with Mg^{2+}; 3 hybrid zones in heterozygote for major zone; hybrid
Major zone	Polymorphic (52) $p_F = 0{\cdot}78$ $p_S = 0{\cdot}22$	zones in heterozygote for minor zone but number is not clear.
Isocitrate dehydrogenase ($NADP^+$) (EC 1.1.1.42); NADP		
Supernatant locus	Polymorphic (52) $p_F = 0{\cdot}78$ $p_{S-1} = 0{\cdot}03$ $p_{S-2} = 0{\cdot}19$	Kidney, liver; F–W, pH 6 P, A–G (acrylamide and starch); single hybrid zone in heterozygote; S-1 and S-2 differ slightly in position.
Mitochondrial locus	Monomorphic (52)	Heart, liver, kidney; some of the enzyme may be in the supernatant but most is in the particulate fraction; number of satellite zones different in different organs; F–W.
Phosphogluconate dehydrogenase (decarboxylating) (EC 1.1.1.44); NADP	Monomorphic (52)	Liver, kidney; F–W; A–G (acrylamide), NADP for best resolution.
Glucose dehydrogenase (EC 1.1.1.47); NAD or NADP	Slightly variable, probably monomorphic (52)	Liver, kidney; F–W, pH 7 P, A–G (acrylamide).
Glucose-6-phosphate dehydrogenase (EC 1.1.1.49); NADP	Monomorphic (52)	Kidney, liver, heart; F–W, pH 7 P, A–G (acrylamide), NADP added.
Glyceraldehyde-phosphate dehydrogenase (EC 1.2.1.12); NAD	Monomorphic (52)	Liver, kidney; F–W, S–K; only sharp zone recorded here.

[A] Abbreviations are explained in Materials and Methods.

Table 1 (*Continued*)

Enzyme or other protein and coenzyme, if any	Genetic variation (and no. of sheep)	Remarks, including organs surveyed and electrophoretic systems used[A]
Glutamate dehydrogenase (EC 1.4.1.2), NAD primarily	Slightly variable, probably monomorphic (52)	Liver, kidney; F–W, S–K; the slight variability is not parallel in liver and kidney samples, probably represents allosteric modification rather than genetic variation.
NADH diaphorases of heart (EC 1.6.4.3 or 1.6.99.3)		Heart; A–G (acrylamide); 24 samples also identical in F–W; at least three of these zones occur in liver and kidney as well; all distinct from the polymorphic NADH diaphorase I of red blood cells whose molecular weight is only 25 000.
Five distinct zones	All 5 are monomorphic	
Hexokinase (EC 2.7.1.1)	Monomorphic (33)	Heart; A–G (acrylamide); should occur in muscle and liver but could not be reliably detected.
6-Phosphofructokinase (EC 2.7.1.11)	Variable (36)	Kidney; F–W.
Pyruvate kinase (EC 2.7.1.40)	'Monomorphic' (33) $p_F = 0{\cdot}03$ $p_S = 0{\cdot}97$	Kidney, liver; F–W; A–G (acrylamide); a second major isozyme occurs in muscle and heart, but its resolution is not as sharp; also some trace isozymes.
Phosphoglucomutase (EC 2.7.5.1)		Kidney has both major and minor zones; F–W; the major zone occurs in other tissues, including, very weakly, erythrocytes.
Minor (faster) zone (PGM II?)	Monomorphic (46)	
Major (slower) zone (PGM I?)	Monomorphic (52)	
Esterases (3.1.–.–)		
Locus I (fastest migrating, trace zone)	Polymorphic (50) $p_F = 0{\cdot}40$ $p_S = 0{\cdot}60$	Liver; F–W, A–G (acrylamide); no hybrid zone in heterozygotes; low activity, but fast, sharp zones.
Locus II (major zone)	Polymorphic (50) $p_F = 0{\cdot}24$ $p_S = 0{\cdot}76$	Liver, kidney; F–W, A–G (acrylamide); more easily typed in kidney, for second major zone in liver; no hybrid zone in heterozygotes.
Locus III (minor zone)	Variable (50)	Liver; F–W; A–G (acrylamide); resolves clear of other esterases but activity varies.
Locus IV (major zone)	Polymorphic (30) $p_F = 0{\cdot}68$ $p_S = 0{\cdot}32$	Liver; F–W; A–G (acrylamide); overlapped by smeary zone in some liver samples, hence only 30 animals scored.
Locus V (slow, minor zone or zones)	Variable (50)	Liver, kidney; F–W; A–G (acrylamide); some individuals have a single zone, others have a set of zones.
Locus VI (slow minor zone)	Variable (50)	Liver; F–W; A–G (acrylamide).
Aminopeptidase (cytosol) (EC 3.4.11.1)	Monomorphic (46)	Kidney; F–W; used L-leucineamide as substrate; has no activity on L-leucyl-β-naphthylamide; occurs at lower levels of activity in liver and heart.
Glucosephosphate isomerase (EC 5.3.1.9)	Monomorphic (47)	Liver, kidney; F–W; A–G (acrylamide)

