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PROTEINS OF THE CAMEL ERYTHROCYTE MEMBRANE

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SUMMARY

Electrophoresis on polyacrylamide gels containing dodecyl sulphate has revealed that the major proteins of the camel erythrocyte membrane are similar to those of the human and bovine species in both electrophoretic mobility and relative abundance. The major difference lies in the major intrinsic membrane protein of molecular weight approx. 100 000. In the camel, this protein has a higher apparent molecular weight than in the human and bovine species.

The very high molecular weight water-soluble protein "spectrin" appears to be very tightly bound to the camel erythrocyte membrane, and is only partially extracted after prolonged low ionic strength dialysis. Total release of spectrin is only achieved by means of more drastic treatment such as incubation with urea, guanidine hydrochloride, sodium hydroxide or *p*-chloromercuribenzoate. Concurrent with the total release of spectrin, the camel cells undergo a shape change from flat ellipsoids to spheres, suggesting an important shape-maintaining role for spectrin in the erythrocytes of this species.

INTRODUCTION

The camel differs from most other mammalian species in the possession of erythrocytes which are not biconcave discs but flattened ellipsoids [1]. In addition, the camel erythrocytes show a remarkable resistance to osmotic lysis [2]. Recently published analyses [2] showed that the lipid composition of the camel erythrocyte membrane is similar to that of other species, but that the protein/lipid ratio is significantly higher in the camel. These authors suggested that the resistance to osmotic lysis may be partially attributed to the increased protein content of the camel membranes, but no analyses of individual proteins were reported.

Recent studies on human red cells have shown that their shape, deformability and resistance to lysis are related closely to the level of ATP and the concentration of calcium ions in the cell [4]. In addition, a water-soluble, high molecular weight protein termed "spectrin", believed to form a fibrous coating on the inner, cytoplasmic surface

Abbreviations: DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); PCMB, *p*-chloromercuribenzoate.

of the membrane [3], has been implicated in maintaining the shape and integrity of the cell [3]. Some workers have postulated an actomyosin-like protein complex to account for the maintenance of the biconcave shape of the erythrocyte [5], but so far firm evidence is still lacking.

The present report describes a comparative study of the membrane proteins of the camel, human and bovine erythrocytes in terms of (a) the protein composition of the membrane, (b) the selective extraction of proteins from the membrane and (c) the effects of metabolic depletion and incubation with various perturbants on the shape and integrity of the membrane.

MATERIALS AND METHODS

Blood samples. Camel blood, from camel "Christine", was kindly supplied by Professor V. Macfarlane of the Waite Agricultural Institute, South Australia. Out dated human packed cells were supplied by the Red Cross Blood Bank, Sydney, and bovine blood was obtained from the Homebush Abbatoirs, Sydney. Citrate anti-coagulant was used in all cases.

Chemicals. *N*-Ethylmaleimide, *p*-chloromercuribenzoate (PCMB), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) were supplied by Calbiochem. Iodoacetamide, Triton X-100 and Specially Pure grade sodium dodecyl sulphate were all obtained from BDH. Iodoacetic acid was obtained from Merck, Vinblastine sulphate from Eli Lilly and colchicine from Sigma. 2-Chloromercuri-4,6-dinitrophenol was prepared by the method of McMurray and Trentham [6]. All other chemicals were of analytical reagent grade. Deionized water was distilled from an all pyrex glass still before use.

Preparation of erythrocyte membranes. Freshly drawn camel blood was transported in ice to the laboratory within 24 h of collection. The erythrocytes were washed free of plasma and buffy layer by means of several washes with cold 0.95 % NaCl containing 5 mM sodium phosphate, pH 7.5, followed by centrifugation at 1200 × g for 10 min at 4 °C. A portion of the washed cells was held in the wash solution for several weeks at 2 °C for metabolic depletion studies. The remainder of the cells were hemolyzed in cold 5 mM sodium phosphate, pH 8.0 [7], the membranes collected by centrifugation at 35 000 × g for 30 min at 4 °C, and washed in a similar manner in the same buffer until free of hemoglobin. The washed membranes were stored at 2–4 °C in the 5 mM phosphate buffer, pH 8.0. For extended periods of storage, 0.02 % sodium azide was added to the storage buffer in order to inhibit microbial growth.

The preparation of human and bovine erythrocyte membranes followed an identical procedure.

