Concanavalin A-agglutinability of membrane-skeleton-free vesicles and aged cellular remnants derived from human erythrocytes

Is the membrane skeleton required for agglutination?

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Vesicles and cell remnants have been obtained by aging of erythrocytes *in vitro*. The vesicles lacking the membrane skeletal proteins and the remnants known to possess a rigid skeleton have been used to assess the role of membrane skeletal proteins in the process of Con A (concanavalin A)-mediated agglutination of erythrocytes. Both the vesicles and the remnants were found to bind Con A at the same density as did intact cells. The vesicles, isolated from normal as well as from the Con A-agglutinable trypsin- and Pronase-treated cells, failed to agglutinate with Con A. They were, however, well agglutinated by WGA (wheat-germ agglutinin) and RCA [*Ricinus communis* (castor bean) agglutinin], indicating that the vesicles are not defective in agglutination. Large, cytoskeleton-free, vesicles prepared by another procedure also gave the same results. The aged remnants from trypsin- and Pronase-treated erythrocytes showed significantly decreased agglutination with Con A, but were agglutinated as well as the fresh cells by WGA and RCA. The agglutination with Con A is thus abolished when the membrane skeleton is absent, and reduced when it is rigid, suggesting that the skeleton may play an important role in the agglutination of erythrocytes by Con A.

INTRODUCTION

For shear-resistant agglutination of cells to occur, it is required that the cells are held together via multiple ligand-mediated bridges [1]. Extensive work with lectin-induced agglutination of tumour/transformed cells has demonstrated that, after lectin binding, the receptor molecules undergo aggregation in the membrane (reviewed in ref. [2]). The aggregation not only precedes the onset of agglutination: its prevention, by chemical cross-linking of membrane proteins or by a decrease in membrane fluidity leads to the abolition of agglutination itself. Normal cells, which do not agglutinate with lectins except at high concentrations, do not show the receptor aggregation, although they do bind the lectin in amounts equal to those bound by transformed cells. It has been suggested that, in the normal cell, the transmembrane lectin receptor is anchored on the cytoplasmic side to a cytoskeletal structure and is hence incapable of lateral movement [3,4]. The treatment of normal cells with a proteolytic enzyme brings about increased agglutinability and, simultaneously, the aggregability of the receptors. According to some reports the proteinase treatment of cells causes the disruption of cytoskeletal elements [5,6], releasing the receptor molecule from mobility restraints. In transformed cells, the receptor is free to move about, owing to the paucity of organized cytoskeletal structures, especially microfilaments [2,7]. Thus the difference in the cytoskeletal control of receptor mobility is believed to be the major determinant of the differential lectin-agglutinability of normal and transformed cells [2,8].

Human erythrocytes show shear-resistant agglutination with Con A only after treatment with a proteolytic enzyme [9]. The mobility of the Con A receptor, band 3 component, is severely restricted by the membrane-associated meshwork of proteins called the membrane (cyto)skeleton [10]. This occurs in two ways: (i) binding of 10–15% of the band 3 molecules to the major skeletal component, spectrin, via ankyrin; and (ii) the entrapment of the long *N*-terminal end of the molecule in the interstices of the skeletal meshwork. The removal of the skeletal proteins from the membrane leads to a 250-fold increase in the mobility of band 3. To investigate the role of the membrane skeleton in the process of Con A-mediated agglutination of human erythrocytes, we have used the vesicles shed by the cells during aging *in vitro*. The membrane of the vesicles is known to be identical in composition with that of the cellular membrane, except in the content of skeletal proteins [11]. Band 3 molecules in the vesicular membrane show greater chemical cross-linking, indicating their higher lateral mobility [12]. The skeleton of the aged cellular remnants is also known to undergo alterations, making it substantially rigid compared with the skeleton of fresh cells [13]. Therefore the agglutinability of the remnants is also likely to be informative and is included in the study.

The results show that not only vesicles isolated from normal cells, but also those obtained from proteinase-treated cells, are unagglutinable with Con A. The aged cell remnants from erythrocytes treated with a proteolytic

Abbreviations used: Con A, concanavalin A; RCA, *Ricinus communis* agglutinin; WGA, wheat-germ agglutinin; TBS, Tris-buffered saline (0.01 M-Tris/HCl, pH 7.4, containing 0.15 M-NaCl); MeMan, α -methyl D-mannopyranoside.

