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UNIQUE PROPERTIES OF THE CAMEL ERYTHROCYTE MEMBRANE
II. ORGANIZATION OF MEMBRANE PROTEINS

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SUMMARY

Camel erythrocyte membranes are distinguished by some unique properties of stability and composition. Notable is their abundance in proteins (protein : lipid ratio of 3 : 1). Membrane proteins of camel erythrocytes were compared with those of human erythrocytes, which have been intensively investigated. Proteins were extracted with various aqueous media (EDTA, alkaline or high ionic strength) and with ionic and non-ionic detergents and were analyzed by gel electrophoresis. In membranes of camel erythrocytes, the peripheral proteins constitute, proportionally, a much smaller fraction of total proteins than in the human erythrocyte, while their distribution is identical per unit of surface area. The camel erythrocyte membrane is particularly rich in integral proteins and in intramembranous particles. The proteins in this membrane are more closely organized than in the human system, as revealed by crosslinking and freeze-etching studies. It is proposed that protein-protein interaction of integral proteins, presumably constituting an "integral skeleton", is a dominant structural feature stabilizing the camel erythrocyte membrane.

INTRODUCTION

Stability and strength of biological membranes are among the major topics currently under study in biomembrane research. The camel erythrocyte membrane is of particular interest in this context in view of its unique properties. The osmotic fragility of camel erythrocytes is outstandingly low and the cells are capable of swelling in hypotonic media to over twice their volume [1, 2], while their osmotic behaviour is markedly anomalous in hypertonic media [3]. Camel erythrocytes are unique among mammalian types in being oval, rather than circular, discs [2, 4]. They are also distinctly more stable to the lytic effects of sonic irradiation and vinblastin [5]. Despite the low degree of extractability of camel erythrocyte phospholipids with ether/methanol mixtures [4], the composition of these lipids does not display outstanding features [5]. Camel erythrocyte membranes differ greatly from those of humans in their higher protein : lipid ratios (3 : 1 in camel erythrocytes as opposed to 1.25 : 1 in the human cells) and also exhibit some differences in amino acid composition [5].

In view of the distinctive properties of the camel erythrocyte membrane, it became of interest to characterize its membrane proteins. The present study compares properties of membrane proteins from camel and human erythrocytes.

MATERIALS AND METHODS

Adult female camels (*Camelus dromedarius*) of a local breed were raised on a diet of grain concentrates and straw, with ample water supply. Heparinized blood samples, collected from camels and from adult human volunteers, were processed within 30 min.

Preparation of erythrocyte ghosts

Ghosts of camel and human erythrocytes were prepared essentially according to the method of Dodge et al. [6]. Erythrocytes were washed four times at 4 °C with 155 mM phosphate buffer (pH 8.0). Human red cells were lysed with 15 vols. of diluted buffer (10 mM) at 2–4 °C. The osmotically resistant camel cells were lysed with 40 vols. of the diluted buffer. The suspensions were centrifuged at 20 000 $\times g$ for 20 min and the pellets were washed twice more in cold 5 mM sodium phosphate buffer (pH 8.0). The resultant ghosts contained less than 1 % hemoglobin.

Polyacrylamide disc gel electrophoresis

The procedure described by Fairbanks et al. [7] was followed, except that the current during electrophoresis was 4 mA per tube. In each sample 100 μg proteins were loaded on the gel. Protein bands were stained with Coomassie blue.

Turbidity measurements

Ghosts were suspended in 155 mM phosphate buffer (pH 8.0) to give an absorbance of about 0.4 at 520 nm (protein concentration 0.18–0.21 mg/ml). Turbidity changes were initiated by the addition of aliquots of 10 % Triton X-100 and monitored at 520 nm by a recording spectrophotometer at 25 °C. Readings were taken 5 min after the addition of the detergent, when turbidity changes had levelled off.

Protein extractions

For extraction of membrane proteins with EDTA at low ionic strength [8], ghosts were suspended in 0.5 mM EDTA (pH 7.5) at 1 mg protein/ml and dialyzed against the EDTA solution for 48 h at 4 °C. For extraction with alkali [9], ghosts were suspended in 0.1 M NaOH (0.4 mg protein/ml) for 15 min at 0 °C. For extraction at high ionic strength [10], the ghosts were suspended in 1 M NaCl (0.5 mg protein/ml) and incubated with constant stirring at 4 °C for 18 h. All extractions were terminated by centrifugation at 100 000 $\times g$ for 1 h at 4 °C. The supernatant and the pellet were separated for protein analysis [11].