Extraction of spectrin. The washed membranes were suspended in an equal volume of 0.2 mM EDTA, pH 7.5, and dialyzed for 24 h at 2 °C against two lots of 0.2 mM EDTA. The membranes were then sedimented at 35 000 × g for 20 min in the SS34 rotor of a Sorvall RC2-B refrigerated centrifuge at 4 °C, and the supernatant withdrawn for protein estimation and electrophoretic analysis. The pelleted membranes were resuspended in 0.2 mM EDTA and the extraction process repeated twice more. After the third extraction, the membrane fragments were dialyzed against distilled water for two days at 2–4 °C.

Extraction was also carried out in 0.1 mM EDTA at 37 °C for one h, essentially as described by Fairbanks et al. [7].

Incubation with protein perturbants. The effect of various protein perturbants [8, 14] on the extractability of the camel erythrocyte membrane proteins was examined by mixing five volumes of the appropriate cold perturbant solution, at pH 8.0 where appropriate, with one volume of the ghost suspension, containing about 0.5 mg/ml protein in 5 mM phosphate buffer, pH 8.0. The mixtures were held in an ice bath for 30 min, after which time the membranes were sedimented at 35 000 × g for 20 min and the supernatants and residues retained for analysis. Portions of each mixture were examined by means of phase contrast microscopy at various times during the incubation period for evidence of structural changes in the membrane.

Protein estimation. Protein content of extracts was estimated by means of the method of Lowry et al. [9], with bovine serum albumin as a standard. For the analysis of whole membranes and membrane residues, 1 % sodium dodecyl sulphate was used to solubilize the suspensions before the addition of the alkaline carbonate reagent. In these cases, 1 % sodium dodecyl sulphate was also added to the standard serum albumin solutions. The presence of the detergent lowered slightly the absorbance of the blue colour produced, but did not affect the estimation of protein concentration in the soluble extracts, providing the appropriate standard control solutions were used.

Polyacrylamide gel electrophoresis. Samples of membrane suspensions and supernatant solutions were analyzed by means of electrophoresis in 5.6 % acrylamide gels containing 1 % sodium dodecyl sulphate, according to the method of Fairbanks et al. [7]. The protein samples were reduced with 1 % mercaptoethanol in 2 % sodium dodecyl sulphate, and bromophenol blue was used as a tracking dye.

Proteins were stained with Coomassie Blue and glycoproteins were located by means of the periodic acid-Schiff (PAS) stain, both as described by Fairbanks et al. [7]. Gels were photographed with Agfa Copex Rapid film, with illumination from a lightbox fitted with a yellow filter. Glycoprotein staining patterns were analyzed at 560 nm with the aid of a Gilson spectrophotometer, model 240, in conjunction with a linear transport attachment.

Analysis of the association state of spectrin. Samples of the extracted water-soluble protein were analyzed by means of gel filtration on Bio-Gel A-15 m (60 × 2 cm) in a buffer consisting of 0.1 M NaCl, 0.01 M sodium phosphate, 5 mM EDTA and 5 mM mercaptoethanol, pH 7.5, as described previously [10]. Supernatants from the protein extracts were also analyzed by means of electrophoresis on Gradipore acrylamide gradient gels described by Margolis and Kenrick [11].

Microscopic examination. Whole erythrocytes and ghosts were viewed with the aid of a Zeiss Photomicroscope, using phase contrast illumination. Photographs were taken with Kodak Recordak microfilm type 5460.

RESULTS

Whole cells

The camel erythrocytes appeared under the microscope as rather flattened ellipsoids, approximately 7 μm long, 4 μm wide, and between 1 and 2 μm thick. This shape was very stable, and was observed in whole blood, in washed erythrocytes and in washed erythrocytes after 4 weeks storage at 2–4 °C in the absence of added metabolizable substrates. No shape change was observed after incubation with 10^{-3} M colchicine, 10^{-3} M vinblastine, or after prolonged incubation with 10^{-3} M calcium. Heating washed erythrocytes to 60 °C for 10 min was without effect on cell shape.

