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THE FORMATION OF VESICLES RETAINING SODIUM-DEPENDENT TRANSPORT SYSTEMS FOR AMINO ACIDS FROM PROTEIN-DEPLETED MEMBRANES OF PIGEON ERYTHROCYTES

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Summary

The process of the formation of vesicles from pigeon erythrocyte membranes was studied. Mildly alkaline solutions of low ionic strength, which reduce human erythrocyte membranes to small vesicles depleted of spectrin and other proteins, have no such effect on pigeon erythrocyte ghosts. A distinct phase of removal of membrane proteins, including spectrin, began to occur only when pigeon erythrocyte membranes were exposed to 0.2 mM EDTA adjusted to pH values above 10.2. Vesicles which demonstrated Na^+-dependent amino acid transport were generated between the pH values 10.8 and 11.4. The results show that peripheral proteins, notably spectrin, maintain the integrity of the pigeon erythrocyte ghost. The interaction of these proteins with the membrane is rather different from that well studied in the human erythrocyte ghost and the possible significance of this for the pigeon erythrocyte is discussed.

Introduction

Following the isolation of the cell membrane, a step often employed during attempts to identify transport components therein is the removal, by the use of mildly perturbing conditions, of peripheral membrane proteins [1]. In several cases, this lipid-enriched residue has been induced to form a population of sealed vesicles that exhibit transport phenomena characteristic of the whole cell [2—4]. Successful procedures are presumably developed by trial and error

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and the correct conditions published. We have found that a closer examination of the conditions causing loss of proteins and vesiculation has not only optimised the transport activities observed but also yielded information about the way the unperturbed membrane is stabilised. Our studies on the pigeon erythrocyte membrane have shown that its integrity is unaffected by conditions which reduce the human erythrocyte membrane to vesicles depleted of most membrane proteins. Peripheral proteins, particularly spectrin, are much more tightly associated with the pigeon erythrocyte membrane.

Experimental Procedure

Materials

Acrylamide and *N,N'*-methylene bisacrylamide, especially purified for electrophoresis, were obtained from British Drug Houses, Poole, Dorset, U.K. L-[^{14}C]Alanine was supplied by the Radiochemical Centre, Amersham, U.K. All other chemicals were of the highest grades available from usual commercial sources. Blood was taken from pigeons that were bred here. They were anaesthetised with ether and bled from a vein in the neck into 20 ml of 0.15 M NaCl, 10 mM Tris-HCl (pH 7.4 at 4°C) containing approx. 500 USP units of heparin.

Methods

Plasma membranes were prepared from pigeon erythrocytes exactly as described previously [5]. Removal of proteins and the formation of vesicles from the membranes were achieved as follows. Samples (0.1 ml) of plasma membranes, suspended in distilled water or the buffer in which they were prepared [5], were added to 25-ml portions of 0.2 mM EDTA, each previously adjusted to various pH values between 10.0 and 12.0 using a combination electrode fitted to a Model 32A pH meter (Electronic Instruments Ltd.). The pH of each mixture was measured again and, after leaving the samples on ice for at least 20 min, they were centrifuged at 90 000 × *g* for 30 min at 4°C. The pellets were resuspended by homogenisation in about 0.1 ml of either distilled water or a buffer suitable for the assay or amino acid transport activity [4,6]. The protein [7] and lipid [8] contents of each pellet were measured as accurately as possible [5] and the ratio plotted against the pH recorded after the addition of membranes to the EDTA medium. The transport activity of the pellets for Na^+-dependent exchange of L-alanine was measured [4,6] and the Na^+-dependent uptake plotted against the pH of extraction. The lipid : protein ratios obtained in two separate experiments are shown in Fig. 1 and the points are seen to be almost superimposable. Samples were solubilised, subjected to electrophoresis and the gels scanned as described previously [5]. DNA was measured by using the method of Kissane and Robbins [9].