Extraction with detergents was carried out by mixture of a suspension of membranes (1.5–2.5 mg protein/ml in 155 mM phosphate buffer, pH 8.0) with an equal volume of detergent, prepared in the same buffer. The mixtures were incubated at 37 °C for 15 min and centrifuged at 40 000 $\times g$ for 1 h at 4 °C. The supernatant and the pellet were separated for protein analysis.

Crosslinking

The crosslinking effects of glutaraldehyde were examined according to the method of Steck [12]. 50 μl of membrane suspension, containing 100 μg proteins in 5 mM phosphate buffer (pH 8.0) were mixed with 50 μl of glutaraldehyde solution, prepared in the same buffer, and then incubated at 25 °C for 30 min. The reaction was stopped by the addition of 0.2 ml of a solution containing 1 % sodium dodecyl sulfate, 10 mM sucrose, 10 mM Tris · HCl (pH 8.0), 1 mM EDTA, 40 mM dithiothreitol and 10 μl/ml pyronine Y. The samples were heated to 60 °C for 20 min and electrophoresed on 4 % polyacrylamide gels.

Freeze-etching

Ghosts were fixed in 5 % glutaraldehyde for 10 min and washed 3 times with 0.1 M phosphate buffer (pH 7.4). The fixed membranes were suspended in 30 % glycerol and further incubated for 30 min. Following freezing in liquid N_2, the membranes were etched at −150 °C and shadowed with platinum/carbon in Balzer's BAF 300 apparatus (Balzer High Vacuum Corp.). The platinum/carbon replicas were cleaned of biological residues by NaOH and by HCl, successively, picked up on grids and examined by a Jeol 100B electron microscope.

Lipid extraction

Lipid extraction of erythrocyte ghosts with a chloroform/methanol mixture was carried out as described earlier [13].

RESULTS

Gel electrophoresis

The profiles of membrane proteins of human and camel erythrocytes were compared by electrophoresis in polyacrylamide gel in the presence of sodium dodecyl sulfate. Fig. 1 presents the distribution of Coomassie blue-stained proteins in 5.6 % acrylamide gels. While the two profiles of membrane proteins show some similarities, several distinct differences are apparent. For comparison, the marking of bands according to Steck and Yu [8] was adopted. The two spectrin bands, 1 and 2, are present in the camel electrophoretogram, with relative mobility as in the electrophoretogram of the human system. However, on the basis of staining intensity estimation, these bands constitute a smaller proportion of the erythrocyte membrane proteins than of the human composite. While band 3 in the human electrophoretogram is diffuse and tailed, the apparently corresponding band of camel erythrocyte membranes is not only well defined but also appears to be the principal membrane protein. In view of the difference in mobilities, the identification of this prominent band with band 3 of the human system is tentative. The electrophoretogram of the camel erythrocyte membrane proteins is typified by a fraction which appears at the top of the gel. This fraction, estimated as less than 7 % of the proteins loaded on the gel, is clearly discernible on a 4 % acrylamide gel (Fig. 2). It may represent native protein(s) of very high molecular weight, but it is also possible that this fraction is comprised of protein aggregates.

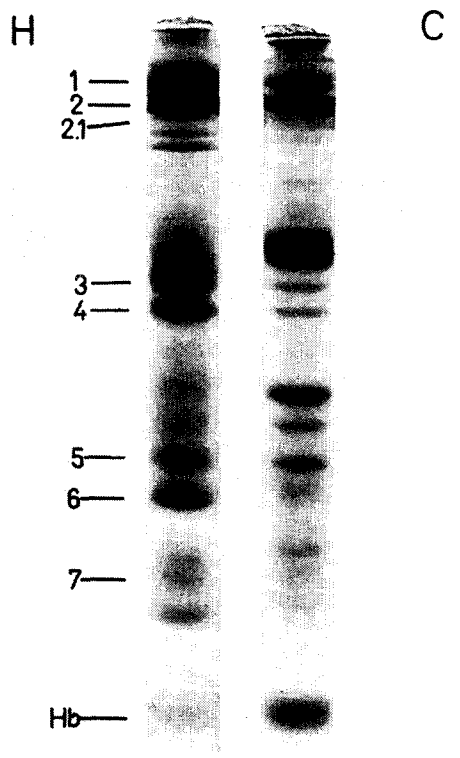

Fig. 1. Distribution of bands of membrane proteins (100 μg) from human (H) or camel (C) erythrocytes following electrophoresis on 5.6 % polyacrylamide gel [7]. The marking of bands is according to Steck and Yu [9].

