Biochemical polymorphism and the 2,3-diphosphoglycerate in the sheep red blood cells

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Summary. There was no significant difference in the level of 2, 3-DPG in the red blood cells of sheep of different haemoglobin types (Hb A and Hb B) or potassium types (HK and LK). However, low glutathione (GSH^L) sheep had significantly higher ($p < 0.01$) level of 2, 3-DPG in their red blood cells than high glutathione (GSH^H) sheep. There was also significant effect of interactions between glutathione, haemoglobin and potassium types ($p < 0.05$) and glutathione and haemoglobin types ($p < 0.01$) on red cell 2, 3-DPG levels.

It is now well known that red cell 2, 3-diphosphoglycerate (2, 3-DPG) moderates haemoglobin function in man and many other mammalian species[1-4]. In sheep, however it has been assumed that 2, 3-DPG plays a negligible role in oxygen transport; firstly, because the level of 2, 3-DPG in the sheep red cell is very low (< 1.0 mM/l) and secondly, because there is relatively little interaction between sheep haemoglobins and 2, 3-DPG. This latter difference has been explained on the basis of the primary structure of the sheep haemoglobin molecule, in which there is a deletion of one amino-acid residue at the N-terminal end of the β-chain; this deletion results in an increased intra-molecular distance which prevents 2, 3-DPG from forming the link that stabilizes the deoxy conformation[5]. The role of 2, 3-DPG in oxygen transport in the sheep has, however, been brought once again into question by Bunn et al.[6] who have reported quite substantial alterations in p50 values when sheep haemoglobins are exposed to high concentrations of 2, 3-DPG in vitro. In addition we have recently observed a 6- to 8fold rise in 2, 3-DPG in the red blood cells of anaemic sheep[7]. These results have led us to a re-examination of the relationship between 2, 3-DPG and haemoglobin function in the red blood cell of sheep. 3 genetically determined biochemical polymorphisms are known in the sheep red blood cell. They are high (HK) and low (LK) potassium types[8], haemoglobin (Hb) types A, B and AB[9], and high (GSH^H) and low (GSH^L) glutathione types[10]. Haemoglobin types and potassium types are known to influence oxygen transport in the sheep red blood cell[11,12] and an effect of GSH on the oxyhaemoglobin dissociation curve has also been shown[13]. We have now investigated the interrelationships between the 2, 3-DPG and these 3 polymorphic characters of the sheep red blood cell.

The experiment was carried out in 2 parts. Firstly, blood was obtained from 67 adult Merino ewes randomly selected from a flock maintained by the Department of Physiology, University of New England. This flock has been selected over a period of about 10 years to provide animals of all genotypes and, as such, may not be a true representative of the Australian Merino sheep. Red cell levels of 2, 3-DPG were estimated using an enzymic method[14].

The distribution of animals according to their phenotypes and the corresponding values for red blood cell 2, 3-DPG are shown in the table. When group means for phenotypes within characters are compared (t-test), it is apparent that GSH^H sheep have significantly lower red blood cell 2, 3-DPG than do GSH^L sheep ($p < 0.01$). No significant differences were found between potassium types or haemoglobin types.

A more detailed analysis of this data, designed to show the effects of interactions between the main polymorphic types was not attempted due to the large differences apparent in sub-class numbers. Instead, a second experiment was undertaken in which 24 ewes were selected from the same source so as to provide a group balanced in numbers for all phenotypes. The red blood cell 2, 3-DPG values obtained from this group again showed significant differences between mean values for phenotypes within characters (t-test). GSH^H animals again had lower values than GSH^L ($p < 0.05$) while in addition LK animals had lower values than HK ($p < 0.01$).

An analysis of variance performed on the data reinforced the above results and in addition showed the significant effect of interactions between GSH, haemoglobin and potassium types ($p < 0.05$) and GSH and haemoglobin types ($p < 0.01$) on red cell 2, 3-DPG levels.

Mean red cell 2, 3-DPG levels (nM/g Hb) in sheep of different polymorphic types

Character	No.	Mean ± S. E. M.	P (t-test)
Hb A	32	137 ± 15	NS
Hb B	35	142 ± 16	
HK	27	160 ± 17	NS
LK	39	125 ± 15	
GSH^H	43	116 ± 12	<0.01
GSH^L	22	187 ± 21	

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The biological significance of these results is not readily apparent. In man, addition of GSH in increasing amounts to either whole blood or haemolysates results in a progressive decrease in the oxygen affinity of the haemoglobin[13], thus producing an effect similar to that produced by 2,3-DPG. Whether this same mechanism exists in sheep is not known. However, since the difference in GSH concentration between GSH^H and GSH^L sheep may be 3- or 4fold, it is possible that the higher levels of 2,3-DPG observed in GSH^L animals are compensating in some way, as yet unknown, for the effect of these low GSH levels, in maintaining the position of the oxygen dissociation curve. The data suggests a negative correlation between GSH and 2,3-DPG and in fact overall $r = -0.44$ ($p < 0.05$). However, within GSH types this relationship is not constant, $r = -0.47$ ($p < 0.05$) within GSH^H and 0.09 (NS) in GSH^L animals.

