A POLYMERIC FORM OF HAEMOGLOBIN-BINDING PROTEIN IN SHEEP FOLLOWING METABOLIC AND HORMONAL DISTURBANCE

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Abstract

The serum proteins of sheep have been examined by concave gradient polyacrylamide gel electrophoresis. It would appear that sheep are usually lacking in haemoglobin-binding proteins (haptoglobins).

However, tissue damage, cortisone, and ACTH administration lead to the appearance in the serum of a polymeric series of haemoglobin-binding proteins resembling haptoglobins. Either no response or a minimal response is observed in adrenalectomized sheep.

The appearance of these proteins after treatment of diabetic sheep with insulin and after re-feeding fasted sheep suggests that a metabolic as well as a hormonal component may be associated with their production.

Provided an appropriate stimulus is given it is apparent that sheep have the genetic potential capable of producing a polymeric series of high molecular weight haemoglobin-binding proteins.

I. INTRODUCTION

In the plasma of most mammals there is normally present a glycoprotein which forms a stable complex with haemoglobin. This protein, referred to as haptoglobin (Laurell and Grönvall 1962), shows polymorphism in humans and a series of haptoglobins of varying molecular weight can be detected (Smithies 1955). Genetic control of the haptoglobin types by a pair of alleles Hp^1 and Hp^2 has been established in humans (Smithies and Walker 1956). A simple haptoglobin component can be shown by electrophoresis for homozygous type Hp^1–Hp^1 whereas type Hp^2–Hp^2 shows a polymeric series of haptoglobins.

In most mammalian species, however, haptoglobin is only present in a monomeric form similar when characterized electrophoretically to human type Hp^1–Hp^1. Studies made on the serum proteins of ruminants indicate that it is difficult to detect any haptoglobin in normal cattle (Bremner 1964), or in goats (Travis, Brown, and Saunders 1970). However, Blakeslee and Stone (1971) have detected haptoglobin in cattle serum by immunochemical means.

During a study of metabolic disturbances in pancreatic insufficiency and diabetes in sheep some aspects of protein metabolism were investigated which led to the examination of serum proteins in sheep by electrophoretic procedures (Jarrett and Robinson 1970). We have now shown that serum haptoglobin cannot normally be detected. On the other hand animals under certain forms of stress show polymeric series of high molecular weight haemoglobin-binding proteins in relatively high concentration.

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II. Materials and Methods

(a) *Experimental Animals*

Adult Merino ewes and wethers 3–5 years of age were used for these studies. They were fed a diet of 750 g wheaten hay chaff and 250 g lucerne chaff per day. Eight sheep were made diabetic by the intravenous injection of alloxan (60 mg/kg) as a freshly prepared solution in 10 ml 0·9% NaCl solution. After 5–7 days (by which time the animals were severely diabetic) a continuous intravenous infusion of insulin was given at the rate of 5–10 units in 20 ml water per day.

Six sheep were fasted for periods of 3–8 days and then re-fed their normal daily ration. Five sheep received a subcutaneous injection of oil of turpentine (2·5 ml/day for 2 days). Four sheep received an intramuscular injection of 250 mg cortisone acetate (Roussell, London) followed by 125 mg the next day. After an interval of several weeks, three of these animals were given an intramuscular injection of 32 mg dexamethasone phosphate (Merck, Sharp, and Dohme Australia Pty. Ltd., New South Wales) followed by 16 mg the next day. Four sheep received an intramuscular injection of 200 i.u. corticotrophin zinc hydroxide (Organon Laboratories, Surrey, England) on 2 consecutive days.

Two sheep were bilaterally adrenalectomized and maintained in good condition for several weeks by daily intramuscular injections of 25 mg cortisone and 5 mg deoxycortisone acetate (Ciba Co. Ltd., Basle, Switzerland). These sheep were treated sequentially with injections of corticotrophin, oil of turpentine, and cortisone at the same dose rate as for the normal animals. Sheep were left for 2–3 weeks between the different treatments.