Extractability of membrane proteins

Extraction of proteins from erythrocyte membrane was carried out with several media in an attempt to characterize the interactions of the proteins in the membrane framework. The effects of a chelating agent, alkali and high ionic strength are presented in Table I. Under all these conditions a smaller portion of the proteins could be extracted from the camel erythrocyte membranes. Extraction in the presence of EDTA, known to remove peripheral membrane proteins [7, 14], solubilized about one third of the proteins from human erythrocyte membranes, but only 12 % from the camel system. Spectrin could not be fully extracted by EDTA from camel erythrocyte membranes. Thus, some of the molecules appearing in bands 1 and 2 are apparently tightly bound to the membrane, similarly to integral proteins. Operationally, this fraction, unextracted by EDTA, is therefore regarded as part of the integral proteins. Based on gel electrophoresis (not shown), the EDTA extract contained, in both cases, three bands corresponding to the spectrin bands and band 5. It appears that the peripheral protein fractions in the two membrane types are similarly composed, but proportion-wise this fraction is smaller in the camel erythrocyte. When calculated on the basis of surface area of erythrocytes, the EDTA-extracted proteins, as well as the lipids, are very similarly represented in the human and camel erythrocyte

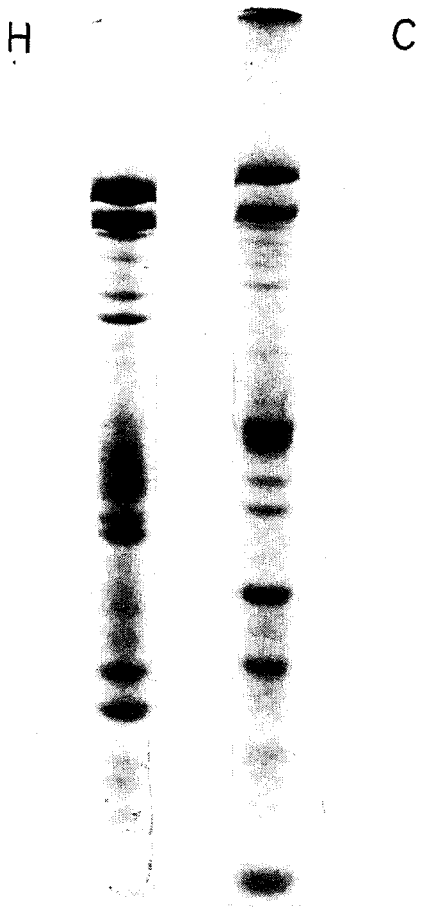

Fig. 2. As in Fig. 1, employing 4 % acrylamide gel.

TABLE I

PROTEIN EXTRACTION WITH AQUEOUS MEDIA FROM MEMBRANES OF HUMAN AND CAMEL ERYTHROCYTES

Treatment*	Extracted proteins (% total membrane proteins)	
	Human erythrocytes	Camel erythrocytes
EDTA, 0.5 mM	32±5	12±3
NaOH, 0.1 M	56±2	29±4
NaCl, 0.1 M	23±3	3±1

* Experimental details are given in Materials and Methods.