General appearance of membranes

After hemolysis and washing, the camel ghosts displayed marked flow birefringence and, on swirling the suspension, a shimmering irridescence was observed, reminiscent of suspensions of tiny flat plate crystals. In the microscope, the membranes appeared as very flat elliptical plates (Fig. 1) which had roughly the same length and width as the whole cells, but in which the upper and lower surfaces appeared to have collapsed together, resulting in negligible thickness. The shape of these membranes was also very stable. Storage in 5 mM phosphate buffer, pH 7.5 for up to 4 weeks produced no detectable shape change, and the swirling irridescence was also unchanged. In contrast to the behaviour of the camel ghosts, human or bovine ghosts stored in 5 mM phosphate for long periods underwent first of all a disc-sphere transformation, and eventually fragmentation to small spherical vesicles.

Gel electrophoresis

The protein staining pattern from camel erythrocyte ghosts was similar to the patterns obtained from the human and bovine ghosts, as shown in Fig. 2. The patterns for the human and bovine proteins are in good agreement with results reported from other laboratories [7, 12, 13]. Bands labelled 1,2 and 5–8 (Fig. 2) appear to be of identical mobility in all three species. However, minor bands between the bands labelled 2 and 3 show wide variation between all three species - three prominent bands that are present in the bovine preparation are absent in both the camel and human patterns. Differences also exist in the region labelled 4 in Fig. 2. However, perhaps the most significant difference between the camel and the other species lies in component

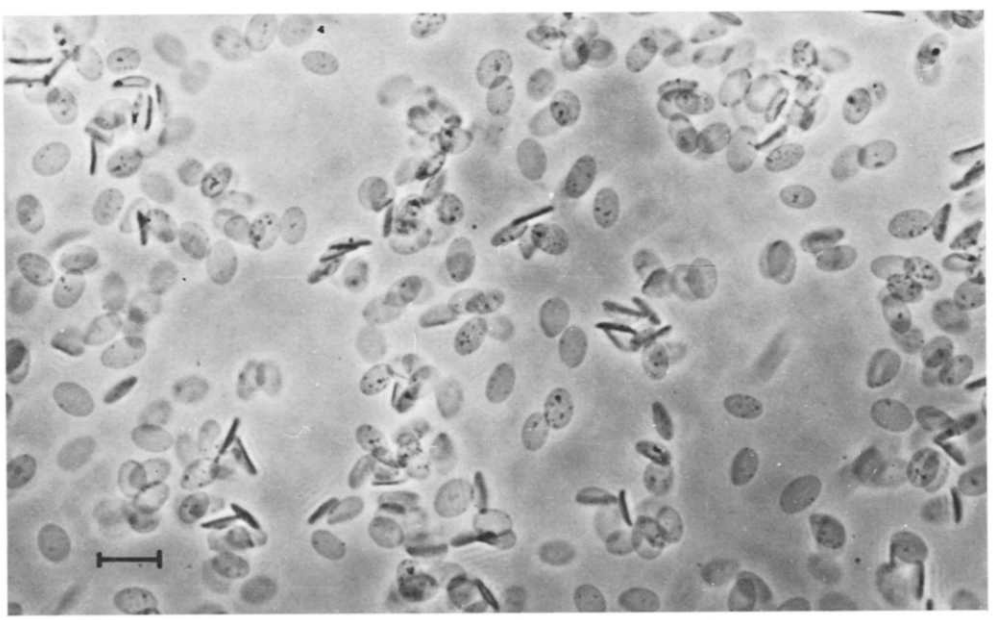

Fig. 1. Camel erythrocyte membranes as viewed under phase contrast illumination. Ghosts were suspended in 5 mM sodium phosphate, pH 8.0. Magnification at film, X 128. Marker indicates 10 μm.

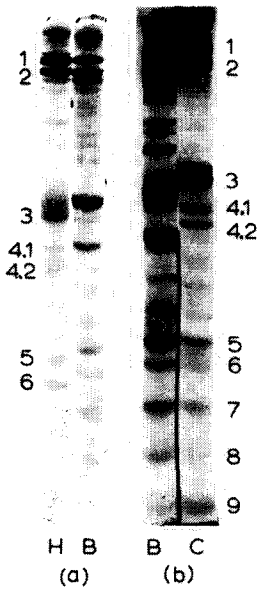

Fig. 2. Electrophoretic patterns of proteins from erythrocyte membranes. Electrophoresis was performed on 5.6 % acrylamide gels containing 1 % dodecyl sulphate as described in Materials and Methods. (a) Human (H) and bovine (B) ghosts, approximately 10 μg protein applied to gels. (b) Bovine (B) and camel (C) ghosts, approximately 100 μg protein applied to gels. Numbers along side the patterns indicate the major proteins according to the nomenclature of Fairbanks et al. [7].