(b) *Analysis of Serum Proteins*

Sera were obtained from all animals prior to treatment and at various stages of treatment. Serum proteins were analysed on a concave gradient slab of 4–26% polyacrylamide gel by the technique of Margolis and Kenrick (1968) using a Gradipore apparatus (Townson and Mercer Pty. Ltd., Sydney).

Proteins were detected by staining the gel with amido black (Fitschen 1964). In most cases duplicate gels were run and stained with a benzidine–peroxide reagent (Ferris, Easterling, and Budd 1963) to detect haemoglobin-binding proteins.

Gel filtration of serum was performed on a Sephadex G200 column 50 by 6 cm to help characterize the various protein fractions and obtain some indication of molecular size. Sheep serum (5 ml) was mixed with 6 mg sheep haemoglobin and applied to the column. Chromatography was effected at 4°C with 2% NaCl solution and a flow rate of 15 ml/hr. The eluate was collected in 5-ml fractions (Ratcliff and Hardwicke 1964). Total protein and haemoglobin-binding protein were determined spectrophotometrically at 280 and 415 nm respectively. The presence of haemoglobin-binding protein in the various fractions was also determined by electrophoresis after concentration of the eluate fractions by freeze-drying.

Sheep haemoglobin was prepared by the method of Dacie and Lewis (1968), freeze-dried, and reconstituted to 100 mg per 1 ml of 40% sucrose for electrophoresis.

Ammonium sulphate fractionation was performed on the serum of a sheep 3 days after it had received a subcutaneous injection of turpentine. The precipitates obtained from the various fractionations between 10% and 70% saturation of ammonium sulphate were dissolved in 0·9% NaCl. The protein concentration was determined on the fractions by absorbance at 280 nm and the fractions examined electrophoretically for haptoglobin.

Single radial immunodiffusion was performed on sheep serum in which haptoglobins were known to be present and on human serum containing type Hp^2–Hp^2 haptoglobin. Human *Hp* antiserum agar-gel immunodiffusion plates (Behringwerke Partigen^R) and standardized human serum (Behringwerke) were used.

III. Results

The electrophoretic pattern of serum proteins of a normal sheep is shown in Figure 1*A*, of the same sheep when diabetic in Figures 1*B* and 1*C*, and at 2, 3, and 4 days after starting an infusion of insulin (5 units/day) in Figures 1*D*, 1*E*, and 1*F*.