[A] Abbreviations are explained in Materials and Methods.

Table 1 (*Continued*)

Enzyme or other protein and coenzyme, if any	Genetic variation (and no. of sheep)	Remarks, including organs surveyed and electrophoretic systems used[A]
Muscle proteins (15 distinct zones, not counting obvious satellites; also includes myoglobin; probably 15 loci total)	All 15 are monomorphic (42)	Muscle; A–G (acrylamide); myoglobin also surveyed in F–W, although for variation it is best surveyed in acrylamide as its mobility is near that of Hb A in several buffer systems in starch gel. The Hb A–B polymorphism shows up in muscle extracts but is not counted here.

[A] Abbreviations are explained in Materials and Methods.

There is also a problem about the subcellular localization of 'malic enzymes'. For a number of species the basic pattern is a more rapidly migrating anodal supernatant 'malic enzyme' and a more slowly migrating anodal mitochondrial 'malic enzyme' in the usual pH 8–8·6 starch gels. However, we found only two 'malic enzymes' in heart extracts, both with independent genetic variation, and yet both being in the supernatant fraction. Although the possibility of leakage from mitochondria exists, we were able to demonstrate the supernatant versus mitochondrial separation of the NAD-dependent malate dehydrogenases and the NADP-dependent isocitrate dehydrogenases. Povey *et al.* (1975) mention that there are differences among some mammalian species in whether or not the major, more slowly moving 'malic enzyme' is mitochondrial or supernatant in location.

The important point from the studies on 'malic enzyme' in sheep is that another potentially useful genetic marker, normally assumed to be lacking in erythrocytes, can be surveyed in those cells (see also Manwell and Baker 1977*b*).

In agreement with Brewer and Sing (1969) we did not find any isocitrate dehydrogenase ($NADP^+$) in erythrocytes; even erythrocytes contaminated with leucocytes yielded so little activity of this enzyme after electrophoresis that this marker could not be used in studies on blood. The pattern of genetic variation, with one hybrid zone in heterozygotes, is typical for this enzyme from a wide variety of animals (Manwell and Baker 1970; McDermid *et al.* 1975). An interesting observation is that the amount of the hybrid zone in the S-2 variant is different for different tissues of the same animal and the rate of development of activity during staining is slower for the electrophoretically slower variant.