3 which is the major integral protein of the membrane [7] and which has been reported to have an apparent molecular weight of 89 000 [7] in the human. In the camel, this protein has a markedly lower mobility than component 3 of the other two species, suggesting that, in the camel, this protein has a higher molecular weight or, alternatively, that it has a different carbohydrate composition from that of the other species.

Marked differences also exist in the distribution of the glycoproteins of the membrane, as shown in Fig. 3. Once again, the patterns for the human and bovine proteins are similar to those presented elsewhere [7, 13]. The glycoproteins of the camel were of higher mobility than those of the other two species and can be assigned approximate values of apparent molecular weight by means of comparisons with the staining patterns of the human membrane proteins and the molecular weight values reported by Fairbanks et al. [7]. The major glycoprotein of the camel ghost, PASC-4, occupies a position in the gels corresponding closely to protein component 7 of the human ghost, and can be assigned an apparent molecular weight value of 28 000. Component PASC-2 corresponds with human protein component 3 of molecular weight 89 000 [7]. The more minor camel glycoproteins PASC-1 and PASC-3 have apparent molecular weights of approximately 140 000 and 40 000, respectively.

Protein extraction

Extraction with 0.2 mM EDTA selectively released protein components 1, 2 and 5 from camel ghosts, together with traces of the components 4.1 and 4.2. Although

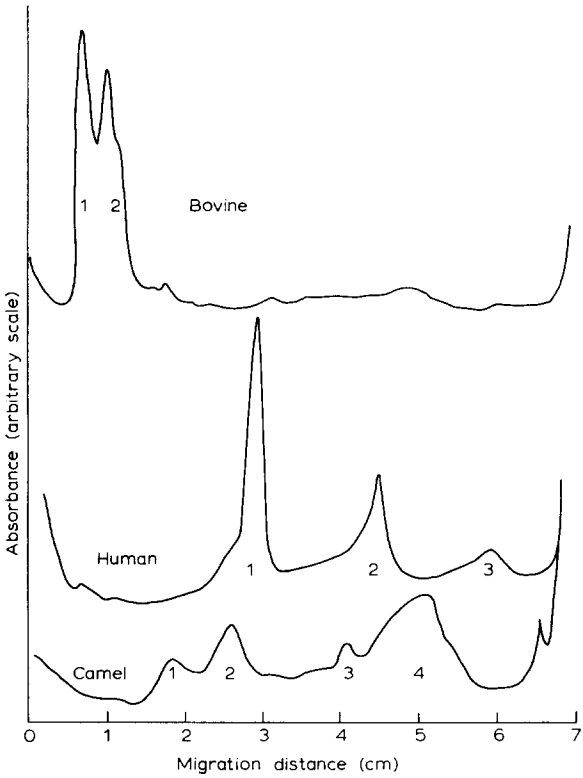

Fig. 3. Glycoprotein staining profiles of the proteins from camel, human and bovine erythrocyte membranes after gel electrophoresis in 5.6 % acrylamide gels containing 1 % dodecyl sulphate. The glycoprotein components are numbered for each species in order of increasing mobility.

TABLE I

EXTRACTION OF PROTEINS FROM ERYTHROCYTE GHOSTS

Proteins were extracted from camel, bovine and human erythrocyte ghosts by means of dialysis against 0.2 mM EDTA, pH 8.0, at 2 °C. Amounts extracted are expressed as mg/ml soluble protein in supernatant, and as the percentage of membrane protein extracted at each step. Results for the whole ghosts refer to the membrane suspension at the beginning of the experiment.

	Camel		Bovine		Human	
	mg/ml	%	mg/ml	%	mg/ml	%
Ghosts	2.4	100	2.5	100	2.6	100
Supernatant 1	0.08	1.5	0.42	9	0.31	7
Supernatant 2	0.12	2.3	0.36	11	0.49	11
Supernatant 3	0.30	5.7	0.18	2	0.29	9
Supernatant 4	0.04	0.7	—		—	
Total % extracted		10.2		22		27