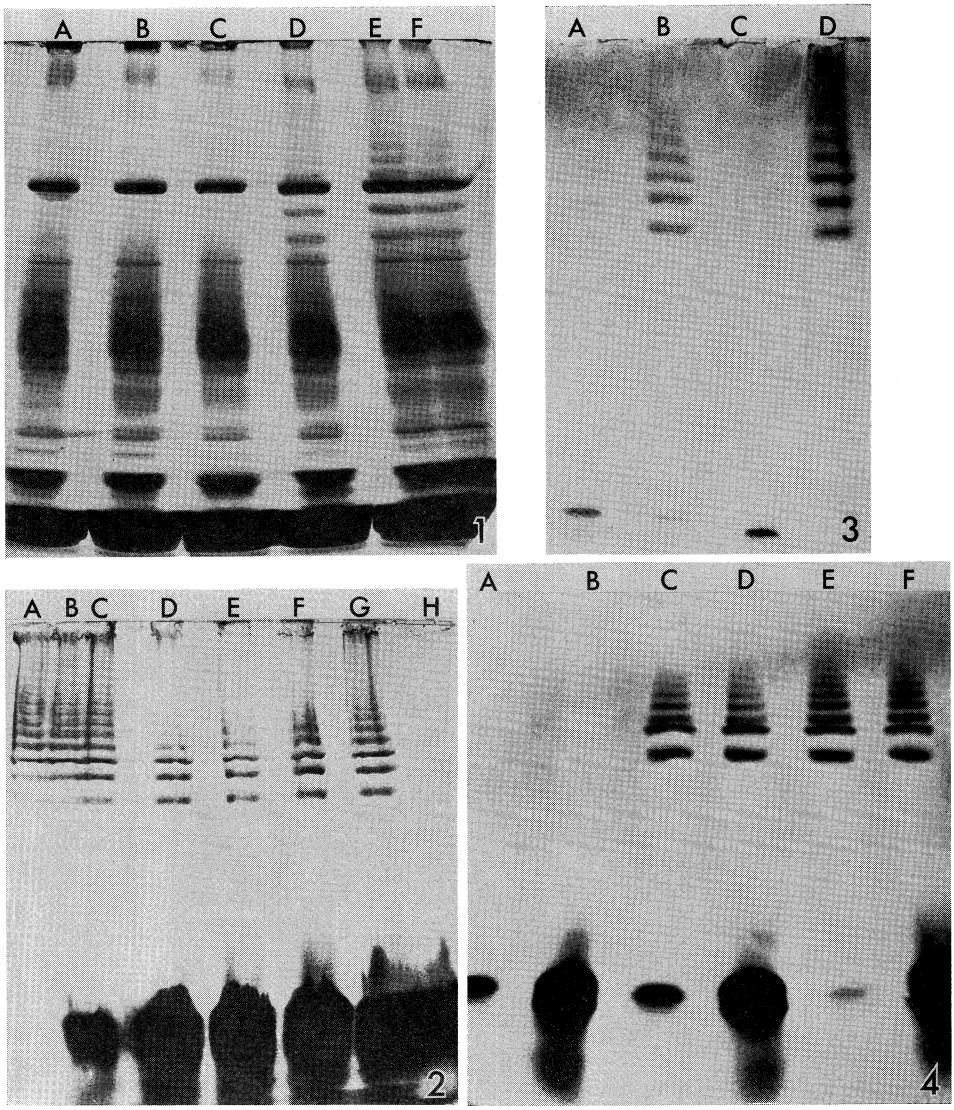

Fig. 1.—Electrophoretic pattern of serum in proteins of normal sheep (*A*) and of the same sheep when diabetic (*B* and *C*) and after insulin (*D*, *E*, and *F*). 7 μl of each serum applied to polyacrylamide gel. Proteins stained with amido black.

Fig. 2.—Electrophoretic separation of serum proteins of insulin-treated diabetic sheep stained with benzidine–peroxide reagent to show haemoglobin-binding capacity. *A* and *B*, fresh serum only; *C–G*, with 0·8, 4, 8, 12, and 16 mg sheep haemoglobin added; *H*, 8 mg sheep haemoglobin only.

Fig. 3.—Electrophoresis of serum of a sheep fasted for 5 days (*A*), the same sheep re-fed for 24 hr (*B*), a normal sheep (*C*), and the same sheep 24 hr after surgery (*D*). Proteins stained with benzidine–peroxide reagent.

Fig. 4.—Electrophoresis of serum of a normal sheep (*A*) and with added haemoglobin (*B*), of the same sheep 2 days after oil of turpentine injection (*C*) and with added haemoglobin (*D*), and 4 days after oil of turpentine injection (*E*) and with added haemoglobin (*F*). Proteins stained with benzidine–peroxide reagent.