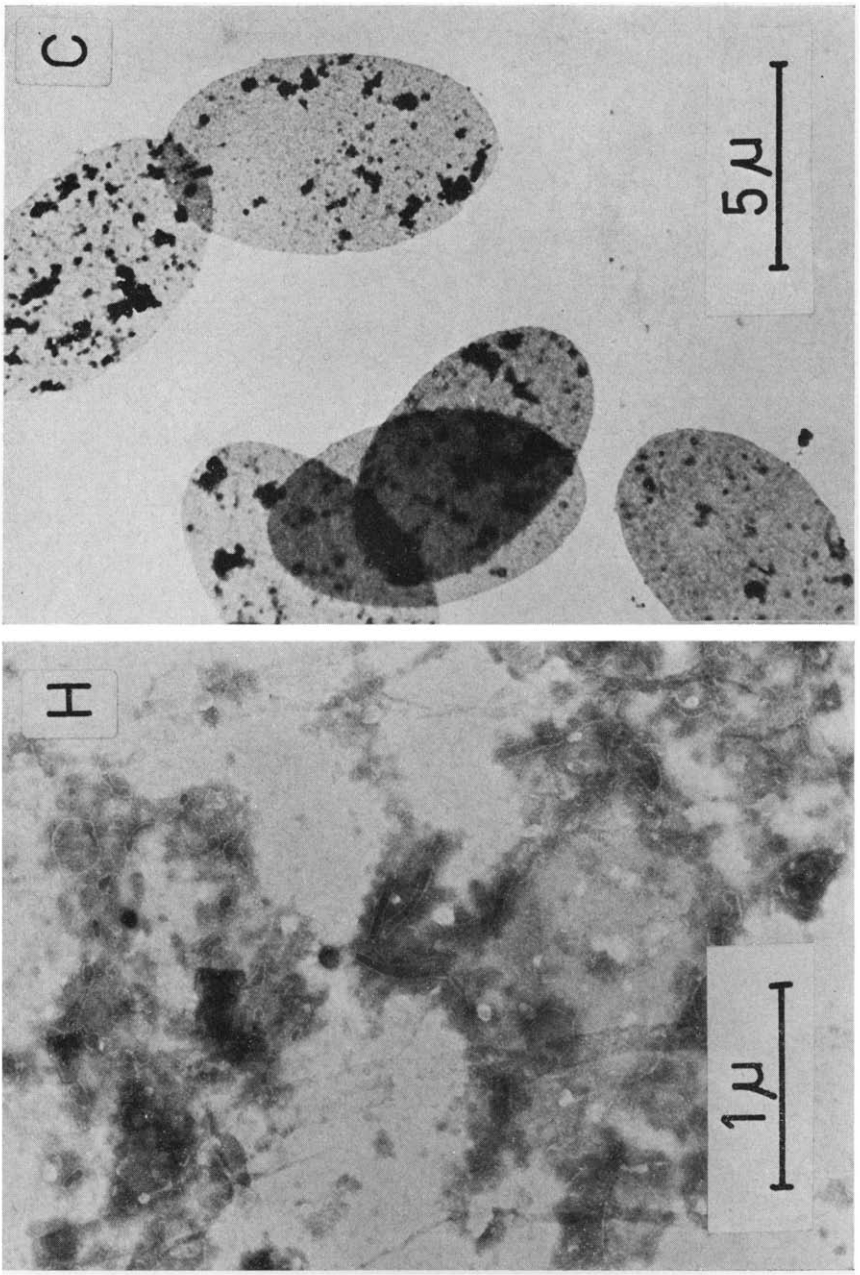

Fig. 3. Electron micrographs of EDTA-treated ghosts of human (H) or camel (C) erythrocytes. Preparations were negatively stained for electron microscopy by 2% phosphotungstic acid.

membrane (Table II). It is obvious, then, that membranes of camel and human erythrocytes differ markedly with respect to the content of integral proteins.

Extraction with EDTA drastically disrupted the membrane of the human erythrocyte, confirming earlier observations [15, 16]. In contrast, the camel erythrocyte ghosts were morphologically unchanged by the EDTA treatment (Fig. 4).

TABLE II

PROPORTIONS OF PROTEINS AND LIPIDS IN MEMBRANES OF CAMEL AND HUMAN ERYTHROCYTES

Proteins and lipids are expressed as $\mu g/\mu m^2$ surface area $\times 10^{-10}$

Erythrocyte membrane	Proteins	Lipids	Protein: lipid ratio	"Peripheral proteins"*	"Integral proteins"*
Human	49	41	1.2	20	29
Camel**	160	47	3.7	19	141

* Proteins extracted with EDTA, as described in Materials and Methods, are regarded as "peripheral proteins", while the residual proteins are termed "integral proteins".