Results

Preliminary observations showed that 'active' vesicles [4,6] were generated only when membranes were exposed to 0.2 mM EDTA at pH 11.0, and not at pH 10.0 or 12.0. The mean value (±S.E.) of the lipid : protein ratio of these

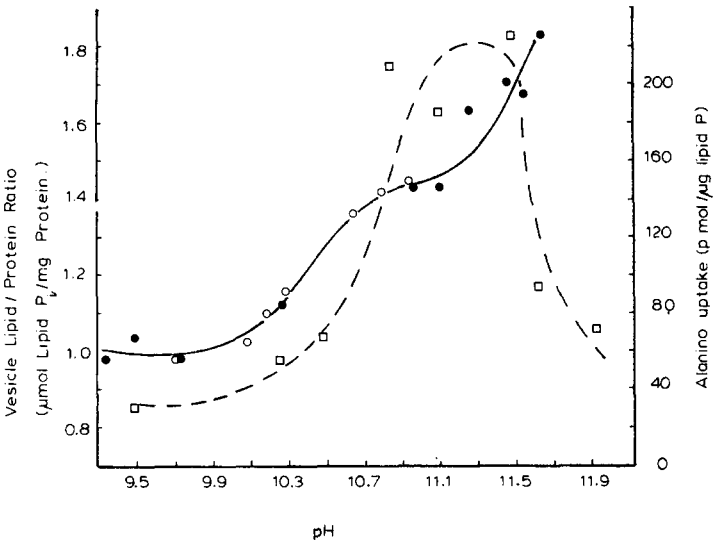

Fig. 1. The effect of the pH of extraction on the lipid : protein ratio of the residue and on the latter's ability to form 'active' vesicles. Experimental details are given in the text and Fig. 2. (○, ●) Lipid : protein ratio of residual membranes after extraction at the pH values indicated. (□) Ability of residual membrane vesicle to take up L-alanine.

vesicles was 1.25 ± 0.03 (6) μmol lipid P_i/mg protein, compared with 0.80 ± 0.02 (4) for the purified membranes, and gel electrophoresis revealed that only band 2 and band 3 proteins remained in the vesicles [4]. Further investigations of the stability of the pigeon erythrocyte membrane revealed the following facts, which are demonstrated by the data in Figs. 1 and 2.

(1) Below pH 10.0, even at low ionic strength, little protein was removed from the membranes. Scans of protein separations on acrylamide gels showed that only band P 4.51 [5] was eluted to any detectable extent (Fig. 2).

(2) None of the spectrin polypeptides were removed at pH 10.0, in marked contrast to the observations of, for example, Bennett and Branton [10] who demonstrated almost complete removal of spectrin from human erythrocyte membranes at pH 7.6, provided the ionic strength was reduced below that given by 10 mM KCl.

(3) As the pH of the EDTA-containing extraction solution was increased a distinct phase of protein removal occurred, which was half-complete at about pH 10.4 and complete at pH 10.8 (Fig. 1). The lipid : protein ratio was then stable for nearly half a pH unit at this point, but raising the pH to above 11.2 resulted in a second phase of removal of protein (Fig. 1). Fig. 2 shows that between pH 10.0 and 11.0, bands 1, 4.1, P 4.52 and 5 were eluted. Residual haemoglobin and bands 7 and 8 were also eluted, though this is not clear from the scans in Fig. 2. As the pH was raised above 11.0, band 2 was eluted, though even at pH 12.3 some remained (Fig. 2).

(4) Membrane material displaying amino acid transport activity was generated only when membranes were exposed to pH values between 10.8 and 11.4 (Fig. 1).

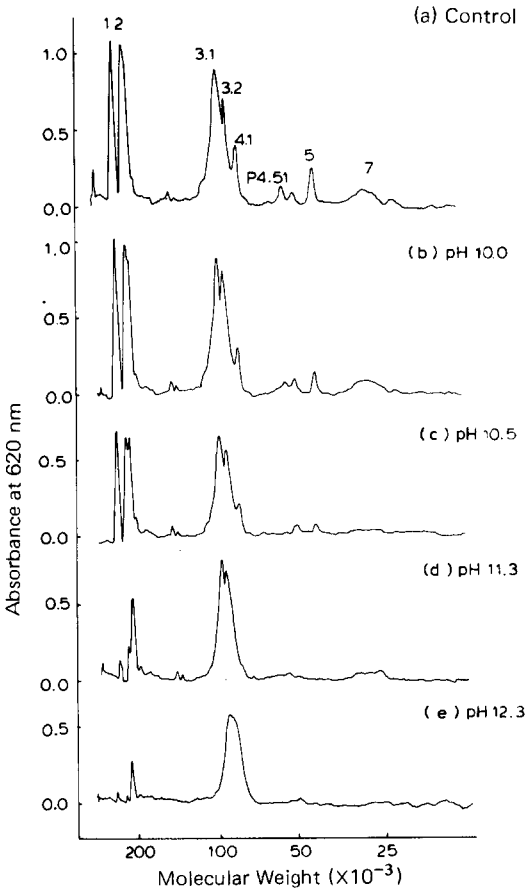

Fig. 2. The effect of the pH of extraction on the removal of proteins from the membrane. Samples of purified membranes were mixed with at least 100 vols. of 0.2 mM EDTA adjusted to the pH values indicated. After centrifugation the pellets were solubilized and subjected to electrophoresis in the presence of sodium dodecyl sulphate. Stained and fixed gels were scanned on a Joyce-Loebl densitometer. Scan a corresponds to untreated plasma membranes. Major bands are labelled in accordance with Ref. 16 as modified in Ref. 5.