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enzyme also show a significant decrease in their Con A-agglutinability.

MATERIALS AND METHODS

WGA, 2-acetamido-2-deoxy-D-glucopyranoside and diamide [diazinedicarboxylic acid bis (*NN*-dimethylamide)] were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Lactose was purchased from BDH, Poole, Dorset, U.K. RCA was prepared from locally available castor beans (*Ricinus communis*) by the procedure of Nicolson & Blaustein [14]. The sources of all other reagents were described in the preceding paper [15].

Chloroform and methanol were distilled before use.

Cell remnants and vesicles

The collection of blood, washing of erythrocytes and the conditions of their enzymic treatment were described in the preceding paper [15].

For the preparation of vesicles and cell remnants, washed erythrocytes, either directly or after enzyme treatment, were aged *in vitro*. The cells were suspended in 10 vol. of TBS containing 250 units of penicillin/ml and 200 μ g of streptomycin/ml, and incubated at 37 °C with intermittent mixing. The glassware and the solutions were sterile, although no special precautions were taken to carry out the washing of cells aseptically.

After 48–56 h the suspension was centrifuged at 400 *g* for 10 min. The supernatant was saved and the pellet of cell remnants was resuspended to the original volume in TBS and re-centrifuged. The combined supernatants served as the source of vesicles. The suspension of vesicles was centrifuged once more at 400 *g* for 10 min, and then left overnight in the cold to remove any contaminating remnants. The vesicles were finally sedimented at 12000 *g* for 15 min in the cold, washed once in TBS, and resuspended in the same buffer to yield an absorbance of 0.15–0.2 at 660 nm. This procedure of vesicle preparation is adapted from that described by Lutz and co-workers [12].

Large, skeletal-protein-free, vesicles were prepared essentially as described by Leonards & Ohki [16].

Agglutination

The agglutination of cells and remnants was assessed microscopically as described in the preceding paper [15]. All lectin agglutination assays were accompanied by appropriate controls employing specific inhibitory sugars: 0.05 M-MeMan for Con A, 0.1 M-*N*-acetylglucosamine for WGA, and 0.1 M-lactose for RCA. Suitable alterations were made in the buffer to adjust for the contribution of the sugar to osmolarity.

The extent of vesicle agglutination was measured by the lectin-induced increase in A_{660} . To 0.5 ml of vesicle suspension, previously adjusted to an absorbance of 0.15–0.20, an equal volume of lectin solution was added.

Fig. 1. SDS/polyacrylamide-gel electrophoresis of membrane proteins of cells, remnants and vesicles

Cell remnants and vesicles were prepared from normal, trypsin and Pronase-treated erythrocytes by aging *in vitro*. Membranes of fresh cells and remnants were obtained by hypo-osmotic lysis and washing. Since the vesicles cannot be lysed, total vesicular protein was applied for electrophoresis. The membranes and vesicles were dissolved in SDS and mercaptoethanol, and electrophoresed in 8.5% -acrylamide running gel. The gel was stained with Coomassie Blue. Lanes a, b and c, membranes from normal, trypsin- and Pronase-treated fresh cells respectively (65 μ g of protein/lane); lanes d, e and f, membranes from remnants of normal, trypsin- and Pronase-digested cells (75 μ g of protein/lane); lanes g, h and i, vesicles isolated from normal, trypsin- and Pronase-treated cells (120 μ g of protein/lane). Note that the bulk of the vesicular protein, namely haemoglobin, moves ahead of membrane proteins under the conditions of electrophoresis, and is not included in the photograph. The arrows indicate the Pronase-cleaved fragment of band 3. The band numbers on the left identify the proteins.

Several lectin concentrations and a control with the inhibitory sugar were used. After incubation at 37 °C for 60 min and vortex-mixing for 10 s, the absorbance of the suspension was measured.

¹²⁵I-Con A binding

Radioiodination of Con A, and its binding to cells, remnants and vesicles, was studied as described in the preceding paper for cells [15].

Diamide treatment of cells

Trypsin- and Pronase-treated cells were treated with 5 vol. of 1–5 mM-diamide dissolved in TBS. After incubation at 37 °C for 30 min, the cells were centrifuged and washed four times in the cold with TBS.

Other methods

The methods of preparation of ghosts, SDS/polyacrylamide-gel electrophoresis and protein determination were as described in the preceding paper [15].