Catalase variation was clearest in diluted erythrocyte samples prepared within 2 or 3 days of haemolysis; organ extracts yielded parallel variation to the erythrocyte samples. Some muscle samples yielded a minor, very sharp, slowly moving zone of catalase activity. Because catalase was best studied in erythrocytes, gene frequency data are placed in another paper (Manwell and Baker 1977*a*) where they were used in comparison of genetic variation between Merinos and Poll Dorsets. Resolution of catalase was not as good as it was for many other proteins, although it was comparable with the results of others (Holmes and Masters 1970; Kelly *et al.* 1971; Numachi 1971). Although it is postulated from the quaternary structure that catalase heterozygotes should show three zones, to our knowledge these have been

visualized only after electrophoresis of catalase from maize, which yields sharp zones (Scandalios 1969).

Esterases were especially active in liver and kidney samples and although the patterns were superficially similar for the two organs, only some of the loci showed genetic variation in both places. Most of the esterases are active on both α- and β-naphthyl acetate, but the relative proportion of activity is different so that a 1α : 3β mixture of substrates, together with a suitable dye coupler (Fast Red Violet LB Salt or Fast Violet B Salt), facilitates visual differentiation of esterases after electrophoresis because of the grey colour with α-naphthol and the red colour with β-naphthol. Those esterases which gave overlapping patterns, or complex multiple patterns in some individuals, were scored as *variable* rather than *polymorphic*; it is likely that the amount of esterase polymorphism has been underestimated—and we have recently found that heart extracts have a major, slowly moving α-naphthyl acetate-specific esterase which appears to have hybrid zones in heterozygotes. Further studies on esterases are presented elsewhere (Manwell and Baker 1977*b*).

Discussion

Heterozygosity of the Sheep

It is clear from these studies on the enzymes in sheep organs, as well as from a study on the enzymes and other proteins in sheep blood (Manwell and Baker, unpublished data) that sheep are by no means deficient in genetic variation. In contrast to the results of Brewer and Sing (1969), it has been found that the sheep has as much genetic variation as man, whether measured as a proportion of polymorphic loci (1% cut-off point used in the various mammalian comparisons) or as the average heterozygosity per locus (see Table 2).

For the present it can be said that man, mouse (*Mus musculus*) and sheep are all approximately equally variable. However, mammals living in more specialized habitats, e.g. mole rats, pocket gophers and elephant seals, have less heterozygosity (Table 2). More precise comparisons, using statistical tests, are not warranted at present because of limitations in the data. Most of these species were studied by different workers using somewhat different techniques and often different protein loci as well. There are few studies where different workers have checked out heterozygosity scoring by exchanging samples. A systematic study of all available variants of egg-white and serum proteins of the domestic fowl involved exchanges of samples between workers in Czechoslovakia and France and the present authors (then in the U.K.); all independently discovered variants were confirmed (Baker *et al.* 1970). However, many of the egg-white variants resolve particularly well on electrophoresis and it remains to be determined how much heterozygosity studies are influenced by technique.

At least for these sheep enzyme polymorphisms, variation detected in one electrophoretic system was confirmed in other systems, providing the enzymes resolved reasonably well. However, there are a number of examples where proteins 'monomorphic' in one electrophoretic system are 'polymorphic' when a more appropriate system is used (Manwell and Baker 1970). It has also been shown that certain enzymes of low activity in sheep erythrocytes [enzymes scored as monomorphic (Manwell and Baker 1977*a*)] were indeed electrophoretically monomorphic when the same enzyme occurring at higher levels of activity in organ extracts is analysed.

Non-random Distribution of Enzyme Polymorphism

Inspection of the present data for sheep, summarized in Table 3, reveals a trend: for three of the seven loci coding for NADP-dependent dehydrogenases there is polymorphism with high heterozygosities (0·50, 0·35, and 0·34), whereas for 10 different loci coding for dehydrogenases primarily or completely dependent on NAD there is no electrophoretically detectable genetic variation. (The slightly variable glucose dehydrogenase of liver, kidney and milk of sheep is not included, for this enzyme uses either NAD or NADP readily.)