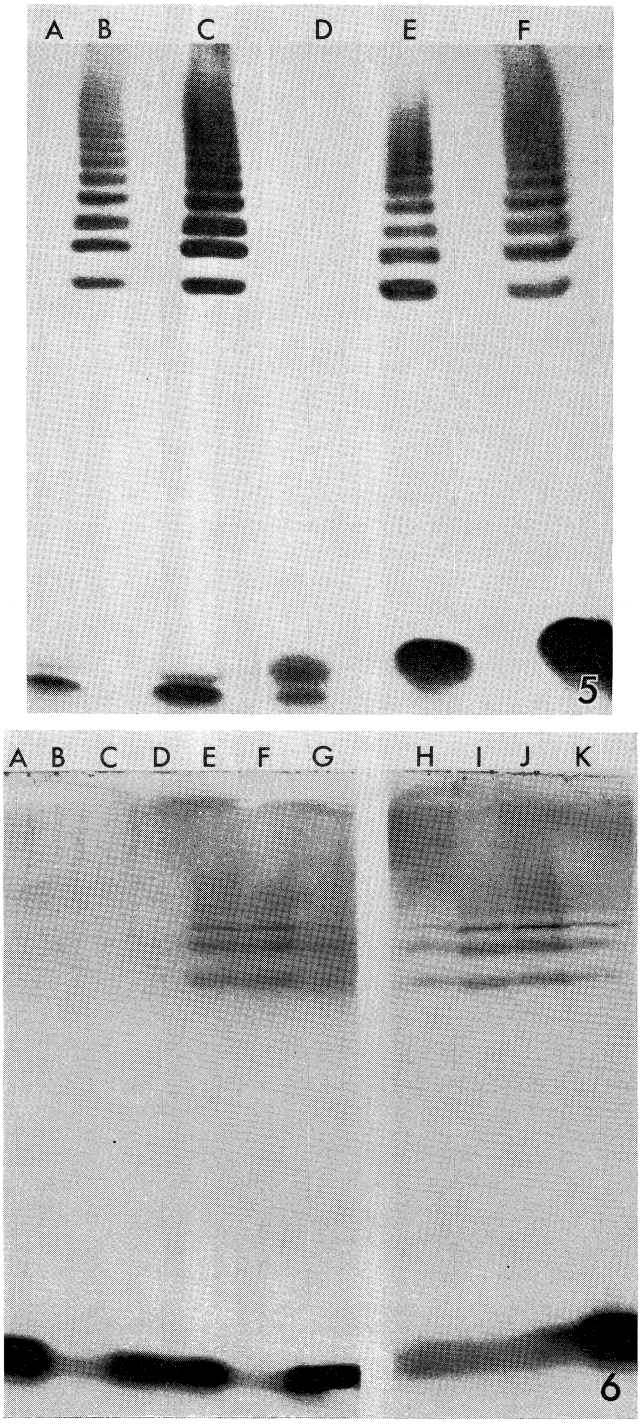

Fig. 5.—Electrophoresis of serum of a normal animal (*A* and *D*), 1 and 2 days after cortisone injection (*B* and *C* respectively), 2 and 3 days after ACTH injection (*E* and *F* respectively). Proteins stained with benzidine–peroxide reagent.

Only minor differences could be detected in animals severely diabetic. However, in all diabetic animals within 48 hr of starting insulin infusions, and occasionally within 24 hr, a polymeric series of proteins of high molecular weight appeared in the region of the α_2 macroglobulins.

These proteins stained with benzedine–peroxide reagent thus indicating a haemoglobin-binding capacity characteristic of haptoglobin. Figure 2 shows these bands from the serum of an insulin-treated diabetic sheep. Separate applications were made of 7·5 μl of freshly prepared serum as such (columns *A*, *B*) and with 0·8, 4, 8, 12, and 16 mg of haemoglobin added respectively (columns *C–G*) and in column *H* 8 mg of haemoglobin in 7·5 μl 40% sucrose was applied to the gel. It is apparent that no high molecular weight haptoglobins were present in sheep haemoglobin (Fig. 2*H*), no haptoglobin of low molecular weight in the freshly prepared serum sample (Figs. 2*A*, 2*B*), and that the addition of varying amounts of haemoglobin to the serum made no difference to the pattern of haptoglobins.

The intravenous cannulation and infusion procedures could not be held responsible for the appearance of these proteins as three sheep similarly cannulated and infused with 20 ml water per day for 5 days showed no such response. Nor did insulin infusion at the rate of 5 and 20 units per day to a normal sheep lead to the appearance of haemoglobin-binding proteins.

Fasting sheep for periods of 3–8 days did not lead to the production of haptoglobins (Fig. 3*A*). However, within 24 hr of re-feeding the fasted sheep haptoglobins were present in the sera (Fig. 3*B*). Figure 3 also shows lack of haptoglobins in a normal sheep (column *C*) and the appearance of haptoglobins 24 hr after surgery (column *D*). The injection of oil of turpentine led to the appearance of intensely staining bands of haptoglobins. Figure 4 shows that haptoglobins are present in sera from animals 2 and 4 days after turpentine injection.

Haptoglobins are present in the serum of a sheep receiving cortisone (Fig. 5: *A*, normal; *B*, 1 day after injection; *C*, 2 days after injection) and also after injection of ACTH (Fig. 5: *D*, normal; *E*, 2 days after injection; *F*, 3 days after injection). The response to cortisone was apparent within 24 hr in all animals treated and within 48 hr after ACTH administration.