(5) Observations made with the phase-contrast microscope showed that over the pH range 10.0—11.0, the appearance of the membranous material changed from a preponderance of rounded ghosts, of about 10 μm diameter, to mostly small fragments — vesicles of about 1.0 μm diameter. These may be seen in the electron micrograph reproduced in Fig. 3.

Fig. 1 demonstrates that vesicles displaying Na^+-dependent amino acid transport activity were generated only under certain conditions and explains the variation in transport activity that we sometimes observed among preparations. Residual Tris buffer and groups on the membranes may lower the pH of the EDTA solution if it is not sufficiently alkaline prior to mixing with the membranes. The subsequent effect of this on transport activity is clearly shown in Fig. 1 and is the result of the failure of the membranes to form vesicles. On the other hand, inactivation of the transport system occurred when the membranes were mixed with EDTA adjusted to too high a pH. We do not know if this was

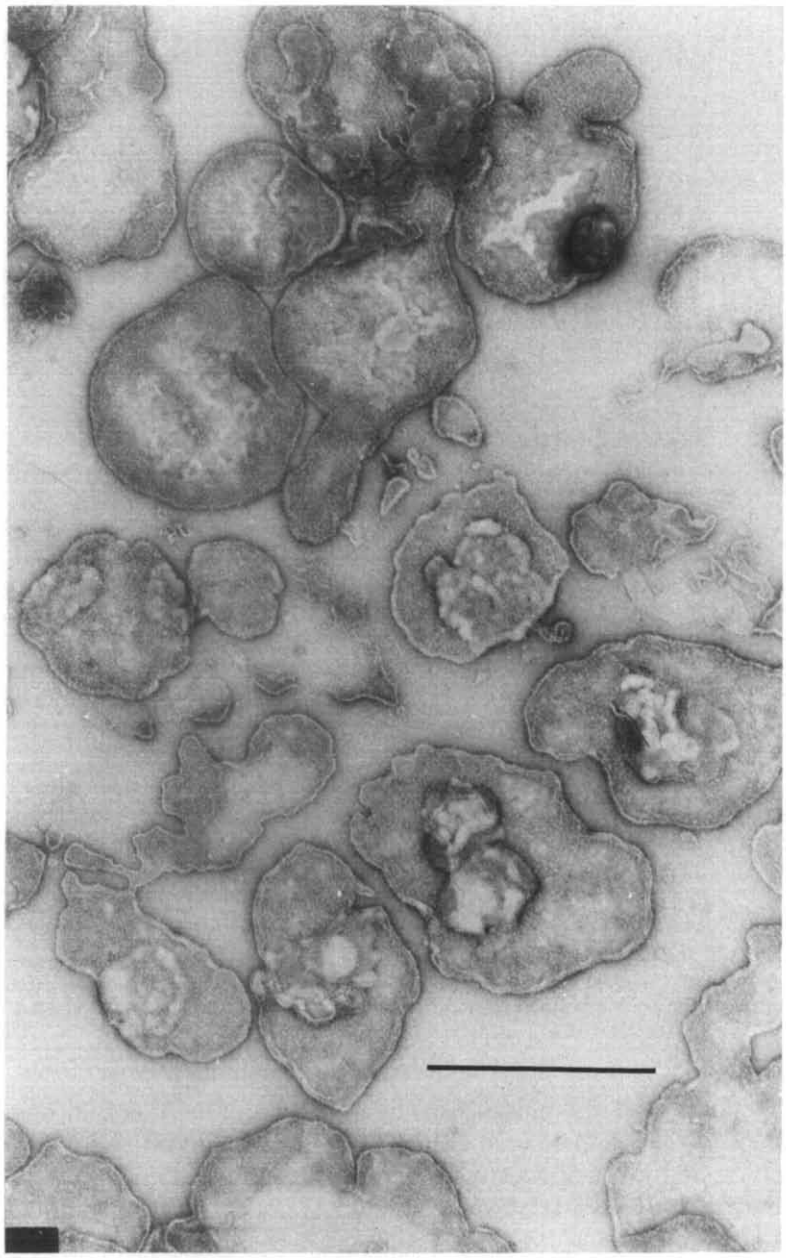

Fig. 3. Electron micrograph of negatively stained membrane vesicles from the pigeon erythrocyte. Vesicles were prepared from protein-depleted membranes of pigeon erythrocytes, as described in the text, and transferred to copper grids, where they were stained with a saturated solution of uranyl acetate. The scale bar represents 1 μm.

due to irreversible denaturation of the carrier system or to the failure of the vesicles to become sealed. Interestingly, band 3 no longer ran as two bands on gels when membranes were treated with EDTA at a pH of approx. 12.0 (Fig. 2).

It is also worth noting here that membranes excessively contamined with material from the cell nucleus did not form an active vesicle population, whichever pH they were subsequently treated with. Three such preparations had a mean (±S.E.) DNA content of 51.2 ± 12.1 μg DNA/mg vesicle protein, whereas the mean (±S.E.) DNA content of three preparatations that displayed Na^+-dependent alanine transport was 10.9 ± 1.2 μg DNA/mg vesicle protein. We were able to detect faint traces of proteins which the mobility of histones [5] when inactive vesicle preparations were subjected to electrophoresis in the presence of sodium dodecyl sulphate. It was quite clear, even to the naked eye, that a major reason for the inactivity of these preparations was their markedly aggregated state.

Discussion

Although low ionic strength is sufficient to destabilise the human erythrocyte ghost [11], in the case of the pigeon erythrocyte a 1000-fold lower H^+ concentration was also required. The data presented are compatible with the notion that the structure of the latter cell membrane is stabilised by interactions involving a group, or groups, with an apparent pK_a of about 10.4. These groups may be located on, or may interact with, skeletal proteins whose presence is required for the stability of the bilayer. One may visualise a stabilising interaction either between the skeletal proteins themselves, or between skeletal proteins and integral structures of the bilayer (i.e., integral proteins or phospholipids). This concept of cytoskeleton involving spectrin is a familar one in the case of the human erythrocyte (Ref. 12 and references contained therein).