Table 2. Measures of protein polymorphism in some mammalian species

1% cut-off point is used for polymorphism *v.* monomorphism

Species	No. of loci (%)	Proportion of loci polymorphic (%)	Average heterozygosity per locus (%)
Man, caucasoid (Lewontin 1974, Table 22)	71	28	6·7
Mouse, *Mus musculus musculus* (Lewontin 1974, Table 22)	41	29	9·1
Mouse, *Mus musculus brevirostris* (Lewontin 1974, Table 22)	40	30	11·0
Mouse, *Mus musculus domesticus* (Lewontin 1974, Table 22)	41	20	6·5
Sheep, *Ovis aries*, organ enzymes and muscle proteins (this paper)	51	14	5·0
Sheep, *Ovis aries*, organ enzymes only (this paper)	36	19	7·1
Sheep, *Ovis aries*, Merino purebred, blood enzymes and other proteins (Manwell and Baker 1977*a*)	30	27	10·9
Sheep, *Ovis aries*, Poll Dorset purebred, blood enzymes and other proteins (Manwell and Baker 1977*a*)	30	20	7·3
Mole rat, *Spalax ehrenbergi* (Nevo and Shaw 1972)	17	19	3·7
Pocket gophers, *Thomomys talpoides*, complex (Nevo *et al.* 1974)	31	23	4·7
Southern elephant seal, *Mirounga leonina* (McDermid *et al.* 1972)	19	21	1·0

In terms of heterozygosity *per se* there is no question of significance: 61 heterozygotes out of 346 opportunities for the loci coding for NADP-dependent dehydrogenases *v.* 0 heterozygotes out of 520 opportunities for the NAD loci.

However, since the sheep studied show two extreme situations—a locus is either polymorphic with one or more alleles at high gene frequencies (0·2–0·5), or monomorphic with little or no genetic variation (gene frequency less than 0·05, essentially monomorphic)—a tougher test of these data is whether or not polymorphism *per se* occurs at random over the major enzyme groupings given in Table 3. This test can be done by treating Table 3 as a 2×9 contingency table, but the usual χ^2 two-tailed heterogeneity test is not suitable because of the small numbers per cell (Siegel 1956); instead, using an appropriate computer program the probability of such a distribution, or a more extreme one, i.e. R. A. Fisher's 'exact test', can be calculated

(Hancock 1975). The probability of such a concentration of polymorphism in two of the nine cells is 0·00216. Thus, were such polymorphisms randomly distributed, the probability of observing the polymorphism concentrated in the NADP-dependent dehydrogenases and in the esterases is approximately only 2 chances in 1000, which is highly statistically significant.

Table 3. Heterozygosity of 52 Merino and Merino crossbred sheep for organ enzymes and muscle protein

Protein category	No. of loci	Heterozygosities (%)
NAD-dependent dehydrogenases	10	all 0
NADP-dependent dehydrogenases	7	50, 35, 34, 2, 0, 0, 0 (average 17)
Glucose dehydrogenase (uses either NAD or NADP)	1	0
NADH diaphorases of heart	5	all 0
Regulatory enzymes of Embden–Meyerhoff pathway (hexokinase, 6-phosphofructokinase, and pyruvate kinase)	3	6, 0, 0 (average 2)
Other glycolytic enzymes (phosphoglucomutase I and II, glucosephosphate isomerase)	3	all 0
Leucine aminopeptidase	1	0
Esterases of liver	6	48, 44, 36, 0, 0, 0 (average 21)
Muscle proteins (including myoglobin)	15	all 0
Total average heterozygosity	51	5·0
Average heterozygosity for enzymes but not muscle proteins	36	7·1

Physiological Significance of this Non-random Distribution?

It has been suggested on the basis of comparisons of genetic variation of birds differing in *r* and *K* selection (Manwell and Baker 1975), that where the emphasis is on high rate of growth and on detoxication of a mixed diet, variation can be expected in the NADP-dependent dehydrogenases—for NADP is largely used in the reduced form as a source of reductive equivalents for growth and for detoxication, whereas NAD is the coenzyme of energy transfer from substrates, to eventually, ATP (Horecker 1969).