Total lipid was isolated from cells, remnants and vesicles by extraction (twice) in 50 vol. of chloroform/methanol (2:1, v/v). The extracts were dried, and the organic phosphate was liberated and determined as described by Ames [17].

RESULTS

Characterization of vesicles and remnants

Incubation of trypsin- and Pronase-treated cells in the absence of an energy source yielded vesicles similar in amounts to those obtained from untreated cells. The yield of vesicles estimated in terms of A_{660} was ~ 0.3 when the vesicle pellet, obtained by aging 2 ml of packed cells, was suspended in 10 ml of TBS. SDS/polyacrylamide-gel electrophoresis of vesicles and Coomassie Blue staining showed the near absence of spectrin and actin and a substantial decrease in the amount of band 4.1 (Fig. 1). Densitometric scans of the gel patterns showed a 4-fold increase in the ratio of band 3 to band 4.1 in vesicles as compared with the ratio in cell membranes. Thus the vesicles are not only spectrin-free (the small amount of spectrin seen is probably due to slight contamination with remnants; see ref. [11]), but are depleted of actin and also (to a significant extent) of band 4.1. The three proteins form the major components of the membrane skeleton [10]. Band 3 (or its corresponding Pronase-derived fragment), band 4.5 and band 4.2 were the predominant Coomassie Blue-stained proteins of the vesicle membrane (Fig. 1). The results agree with those of Müller *et al.* [18]. In some experiments phosphate or glycylglycine buffer was used in place of Tris. The yield of vesicles and their agglutination behaviour (below) were the same in both cases.

The membrane proteins of the remnants showed qualitative identity with the proteins of the ghosts of corresponding fresh cells (Fig. 1). The crenated remnants from normal or enzyme-treated cells, after 48 h of incubation, could not be reversed to the disc shape by incubation in glucose, adenosine, inosine and phosphate, indicating irreversible alterations in their membrane skeleton [13].

Con A binding to cells, remnants and vesicles

The ability of fresh normal, trypsin- and Pronase-treated cells, and the remnants and vesicles derived from

them, to bind Con A was assessed by using ¹²⁵I-labelled lectin. In all cases the maximal binding was observed at and above 500 μ g of Con A/ml (results not shown). Subsequent binding studies were carried out with Con A at 500 μ g/ml.

In each binding experiment 10⁷ fresh cells or 10⁷ remnants and vesicles obtained from approx. 8×10^8 cells were used. Since the surface areas of the cells, remnants (which are highly crenated) and vesicles (which possess a very small mean diameter, namely 185 nm; ref. [11]) would be variable, the number of Con A-binding sites could not be directly compared. For this reason, phospholipids were extracted from the same number of fresh cells and remnants and from a portion of the vesicular suspension used for lectin-binding studies. From the amount of lipid phosphorus found, the Con A bound to remnants and vesicles was recalculated in terms of the phospholipid present in 10⁷ fresh cells.

Table 1 presents the Con A-binding abilities of cells, remnants and vesicles of the three types: untreated, and treated with trypsin or Pronase. No significant differences are found in the densities of Con A-binding sites per unit surface area, expressed in this case on the basis of phospholipid content.

Agglutination with Con A

The skeletal-protein-free vesicles obtained from normal cells, like intact cells, failed to show any agglutination with Con A. What was surprising, however, was the total lack of agglutination of the vesicles derived from the Con A-agglutinable trypsin- and Pronase-treated cells (Fig. 2a). The results were reproducible in six different experiments and were not

Table 1. Quantification of Con A binding to erythrocytes and their aged remnants and vesicles

Normal, trypsin- and Pronase-treated cells were subjected to aging *in vitro*, and vesicles and remnants were isolated. Fresh cells (10⁷), remnants (10⁷) and vesicles obtained from approx. 8×10^8 cells were incubated with different concentrations of ¹²⁵I-labelled Con A from 0 to 1000 μ g/ml. After 60 min at 37 °C, the membrane-bound and free Con A were separated by centrifugation at 8000 *g* for 2 min and the radioactivity in the pellet was determined. Maximal binding was observed at [¹²⁵I-Con A] values of 500 μ g/ml and above. In two subsequent experiments the binding was studied at a Con A concentration of 500 μ g/ml. The averages $\pm$ s.d. for the three experiments are given below. The amount of Con A bound (in ng) is expressed on the basis of phospholipid equivalent to 10⁷ fresh cells. Under these conditions, 10⁷ cells yielded 3.9 nmol, 10⁷ remnants 3.3 nmol, and vesicles from 8×10^8 cells 11.0 nmol of organic phosphate in the phospholipid extracts (averages for three experiments). Similar values were found for the cells, remnants and vesicles of trypsin- and Pronase-treated samples. $P > 0.1$ in all cases.