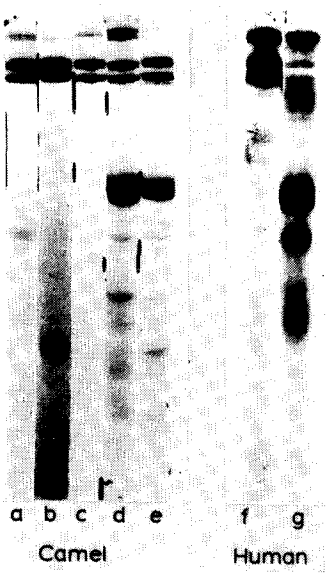

Fig. 4. Electrophoretic patterns showing the effects of dialysis of ghosts against 0.2 mM EDTA, pH 8.0, 2 °C. a–e, camel; a, 37 °C supernatant; b, supernatant 2; c, supernatant 4; d, residue after 4 extractions; e, whole ghosts. f, g, human; f, supernatant 2; g, residue after 2 extractions.

four extractions depleted the membranes of most of the component 5, the amount of spectrin (components 1 and 2) extracted from the camel ghosts by this procedure was considerably less than the amount extractable from human or bovine ghosts. Table I shows the total protein extracted from the three types of membrane, and it is apparent that only about half the amount of protein extractable from bovine and human ghosts can be extracted from camel membranes. Fig. 4 shows that the prolonged extraction of the camel ghosts does not deplete the membrane of all the spectrin, while only two extractions of the human ghosts are required to remove almost all of the human spectrin.

Incubation of either fresh or partially extracted camel ghosts in 0.1 mM EDTA at 37 °C for 1 h also failed to release more than about half of the spectrin from the membranes, although similar treatment of bovine or human ghosts resulted in almost total loss of spectrin, in agreement with the results of Fairbanks et al. [7]. However, the 37 °C incubation did appear to increase the amount of components 4.1 and 4.2 released from the membrane, as shown in Fig. 4.

Even after prolonged extraction of the camel ghosts, and the removal of about half of the original spectrin content, the ghosts showed no obvious shape change. There was no sign of the fragmentation that is seen with human ghosts after similar treatment [3].

Extraction of camel ghosts with protein perturbants

The effects of various protein perturbants were examined in order to relate possible changes in the shape of the camel ghosts to changes in specific proteins or to specific extraction of some of the protein components.

Calcium chloride and magnesium chloride at concentrations up to 5 mM had little effect on the shape of camel ghosts, although some clumping of the membranes was observed at concentrations above 1 mM.

The microtubule disrupting agents colchicine and vinblastine had no observable effect on membrane morphology at concentrations between 10^{-6} and 10^{-3} M. No protein was extracted by these agents.

However, a drastic shape change from flat elliptical plates to small spheres did occur on incubation with urea or guanidine hydrochloride at concentrations above 2 M, with 0.01 M NaOH and with 5 mM *p*-mercuribenzoate. The effect of sulphydryl reagents in general was examined by means of incubation with up to 5 mM *N*-ethyl maleimide, iodoacetate, iodoacetamide, chloromercuri-2,4-dinitrophenol and DTNB. None of these agents was able to induce the shape change of the camel ghosts brought about by PCMB, and none of them caused the release of significant amounts of soluble protein. Moreover, preincubation of the ghosts with 5 mM *N*-ethylmaleimide was unable to block the effects of PCMB on the shape of the membrane.

The spherical ghosts induced by urea and PCMB were about 4 microns in diameter, while those brought about by guanidine hydrochloride were smaller, about 3 microns, and appeared denser under phase contrast.

Electrophoresis of the supernatants after incubation showed that, in all cases where a marked change in shape had occurred, spectrin was extracted almost totally from the ghosts. In addition, however, component 5 and traces of components in the 4 region were also extracted. For the case of urea, a series of concentrations was examined. It was found that at 2 M urea, at which concentration the shape change begins to occur, spectrin and component 5 were virtually the only proteins extracted (see Table II). As the urea concentration was increased, the two proteins 4.1 and 4.2 also became soluble, until at 6 M urea, when the ghosts began to fragment, some traces of component 3 are also extracted.

When these experiments were repeated with ghosts that had previously been extracted with 0.2 mM EDTA, and were thus largely depleted of component 5, the

TABLE II

EFFECTS OF UREA ON CAMEL GHOSTS

The effects of urea on the shape of camel erythrocyte membranes and on the extraction of specific proteins was examined as described in Materials and Methods.