One cannot, from the data presented above, rule out the possibility that removal of proteins from the pigeon erythrocyte ghost occurred at lower pH values, as it does in the human erythroctye ghost. However, if this were the case, it should be assumed that they became trapped inside the rounded ghost until the form of the latter was disrupted by perturbations occurring at higher pH values. Trapping of this kind seemed very unlikely in view of the large and presumably unresealable hole left by the enucleation process [5]. Thus spectrin, and probably other 'skeletal' proteins, appear to stabilise the pigeon erythrocyte membrane as they do the human erythrocyte membrane [12]. However, the interactions conferring stability appear to be quite different in the two cell types; those in the human cell are more easily perturbed. In particular, band 2 is much more tightly bound to the pigeon erythrocyte membrane and was only partially removed at pH 12.3. In the absence of other components, however, its presence was not sufficient to ensure the integrity of the cell ghost.

Interestingly, the camel erythrocyte, although enucleated, exhibits on ellipsoid shape (like the pigeon erythrocyte) and its spectrin is removed only by strongly perturbing conditions; for example, by 0.01 M NaOH [13]. It seems likely that this vary stable interaction of spectrin with the camel erythrocyte membrane permits the animal to withstand the extreme osmotic conditions that it encounters. The resistance of camel erythrocytes to osmotic lysis has been established [14]. When avian erythrocytes are exposed to

osmotic stress, restoration of cellular volume is achieved by modification of the membranes' permeability to K^+ [15]. Prior to and during this restoration of volume, the membrane must be able to maintain its integrity under the stress imposed by swelling or shrinkage. The biconcave disc form, shown by erythrocytes of most mammalian species, allows volume change to occur without stretching the membrane. The more stable interaction between spectrin and the bilayer observed in the ellipsoid-shaped cells may reflect a different evolutionary strategy for coping with volume change. Thus, a different, more subtle and more easily perturbed, interaction between spectrin and the membrane may be necessary to control the expansion of the biconcave disc form, whereas a rigid and tightly associated spectrin framework may confer mechanical strength to the membranes of expanded, elliptically shaped, erythrocytes.

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