Cells . . .	Con A bound (ng)		
	Normal	Trypsin-treated	Pronase-treated
Fresh cells	239 $\pm$ 38	200 $\pm$ 56	186 $\pm$ 28
Remnants	221 $\pm$ 24	211 $\pm$ 3	190 $\pm$ 17
Vesicles	223 $\pm$ 82	166 $\pm$ 45	188 $\pm$ 31

altered by increasing the Con A concentration, vesicle density and the period of incubation, or by agitation of the suspension during incubation. Moreover, the vesicles obtained from papain- or neuraminidase-treated cells (agglutinable with Con A) also failed to show any agglutination with the lectin (results not shown). Isolation of vesicles from normal cells and their subsequent treatment with trypsin also did not produce agglutination with Con A (results not shown).

The remnants of normal cells were not agglutinated by Con A. Those obtained from trypsin- or Pronase-treated cells were agglutinated, but to the extent of 45–75% of the fresh-cell value at different concentrations of the lectin (Fig. 2b). The decrease in agglutination was statistically significant ($P < 0.01$ – 0.001) at all concentrations of Con A. The rigid membrane skeleton therefore appears to influence the agglutination of cells by Con A. Since metabolic reinstatement was not possible for the remnants, we could not test whether derigidification of the skeleton would restore the level of agglutination.

Agglutination by WGA and RCA

Since the vesicles and remnants bind Con A to an extent almost equivalent to that shown by fresh cells (Table 1), the lack of vesicle agglutination and the

decreased agglutination of remnants with Con A could be a 'deleterious' effect of aging. In that event their agglutinability by other lectins should also be affected. Hence the agglutinability with WGA and RCA was studied. Both these lectins, unlike Con A, agglutinate normal human erythrocytes. The agglutination of trypsin- and Pronase-treated cells was slightly higher than that of untreated cells by each of them (Figs. 3a and 3b, insets). The vesicles derived from normal and enzyme-treated cells were not only agglutinated by the two lectins, but showed the preservation of the agglutination differences exhibited by the respective intact cells (Figs. 3a and 3b). However, the vesicles apparently required a somewhat higher lectin concentration for agglutination than did the cells.

Although the agglutination response of the vesicles appears small (ΔA_{660} of about 0.12), the 'low' value must be ascribed to relative insensitivity of the method used for the quantification of agglutination. The clumps formed are large enough to be visible by the unaided eye, and become so large at lectin concentrations above $20 \mu\text{g/ml}$ that they settle down rapidly in the cuvette, precluding the proper determination of absorbance.

The remnants of normal, trypsin- and Pronase-treated cells agglutinated very well with WGA and RCA (Figs. 4a and 4b); in fact, a little better than the corresponding

Fig. 2. Con A-agglutinability of vesicles and cellular remnants isolated from normal, trypsin- and Pronase-treated cells

Cell remnants and vesicles were isolated from normal, trypsin- and Pronase-digested cells and were treated with different concentrations of Con A and incubated at 37°C for 60 min. (a) The vesicle suspension was vortex-mixed for 10 s and the A_{660} was measured. The increase in A_{660} is taken as the measure of agglutination. The inset shows the agglutination of fresh cells. (b) The suspension of remnants was tapped several times and the extent of agglutination was determined microscopically. Inclusion of 0.05 M-MeMan with Con A ($250 \mu\text{g/ml}$) abolished the agglutination completely (result not shown). —, Fresh cells; ----, remnants. The vesicles, remnants and free cells have the same symbols: Δ, normal; ○, trypsin-treated; ●, Pronase-treated. Each point is an average for six individual experiments. The vertical bar denotes $\pm$ s.d.