Urea (M)	Proteins extracted	Shape
(a) Fresh ghosts		
0	(1, 2)	Flat ellipses
2	1, 2, 5	Swollen ellipses
4	1, 2, 4.1, 4.2, 5	Spheres
6	1, 2, (3), 4.1, 4.2, 5	Spheres, fragments
(b) Partially extracted membranes, depleted of component 5.		
0		Flat ellipses
2	1, 2	Spheres, swollen ellipses
4	1, 2	Spheres
6	1, 2, (3)	Spheres, fragments

change in shape occurred over the same range of urea concentrations. However, in these experiments, spectrin was virtually the only protein extracted at the urea concentrations required for the shape change (Table IIb). The effects of urea and guanidine on the shape of the ghosts were not reversed after removal of the perturbant by dilution or dialysis.

Extraction with Triton X-100

The non-ionic detergent, Triton X-100, has been shown to extract a large fraction of the integral proteins from the human erythrocyte membrane, leaving a residue of spectrin and other peripheral proteins [14]. Incubation of both human and bovine ghosts in 1 % Triton X-100 at an ionic strength of 0.01 in the present study resulted in extensive solubilization of the membranes and extraction of the integral proteins, in agreement with a published report [14]. However, treatment of the camel ghosts in a similar manner resulted in only a slight release of spectrin and a trace of component 3. No glycoproteins could be detected in the supernatants after extraction, in marked contrast to the behaviour of human ghosts [14]. Furthermore, no shape change was induced in the camel ghosts, which remained visible as flat elliptical plates. In this respect the camel ghosts behaved quite differently from the human and bovine ghosts, which became almost invisible under phase contrast illumination after the detergent treatment.

Association state of spectrin from camel erythrocyte ghosts

Spectrin from bovine erythrocyte ghosts has recently been found to exist in a series of stable aggregates which can be separated by gel filtration on a column of 4 %

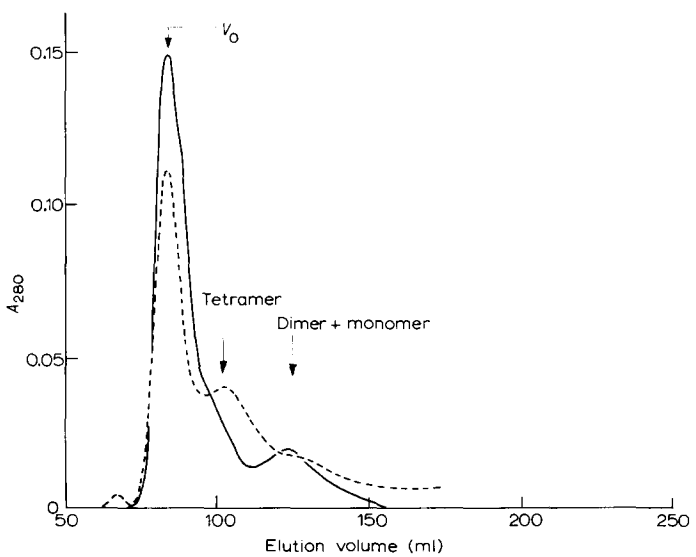

Fig. 5. Gel filtration profiles for the water soluble proteins of camel and bovine erythrocyte membranes after chromatography on Biogel A-15m. The void volume (V_0), tetramer and dimer peaks are indicated. (—), camel; (- - -), bovine.

agarose beads [10]. These aggregates can also be separated on gels containing a gradient of acrylamide concentration (Ralston, G., unpublished results). Fig. 5 shows that the elution profile of spectrin extracted from camel ghosts is basically similar to that from bovine ghosts. There is a large proportion of highly aggregated material in the void volume peak, a shoulder corresponding to the elution volume of the bovine tetramer, and a well defined peak corresponding with the bovine dimer and monomer. Electrophoresis on Gradipore gels confirmed the gel filtration results, and showed more clearly that the ratio of dimer to tetramer is somewhat greater in the camel than in the bovine. In addition, the amount of monomer is higher in the camel than in the bovine, and in this regard seems to resemble the human protein more closely than it does the bovine.

DISCUSSION

The shape of the camel erythrocyte membrane, unlike that of the human or bovine, is particularly stable, not only in whole erythrocytes, but also in the isolated ghosts. Metabolic depletion [4], treatment with calcium [4, 15] and heating to 60 °C [16] all induce disc-sphere changes or fragmentation of human erythrocytes and ghosts, but appear to be without effect on camel erythrocytes, according to the results of the present study. This remarkable stability is consistent with the resistance of camel erythrocytes to osmotic lysis, observed by other workers [2].