After adrenalectomy haptoglobins were present during a post-operative period of about 4 days and thereafter when the animal was maintained in good condition on cortisone and deoxycortisone acetate no haptoglobins were detected (Figs. 6*A*, 6*B*). The administration of ACTH at this stage did not lead to haptoglobin production (Figs. 6*C*, 6*D*). Both turpentine and cortisone injections, treatments which led to a marked response in normal animals, only gave a minimal response in the adrenalectomized sheep (Fig. 6: *E*, *F*, *G*, and *H–K*, respectively).

When serum with added haemoglobin from a normal sheep was run on a Sephadex G200 column, only the double peaks characteristic of the dimeric form of sheep haemoglobin could be detected (by reading the absorbance of the eluates at 415 nm). However, serum from the turpentine-treated animal showed both the

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Fig. 6.—Electrophoresis of serum of an adrenalectomized sheep maintained in good condition on cortisone and deoxycortisone acetate injections (*A* and *B*), 2 and 3 days after ACTH injections (*C* and *D*), 1, 2, and 3 days after oil of turpentine injections (*E–G*), and 1–4 days after 250 mg cortisone per day for 2 days (*H–K*). Proteins stained with benzidine–peroxide.

haemoglobin peaks at an eluate volume of 270 and 310 ml and an earlier peak at eluate volume of 100 ml (Fig. 7). The eluate corresponding to this peak (after concentration and electrophoretic examination) showed the polymeric series of haemoglobin-binding proteins characteristic of haptoglobins seen in the sera of the treated animals.

Subsequent electrophoretic examination of fractions of ammonium sulphate precipitation of normal sheep serum did not show any detectable haptoglobins. Similar treatment of serum from a turpentine-injected animal did reveal haemoglobin-binding proteins in fractions of 50–60% saturation of ammonium sulphate.

Immunoprecipitin rings were detected for human serum and standardized human serum when tested on human haptoglobin antiserum agar-gel immunodiffusion plates. However, no indication of any reaction could be obtained with either normal sheep serum or with serum from sheep treated with turpentine and known to have haptoglobin polymers present.

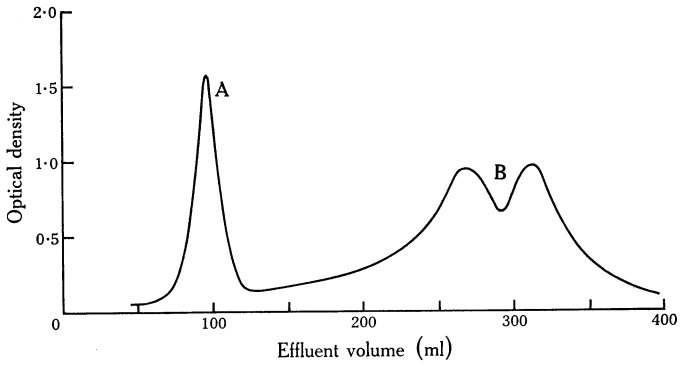

Fig. 7.—Separation of haemoglobin and haemoglobin-binding proteins in serum from a turpentine-treated sheep. Chromatography of serum proteins on a Sephadex G200 column was carried out using 2% NaCl as the eluting buffer, and the optical density of each 10 ml fraction was measured at 415 nm. The void volume of the column was approximately 90 ml. *A*, haptoglobin–haemoglobin complex; *B*, haemoglobin.

IV. Discussion

It would appear that lack of detectable serum haemoglobin-binding proteins may be a normal feature of ruminants. Fifty sheep of varying ages, sex, and breed have been examined in this current study and no evidence of a haemoglobin-binding protein has been found in sera from untreated animals. Goodger (1970) reported the presence of a polymeric haemoglobin-binding protein in calves following tissue damage, but was not able to detect the polymeric protein in normal calves. Travis, Brown, and Sanders (1970) claim that goats are usually ahaptoglobinaemic. However, after γ-irradiation a polymeric series of haemoglobin-binding proteins similar to human haptoglobin Hp^2–Hp^2 can be readily detected in serum.