Fig. 3. Agglutination of vesicles with WGA and RCA

Vesicles were isolated from normal (Δ), trypsin ($\circ$) and Pronase-digested ($\bullet$) cells after aging *in vitro*. The vesicles were incubated with various concentrations of WGA (a) and RCA (b). After 60 min incubation at 37 °C and vortex-mixing for 10 s, the A_{660} was measured. Agglutination is expressed in terms of increase in A_{660} . '(+ Sugar)' indicates 0.1 M-*N*-acetylglucosamine for WGA and 0.1 M-lactose for RCA. The insets show the agglutination of fresh, normal, trypsin- and Pronase-treated cells. All values represent averages of six determinations $\pm$ S.D.

fresh cells at lower concentrations of the lectins. The increase, however, was not statistically significant. Remnants from the Pronase-treated cells were agglutinated better than those from trypsin-treated cells, whereas the normal cell remnants were the least agglutinable. Thus the remnants exhibited the same agglutination pattern as the respective fresh cells. To avoid overcrowding the Figures, the results for proteinase-treated cells and their remnants are not presented.

Agglutination of diamide-treated cells

The Con A-mediated agglutination of trypsin- and diamide-treated cells was nearly as good as the agglutination of trypsin-treated cells (Fig. 5). Similar results were obtained with Pronase-treated cells (results not shown). The diamide-treated cells are known to undergo loss of phospholipid asymmetry [19] and of cellular deformability [20]. The two characteristics thus seem to be unimportant for Con A-mediated agglutination of erythrocytes.

Agglutination of large vesicles

After the completion of the above experiments, another procedure for the isolation of skeletal-protein-free vesicles was published [16]. The method involves the treatment of cells with Ca^{2+} and EDTA at 45 °C and yields vesicles that are large enough to be seen under the optical microscope. The vesicles prepared from normal and proteinase-treated cells were tested for their Con A-,

WGA- and RCA-agglutinabilities. Essentially the same results were obtained as with the aged vesicles (results not shown).

DISCUSSION

If the restriction of band 3 mobility were responsible for the lack of agglutinability of human erythrocytes with Con A, the removal of skeletal proteins should have brought about the agglutinability. However, from the study described in the preceding paper [15] the removal of glycophorin (or the sialic acid attached to it) has been found necessary for Con A-mediated agglutination to occur. The lack of agglutination of vesicles from trypsin- or Pronase-treated cells, devoid of both glycophorin and the skeleton, was therefore surprising. Since the vesicles bind Con A to the same extent as intact cells and are also agglutinable with WGA and RCA, the absence of agglutination with Con A appears to be a specific loss. The same results were obtained with vesicles prepared by two different methods. Since the membranes of proteinase-treated cells and their corresponding vesicles differ mainly in respect of the skeleton, the latter would appear to be the major candidate responsible for the loss of agglutinability. The decreased agglutinability of the remnants, possessing a rigid skeleton, would tend to support such a possibility. The diminished agglutinability of the remnants, again, occurs only with Con A and not with WGA or RCA.

Fig. 4. Agglutination of cellular remnants by WGA and RCA

Cellular remnants obtained by aging *in vitro* were treated with various concentrations of WGA (a) and RCA (b). After incubation of 60 min at 37 °C, the suspensions were tapped several times and the extent of agglutination was determined microscopically. The agglutination of fresh cells and their remnants is given. The average $\pm$ S.D. of six determinations is given. The inclusion of 0.1 M-N-acetylglucosamine and 0.1 M-lactose totally abolished the agglutination due to WGA and RCA respectively. The higher agglutination of remnants at lower concentrations of lectins is not statistically significant ($P > 0.1$). Similar results were obtained with the remnants of trypsin- and Pronase-treated cells. These results are not shown. —, Agglutination of fresh cells; ----, that of the remnants.

Fig. 5. Con A agglutination of diamide-treated erythrocytes

Trypsin- and Pronase-digested erythrocytes were treated with TBS or 1–5 mM-diamide for 30 min at 37 °C. The cells were washed and their agglutinability with Con A was assessed. The agglutination of untreated, trypsin-treated (Δ) and those treated with 1 mM ($\square$)- and 5 mM ($\bullet$)-diamide is shown. Other data are omitted for the sake of clarity.

Apart from the absence of skeletal proteins, the vesicular membrane has been reported to undergo the loss of its phospholipid asymmetry [21]. Also, the ATP-depleted remnants would be expected to be non-deformable. Deformability has been advanced as an important property determining the agglutinability of cells [22]. The unimpaired agglutinability of the diamide-treated cells (Fig. 5), which undergo both the loss of membrane phospholipid asymmetry [19] and deformability [20], has shown that neither characteristic is crucial to Con A-mediated agglutination of erythrocytes.