** Camel erythrocyte surface area according to Yagil et al. [3].

The extractability of erythrocyte membrane proteins by an anionic and a cationic detergent at 37 °C is presented in Fig. 4. Sodium dodecyl sulfate was similarly effective in solubilizing the membrane proteins of both camel and human erythrocytes. However, cetyltrimethylammonium bromide exhibited a differential effect and extracted camel ghost proteins to a lesser extent at the indicated range of detergent: membrane protein ratios. At a higher detergent: protein ratio (14 : 1), cetyltrimethylammonium bromide fully solubilized both membranes. A more pronounced differential effect was exerted by the non-anionic detergent Triton X-100. When membrane suspensions were monitored for turbidity changes [17], it was clearly apparent that Triton X-100 was much more effective in solubilizing the membranes of human erythrocytes than those of camel erythrocytes (Fig. 5). Protein determinations indeed established that the extraction of proteins by Triton X-100 from camel erythrocyte membrane was limited, while protein extraction from the membranes of human

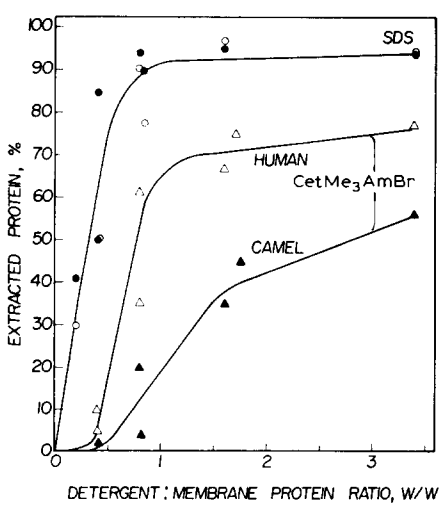

Fig. 4. Extractability of proteins from human and camel erythrocyte membrane with sodium dodecyl sulfate (SDS) and with cetyltrimethylammonium bromide (Cet Me_3Am Br)

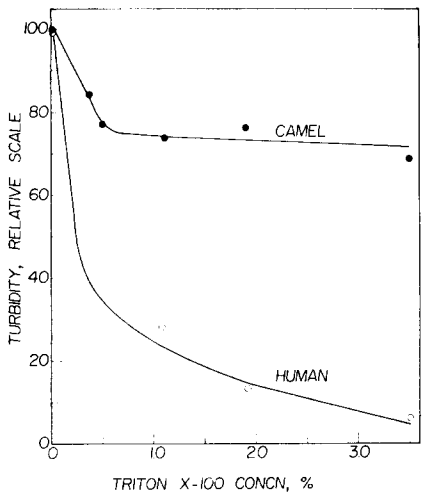

Fig. 5. Turbidity of suspensions of human and camel membranes as affected by Triton X-100. The turbidity of untreated suspensions ($A_{520nm} = 0.4$) is taken as 100 on a relative scale while zero is related to the reading of the suspending medium.

erythrocytes was several-fold greater (Fig. 6). Despite these differences in protein extraction, phospholipids were extracted to a large extent (> 90 %) from both membrane types by Triton X-100 (3 mg/mg protein). It is noteworthy that the pretreatment of camel erythrocyte membranes with EDTA did not promote the extractability of proteins with Triton X-100.

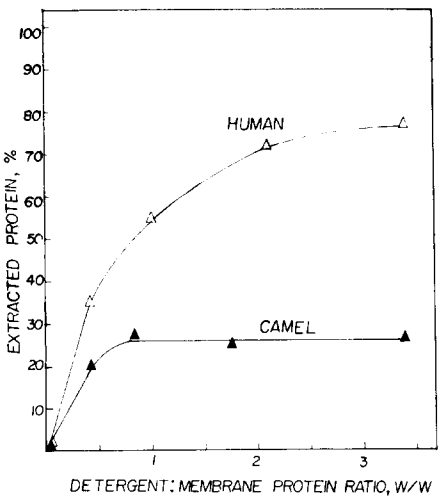

Fig. 6. Extractability of proteins from human and camel erythrocyte membrane by Triton X-100.

Freeze-etching

Erythrocyte membranes were freeze-cleaved to follow the distribution of membrane particles. It appears from Fig. 7 that particles in surface A (oriented toward the extracellular space) are larger in camel membrane than in the human

erythrocyte. They are also more densely distributed than in the human membrane by about 60 %. Surface B of the camel erythrocyte is also distinguished by larger particles than the human system, but the distribution is similar for membranes.