Although microtubules have been reported to be involved in shape maintenance of avian erythrocytes [17], it is unlikely that microtubules are responsible for the stability of camel erythrocytes, as the potent microtubule disrupting agents colchicine and vinblastine both failed to alter the shape of camel red cells and ghosts at concentrations sufficiently high to disrupt the microtubules of the avian cells [17]. Furthermore, in the present study, colchicine and vinblastine were unable to cause the transition to spherocytes in camel red cells that had been observed in human red cells treated with these agents, and which was presumed to have been the result of interaction with microfilamentous proteins [18].

In addition to the stable shape of the camel erythrocyte membrane, the water-soluble protein spectrin appears to be more tenaciously bound to the camel erythrocyte membrane than it is in other species. Prolonged extraction at low ionic strength induces the release of only half of the spectrin in the camel ghost, while almost all of the spectrin in human and bovine ghosts can be removed after two or three similar extractions. Even at 37 °C, at which temperature bovine and human ghosts release all of their spectrin within 1 h, camel ghosts released only a fraction of the spectrin content, and no obvious shape change could be detected.

More drastic treatment, such as incubation with urea, guanidine hydrochloride or PCMB caused the release of most of the camel spectrin from the ghost, as well as a concurrent change in shape from flat ellipses to small spheres. However, even under these drastic conditions, the membrane did not fragment until the urea concentration was raised above 6 M.

An interesting aspect of this study was the high degree of specificity of PCMB as a spectrin-releasing agent. No other sulphydryl reagent examined was able to duplicate the effect of PCMB. Even the other organomercury compound that was used, chloromercuri-2,4-dinitrophenol, was unable to mimic the effect of PCMB.

Similar specific effects of PCMB and *p*-chloromercuribenzene sulphonate were observed by Steck and Yu [8] on the release of proteins from human erythrocyte membranes. Presumably a charged group at the para position is essential for the particular reactivity of these reagents towards the membrane proteins.

Although the present results implicate spectrin in the maintenance of membrane shape in the camel erythrocyte, other proteins of the membrane presumably play a vital role. The failure of the camel ghosts to fragment after spectrin release suggests that, even in the absence of spectrin, the membrane shows greater stability than those of the bovine and human species. Furthermore, the failure of the nonionic detergent, Triton X-100, to extract significant amounts of components 3 and 4 from camel ghosts at concentrations high enough to cause gross disruption of the human ghost, suggests that, in the camel, components 3 and 4 are different from the corresponding human and bovine proteins. This conclusion is supported by the electrophoresis patterns which show that component 3 in the camel is apparently of higher molecular weight than component 3 from human and bovine ghosts. The proteins in the component 4 group also appear to be different from the bovine and human corresponding proteins, in terms of both mobility and solubility. The glycoproteins of the camel show marked differences from the human and bovine glycoproteins in both mobility and extractability with Triton X-100. However, the glycoproteins of the human and bovine ghosts also display large differences from one another in terms of their electrophoretic distribution, as shown in both the present report and the work of Kobylka et al. [13], yet the membranes of both species are similar in physical properties.

Nicolson and Painter [19] have shown that spectrin and the integral proteins of the membrane are closely associated in the human erythrocyte membrane. Singer [5], in a recent review, has suggested that the presence of spectrin on the membrane prevents the lateral movement of integral proteins in the membrane. It is likely, then, that the glycoproteins, component 3 and spectrin may all interact in order to maintain the integrity of the membrane. Removal of spectrin may then allow the lateral movement of integral proteins, and disruption of the shape and integrity of the membrane. In the bovine and human membrane this is apparently sufficient to allow the disruption and fragmentation of the membrane. In the camel, however, it is envisaged that the changes in the integral proteins and their greater abundance relative to the lipid content of the membrane allow the camel membrane to resist gross disruption.

On the basis of the present evidence, it is not clear whether spectrin is firmly bound to the membrane because of intrinsic differences in its own structure, or whether the differences in integral proteins, in particular component 3, give rise to the increased binding of spectrin. However, the similarity of the association pattern of human, bovine and camel spectrin, and the marked differences between the glycoproteins and between the component 3 proteins, suggest that the major difference lies not in spectrin, but in the integral proteins.

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