Tosteson[15] reported an increased activity of sodium-potassium activated adenosine triphosphatase (S-ATPase) in red blood cell membranes from HK sheep. Eaton et al.[16] showed that HK red blood cells had, on average, 31% higher levels of ATP than did LK red blood cells and commented that, with an increased level of S-ATPase activity, HK red cells would be expected to have less ATP than LK cells because of the increased utilization of ATP for the electrolyte pump. Our finding that HK red blood cells have higher levels of yet another important organic phosphate compound raises the possibility that 2,3-DPG might also, directly or indirectly, be associated with ion transport in the sheep red blood cell. This suggestion is strengthened by the observations of Gardos[17] and Parker[18] who have suggested that in man, 2,3-DPG plays a role in potassium transport, possibly by way of 2,3-diphosphoglycerate-phosphatase, the enzyme that breaks down 2,3-DPG. Further investigation into the role of 2,3-DPG in the sheep red blood cell should be of significance.

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A simplified assay for cyclic AMP using protein kinase binding[1]

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Summary. The protein kinase binding assay for cAMP was modified by substitution of adsorption by QAE cellulose for the membrane filtration. This modification obviates the variation of recovery of cAMP with the volume of buffer used to wash the filter. The assay is reproducible and technically simpler than those currently employed.

Introduction. One of the most sensitive methods for the determination of the concentration of adenosine 3′,5′ cyclic monophosphate (cAMP) employs cAMP-dependent protein kinase binding[2–4]. Of all these methods, the method described by Gilman[2] is perhaps the most popular. However, the use of cellulose acetate membrane filters in this method to separate cAMP bound to protein kinase from the free nucleotide presents several problems. Membrane filtration is time consuming, and, more importantly, we found that the recovery of bound cAMP varied with the volume of phosphate buffer used for washing the samples.

Materials and methods. 2 assay systems for the determination of cAMP by the protein kinase binding method were employed. The first was identical to that described by Gilman[2]. Typically, different amounts of cAMP were incubated with 4 µg of cAMP dependent protein kinase (Sigma Chemical Co., St. Louis, Mo.); 28 µg of protein kinase inhibitor (Sigma Chemical Co., St. Louis, Mo.); 2 pmoles of (^{3}H)-cAMP with a specific activity of 27.5 Ci/mmole (New England Nuclear, Boston, Mass.) and 50 mM sodium acetate buffer, pH 4.0 in a total volume of 0.1 ml. The assay mixtures were incubated at 0 °C for 60 min. In the original method[2] millipore filters were used to separate free from bound nucleotide. This step was performed exactly as described, but the volume of sodium phosphate buffer used to wash the membrane was varied. In the present method a 5% (dry wt/vol) QAE cellulose (N,N-diethyl-N-2-hydroxypropylamino cellulose; exchange capacity, 0.55–0.52 meq/g; Biorad, Richmond, Calif.) suspension in water was added to the assay mixture to separate free cAMP from cAMP bound to protein kinase.

Since QAE cellulose is a strongly basic ion-exchanger, it can adsorb various anions including cAMP. However, neither protein kinase nor cAMP bound to protein kinase is adsorbed by the cellulose and this property of the exchanger has been exploited to separate free cAMP from bound cAMP. Before use, the QAE cellulose was soaked in water at least for 24 h at room temperature. The cellulose suspension (2 ml) was thoroughly mixed with the

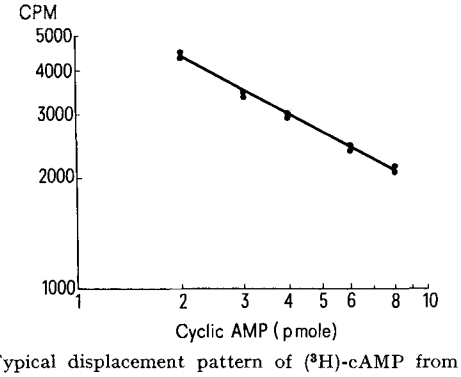

Fig. 1. Typical displacement pattern of (^{3}H)-cAMP from protein kinase by increasing concentration of unlabelled cAMP. Free cAMP was separated from protein kinase bound cAMP by QAE cellulose suspension as described in the text.

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