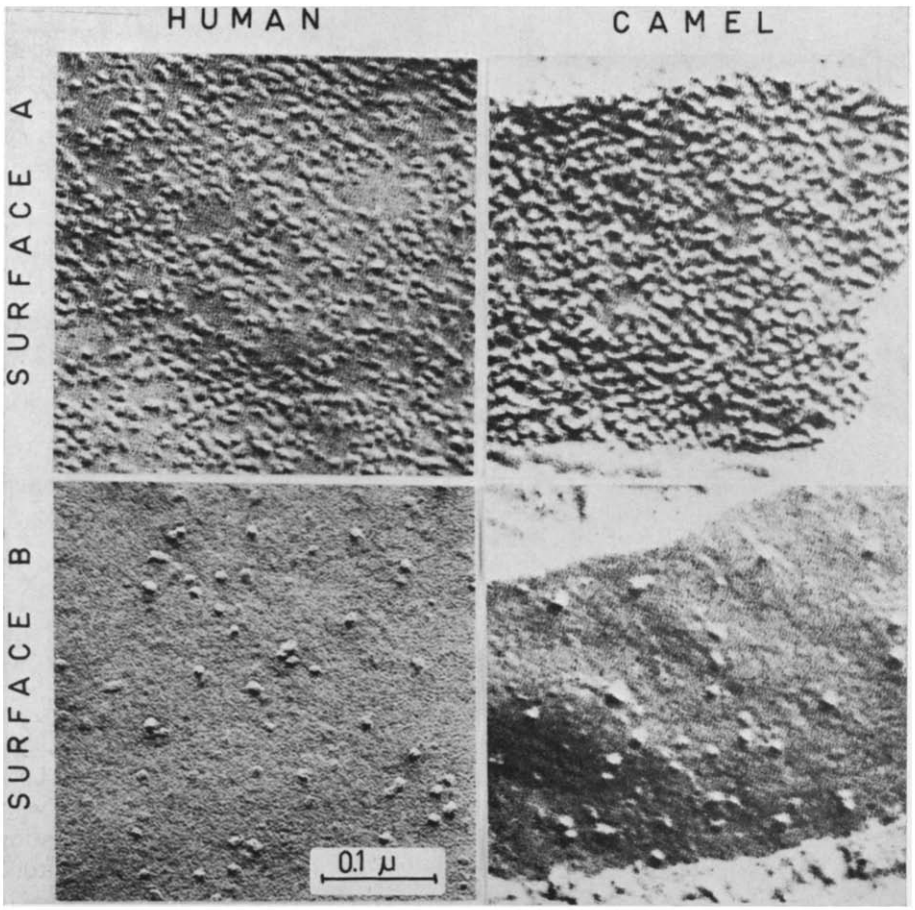

Fig. 7. Distribution of intramembraneous particles in ghosts of human and camel erythrocytes, as revealed by freeze-etching.

Crosslinking

The data suggest a closer packing of membrane proteins in camel erythrocytes than in human ones. One can examine this tentative conclusion by crosslinking ghost proteins with glutaraldehyde and by resolving them electrophoretically in the presence of sodium dodecyl sulfate. Fig. 8 shows that while membrane proteins of human erythrocytes are only partially crosslinked by 1–2 mM glutaraldehyde, this reagent, even at 1 mM, completely crosslinks the proteins from camel erythrocyte membranes.

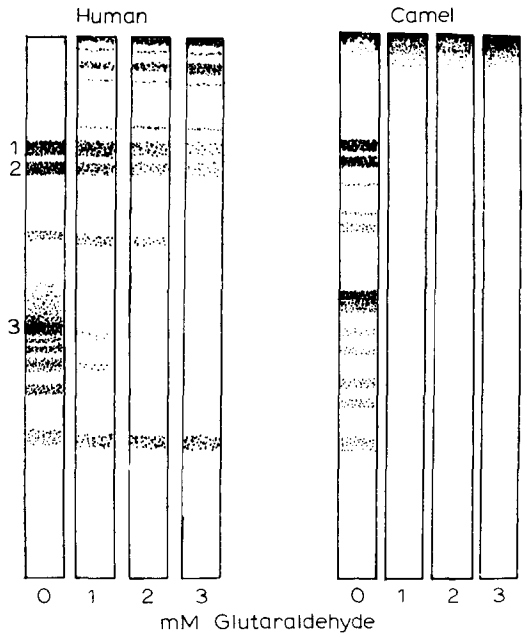

Fig. 8. Gel electrophoresis of membrane proteins from human and camel erythrocytes following crosslinking by glutaraldehyde. The distribution of bands is diagrammatically presented and the three major bands of human erythrocytes are identified.