Thus the absence of membrane skeleton remains the best candidate for explaining the loss of Con A-agglutinability of erythrocytes. How could it help in the process of agglutination? Since ligand-mediated multiple bridges are necessary for shear-resistant agglutination [1], either the receptors must aggregate in the membrane of each cell or the interacting cells must form multiple contacts between discretely located receptors. The latter mechanism, requiring substantial cell-surface contacts, can operate if the cells are highly deformable. But, since the abolition of deformability (in diamide-treated cells) does not affect Con A-mediated agglutinability, obviously the agglutination should involve the aggregation of Con A receptors.

In nucleated cells after ligand binding the receptor molecules often interact with cytoskeletal structures and later undergo redistribution together with them (see ref. [23] and references therein). The contractile force of the skeletal elements, especially microfilaments, is believed to bring about the aggregation of the ligand-receptor complexes. Tumour/transformed cells, which show the aggregation of lectin-receptor complexes [2], however, are completely or greatly depleted in microfilaments and possess microtubules which may be abnormal or few in number [7]. Many eukaryotic cells have recently been found to possess proteins similar to those present in the erythrocyte membrane skeleton [24], and, in all likelihood, also contain a skeleton as part of their plasma membrane [25]. The skeleton may also restrict the mobility of integral proteins [25,26], as in the case of band 3 in the erythrocyte membrane [10]. There are several instances where the ligand-induced aggregation of receptor clustering is reported to be accompanied by a superimposable clustering of spectrin- or ankyrin-type proteins (see, e.g., refs. [27-29]) in these cells. In at least one instance this clustering has been shown to be unaffected by cytochalasin B [28], the microfilament-depolymerizing agent. Thus, as in the case of transformed cells, the aggregation of receptor molecules and membrane skeletal proteins can occur in the absence of microfilaments. The erythrocyte membrane skeleton, the prototype of the cell membrane skeleton, has recently been found to contain myosin [30,31], tropomyosin [32] and troponin I [24], which suggests that the skeleton very likely has contractile properties [24], as was indicated previously [33-36]. The skeleton would thus have the ability to bring about the aggregation of integral proteins attached to it. In the case of Con A-mediated agglutination of erythrocytes, lectin binding would be expected to induce new interactions between band 3 and skeletal proteins before the suggested aggregation (we provide some evidence for this in the following paper [37]).

In previous reports [9,38] the formation of Con A-receptor aggregates during Con A-mediated agglutination of erythrocytes was ruled out. This was based on the observation that the agglutination was not affected at low temperatures, where the fluidity of the membrane was expected to be abolished. However, it is now known that the erythrocyte membrane remains fluid down to -5°C [39]. In another report the aggregation of intramembranous particles (which contain band 3 protein) was observed in the membranes of erythrocytes treated with both Pronase and Con A [40]. Those authors, however, did not consider this pertinent to agglutination, since succinyl-Con A (a weakly agglutinating derivative of Con A) was also able to induce the aggregation. If, as suggested above, the aggregation is primarily caused by the skeleton after the Con A (succinyl-Con A)-induced interaction of band 3 with the skeleton, rather than by the lectin, the results obtained [40] would be expected.

We envisaged that the aggregation of lectin-bearing receptor molecules, caused by the skeleton, enhances the possibility of cross-linking of receptor molecules by the lectin by holding them together for sufficient time. In the ATP-depleted remnants, although the skeleton is rigid, mere lectin-induced interaction of band 3 with the skeletal elements would help to 'concentrate' the receptors in 30% of the membrane area, since this is

approximately the area covered by spectrin strands [34]. If the skeletal structure is partly aggregated [13], as a result of ATP-depletion, the 'concentration' would be even greater. This would explain the agglutination of the proteinase-treated remnants by Con A, although to a decreased extent.

It may be pointed out that band 3-incorporated liposomes are agglutinated by Con A [41,42], despite the absence of skeletal proteins in them. However, band 3 molecules exist in an already aggregated state [41,43] in the membranes, possibly as an artefact of the purification procedure.

Both WGA [44] and RCA [45] binding to erythrocytes have been reported to induce alterations in the membrane skeleton. Obviously the effects are not relevant to the agglutination caused by them. The two lectins, apart from causing agglutination of the skeletal-protein-free vesicles, differ from Con A in another respect: they agglutinate human erythrocytes without prior proteinase treatment.

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