In comparison with closely related artiodactyls, the sheep has been '*r*-selected'. Whereas most wild ungulates have a single offspring per pregnancy, twins are quite common in a number of sheep breeds and a few breeds have even larger litters; ewes of domestic sheep also reach sexual maturity sooner, often breeding within 1 year of birth. Man has selected sheep for fast growth, either of carcass or of wool.

Two possible explanations can be suggested for the biological significance of the NADP-dependent dehydrogenases and their polymorphism.

Firstly, all three of these polymorphisms have hybrid zones in the heterozygote, allowing the possibility of positive heterosis by complementation at the level of quaternary structure; indeed, nearly all of the activity of the major 'malic enzyme' in heterozygotes is in the hybrid zones. Whether the hybrid molecules have a faster rate of action, or a different degree of feedback regulation, remains to be determined.

Secondly, polymorphism of enzymes involved in growth rate would be a reasonable adaptive strategy of '*r*-selected' generalists in a patchy environment, reducing *intra*-specific competition. Genetic load would be reduced if animals with different genotypes sought out different niches optimally suited to their genotype, i.e. if sheep growing more slowly grazed the more marginal parts of a paddock. It is known that

dominance hierarchies in mammals often result in the larger individuals obtaining the better quality food, the smaller individuals being relegated to more marginal food and territory (Wilson 1975). Individuals growing more slowly often survive better during periods of scarcity. The evolutionary strategy is often to preserve this variability in growth rate and this would explain why, contrary to previous theorizing, dominant males do not always achieve greater reproductive success than males at the bottom of the hierarchy (see review by Kolata 1976).

The greater incidence of polymorphism among esterases may be related to that for NADP-dependent dehydrogenases in that both enzymes play roles in detoxication. NADP-dependent dehydrogenases supply the NADPH needed for the 'microsomal' P-450 enzyme system which detoxifies many aromatic compounds. We know less about the precise roles of the multiplicity of esterases, for the localization method after electrophoresis is based on hydrolysis of naphthyl esters, which are only occasionally natural compounds. Evidence from studies on pesticide resistance in insects suggests that some of these active, broad substrate specificity esterases detected after electrophoresis play important roles in detoxication; the serum H-esterase of sheep was originally discovered when some sheep died after being given an anti-helminthic judged to be 'safe' (see review by Manwell and Baker 1970). Sheep that have a serum esterase active on α-naphthyl acetate are able to detoxify the anti-helminthic, whereas those sheep with a serum esterase that cannot hydrolyse this ester are also unable to hydrolyse the poison.

Detoxication is especially important to sheep husbandry, for man has frequently taken sheep into new marginal environments where some plants have evolved toxic compounds as counter-measures to grazing.

Our results now allow the formulation of some testable hypotheses relating protein polymorphisms and animal production—hypotheses which rest on better biochemical rationale than the previous random searches for correlations. Do sheep which are heterozygous for NADP-dependent dehydrogenase polymorphisms grow more quickly? Have they better capacities for detoxication? If esterase polymorphism is related to differential tolerance to toxic compounds, then where sheep are dying from eating toxic plants, differential survival of esterase genotypes should be observed and should be able to be correlated with the poisons in the diet.

Note Added in Proof

Mr Tom Mann, Roseworthy Agricultural College, has supplied growth rate data for 27 crossbred sheep which we have typed for all of the known protein polymorphisms. The one statistically significant correlation (5% level) is that heterozygotes for $NADP^+$ dependent dehydrogenases grow on average 10·4% faster than homozygotes for these enzymes, accounting for 14·6% of all variation in growth rate. Obviously, crossbred animals are heterozygous for many loci, but the preliminary results are sufficiently important to warrant further study, with larger numbers of individuals, on different planes of nutrition, and on populations with different breeding structures.

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