DISCUSSION

A general scheme for membrane organization includes integral proteins, which are embedded in a lipid bilayer, and peripheral proteins which are loosely bound to the membrane. The present study indicates that this scheme is applicable to membranes of both human and camel erythrocytes. Qualitatively, despite some differences, both membrane types are comprised of similar profiles of polypeptides. Quantitatively, however, the two membranes differ markedly with regard to the relative distribution of peripheral and integral proteins. Peripheral proteins comprise 30–40 % of total membrane proteins in human erythrocytes [7, 16, 18–20], while an overwhelming majority of the proteins in camel erythrocyte membranes are integral. These quantitative differences are accentuated by the striking protein abundance in the camel erythrocyte membrane [5].

Proteins are effectively solubilized from camel erythrocyte membranes by ionic detergents, notably by sodium dodecyl sulfate, while extraction by the nonionic detergent Triton X-100 is limited (Figs. 5 and 6). It may be concluded that protein-protein interaction of integral proteins is a dominant structural feature of the camel erythrocyte membrane. Interactions within membranes in other systems have also been interpreted in the light of the effect of detergents. For example, plasma membrane junctions in animal cells [21], synaptic membranes [22, 23] and the outer membrane layer of Gram-negative bacteria [24] are all characterized by protein-protein interactions and are relatively resistant to nonionic detergents.

Removal of the peripheral proteins from the human erythrocyte membrane brings about a complete disruption of the membrane structure [15, 16]. It appears that bands 1, 2 and 5 of this membrane constitute a network of microfilaments adherent to the cytoplasmic surface of the erythrocyte membrane, offering the membrane a skeletal support [25]. For convenience we refer to this network as the "peripheral skeleton". It is apparently also typical of the camel erythrocyte membrane, but we propose that the camel's system is distinguished by an additional, more prominent network, termed tentatively "integral skeleton". This presumed network is stabilized by protein-protein interaction of closely distributed integral proteins and possibly also by interaction with lipids. The unusual ratio of solvents required for effective extraction of lipids from camel erythrocytes [4] may indeed reflect some unusual lipid-protein interaction, since the lipid composition of camel erythrocytes is essentially similar to that of other species [5].

Camel erythrocyte membranes exhibit properties reminiscent in some respects of human erythrocyte membranes treated with glutaraldehyde. Fragmentation of human erythrocyte membranes by EDTA and by sonication at high pH are prevented by crosslinking with glutaraldehyde [26]. Glutaraldehyde-treated intact erythrocytes show modified osmotic behavior and altered cell deformability [27]. Furthermore, Ehrlich ascites tumor cells were stabilized against osmotic, sonic and thermal stresses following treatment with glutaraldehyde [28]. Indeed, camel erythrocytes are not only uniquely stable, osmotically-speaking, but are also particularly resistant to the lytic effect of sonication [5]. The analogy between the camel erythrocyte membrane and the glutaraldehyde-treated membrane of human erythrocytes is of course limited by a major difference. While crosslinking by glutaraldehyde is irreversible, involving covalent bonds, the interactions stabilizing the "integral skeleton" are essentially reversible and apparently dynamic, allowing the camel cells to swell in hypotonic media, albeit differently from erythrocytes of other species [2]. The interactions related to the presumed "integral skeleton" are expected to be dynamic, to cope with the dynamic properties of the erythrocyte membrane.

Two of the alternative explanations formerly offered for camel erythrocyte resistance to vinblastin, namely that camel erythrocytes are either devoid of, or particularly rich in, spectrin, may now be rejected, in view of the present data. Furthermore, we verified that the EDTA extract of camel erythrocyte membranes contained proteins that formed typical complexes with vinblastin, as already described for human erythrocytes [29]. We suggest that despite the interaction of vinblastin with spectrin *in situ*, the overall structure of the camel erythrocyte membrane is not disrupted, owing to the dominant role of the "integral skeleton".

The relatively high density of intramembranous particles and the close proximity of membrane proteins further attest to the unique structural features of the camel erythrocyte membrane.

The uniqueness of camel erythrocyte membrane has been recently demonstrated, independently, by Ralston [30]. In his detailed study, Ralston points to an important shape-maintaining role for spectrin in the camel erythrocyte, but concludes that the major difference concerning membrane proteins lies not in spectrin but in the integral proteins.

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