Spooner (1969) reported the production of auto-antibodies to haemoglobin in cattle infected with haemolytic bacteria and discussed their possible role in relation to the presence and clearing of haemoglobin from the blood. However, he later

(1970) showed that in both cattle and sheep the haemoglobin-reactive protein, produced as a result of an infection with haemolytic bacteria, is not an immunoglobulin. By studying the auto-reactivity to haemoglobin he was able to show that while the haemoglobin-reactive protein has many similarities to haptoglobin, it differs from human haptoglobin in size, specificity, and ability to precipitate with haemoglobin.

Apparently cattle, goats, and sheep respond in a similar manner to tissue damage with the production of a polymeric series of high molecular weight haemoglobin-binding proteins. However, the studies reported here indicate that factors other than tissue damage *per se* must be involved. Marked synthesis occurs after a metabolic disturbance such as re-feeding a fasted animal, or following the early effects of insulin administration to a diabetic sheep. In both of these situations effects associated with altered intermediary metabolism may be important. For example synthesis of these polymers may be part of a more generalized protein synthesis taking part in the liver and other sites.

Hormonal interrelationships, however, should not be overlooked and it is possible that the adrenal glands are involved in many of the stress situations which apparently lead to haemoglobin-binding protein synthesis in ruminants. Certainly both adrenal corticosteroids and ACTH act as powerful stimuli to the production of these proteins in sheep. Moreover the failure of ACTH to give a response in the adrenalectomized animal and the low level of response in these animals following tissue damage would appear to indicate some role for the adrenal steroids. Krauss (1963) reported a considerably reduced response in the level of circulating haptoglobins in adrenalectomized rats after a subcutaneous injection of turpentine. He considered that the adrenals play a permissive role as the response to tissue injury was not completely abolished in adrenalectomized animals.

The results derived from gel filtration and polyacrylamide gel analysis indicate that the polymeric series of proteins which appear after stress have a molecular weight greater than 200,000. This value agrees with that reported for calves by Goodger (1970) and for goats by Travis, Brown, and Sanders (1970). All these authors have commented on the similarity between these haemoglobin-binding proteins in ruminants and human Hp^2–Hp^2 haptoglobins. This current study with sheep showed no cross reactivity between sheep serum and anti-human haptoglobin serum indicating a marked dissimilarity between the haemoglobin-binding proteins in the two species. Similarly, Travis, Brown, and Sanders (1970) have reported that goat serum haptoglobins do not cross react with Hp^2–Hp^2 haptoglobin antisera from humans.

While Spooner (1969, 1970) refers to these proteins when present in ruminants as haemoglobin-reactive protein, Blakeslee and Stone (1971) refer to them as haptoglobin and reported that cattle sera can be assayed for the presence of haptoglobin using specific precipitating rabbit antiserum. They also found that haptoglobin is usually undetectable in the sera of normal healthy cattle. Its appearance can follow the stress of an infection or an inflammation.

In most animal species and in man ahaptoglobinaemia is usually secondary to increased release of haemoglobin into the circulation and subsequent complexing of haptoglobin with haemoglobin. It does not appear to be due to a congenital

lack of haptoglobin (Allison and Rees 1957). There would appear to be no evidence that the ahaptoglobinaemia in normal cattle, goats, and sheep is associated with a haemolytic crisis.

It is apparent that these animals have the genetic potential to produce a series of high molecular weight haemoglobin-binding proteins if given an appropriate stimulus. Thus, as indicated by Blakeslee and Stone (1971) care should be taken not to conclude that a new polymorphism exists in the serum proteins of ruminants.

As Spooner and Miller (1971) could only find haemoglobin reactive proteins in 0·6% of supposedly normal cattle and their presence was detected in a wide range of sick animals, they have suggested that the measurement of haemoglobin-reactive proteins could be used as an aid to the diagnosis of acute inflammation. However, from these current studies with sheep it would appear that the presence of high molecular weight haemoglobin-binding proteins is likely to be associated with a stress situation in which metabolic and hormonal factors may be involved.

V. Acknowledgments

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