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FORMATION OF PROTEIN POLYMERS IN ERYTHROCYTE GHOSTS INCUBATED WITH SONICATED LIPID VESICLES

EFFECTS ON SPECTRIN EXTRACTIBILITY, PERMEABILITY OF GHOSTS TO VESICLES, INTRAMEMBRANE PARTICLE DISTRIBUTION AND BLEB FORMATION

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The incubation of human erythrocyte white ghosts with phosphatidylcholine (PC) vesicles or cholesterol/phosphatidylcholine (C/PC) vesicles under hypotonic or isotonic conditions generated membrane protein cross-links. The latter appeared in the form of a high molecular weight polymer after SDS-polyacrylamide gel electrophoresis. The polymer started to develop within a few minutes of incubation, arising largely from spectrin, and required 24 h or more for completion. It occurred regardless of cholesterol depletion undergone by the ghosts in the presence of PC vesicles. It was not reversed upon further incubation in a hypotonic, vesicle-free medium. When initial incubation was carried out under hypotonic conditions, a number of other alterations were recorded: (i) spectrin extractibility was abolished; (ii) ghosts became gradually impermeable to vesicles within a few hours, a process referred to as slow 'resealing' and generating an irreversible sequestration of the vesicles; (iii) intramembrane particles aggregated and blebs free of intramembrane particles pinched off inward or outward. When initial incubation was conducted under isotonic conditions, the following was observed: (i) spectrin was unextractible, as could be expected; (ii) vesicles did not enter the ghosts, a fact indicating an immediate and complete impermeabilization of ghosts to vesicles, a process referred to as fast 'resealing'; (iii) intramembrane particle aggregation and blebs free of intramembrane particles were also present. When initial incubation was performed under isotonic conditions, but in the absence of vesicles, the polymer failed to be associated with spectrin inextractibility. These data support the view that lipid vesicles generate a high molecular weight polymer-associated, slow resealing of erythrocyte ghosts that differs, at least in part, from the polymer-free, fast resealing induced by a vesicle-free isotonic medium. Resistance to β-mercaptoethanol of the polymer makes unlikely the sole participation of disulfide bonds. Absence of added Ca^{2+} in the medium is inconsistent with the transglutaminase-catalyzed formation of amide linkages. When ghosts were separated from the vesicles by a cellophane membrane upon hypotonic incubation, spectrin remained extractible and no polymer developed. Sonication of the vesicles under nitrogen and in the presence of butylated hydroxytoluene did not prevent the formation of the polymer.

Abbreviations: *C/P*, cholesterol-to-phospholipid molar ratio; C/PC, cholesterol/phosphatidylcholine; PC, phosphatidylcholine; SDS, sodium dodecyl sulfate; TEMED, *N,N,N',N'*-tetramethylethylenediamine.

Therefore, one can rule out the involvement of such bifunctional, diffusible compounds as malonyldialdehyde that are likely to arise from lipid peroxidation and to cross-link membrane proteins through Schiff base formation. Thiobarbituric acid-reactive material remained hardly detectable. At the present time, the vesicle-induced cross-links do not clearly resemble any of those commonly encountered between erythrocyte membrane proteins.

Introduction

Cross-linking of proteins refers to the formation of covalent bonds between distinct polypeptide chains, generating high molecular weight polymers. Erythrocyte membrane proteins are likely to undergo cross-linking under a variety of conditions. Oxidant stress may induce disulfide bonds [1–3] that are recognizable since they are reducible with β-mercaptoethanol. In some instances, oxidant stress may also lead to non-reducible cross-links [4,5]. In these cases, it has been postulated that lipid peroxidative breakdown products, such as malonyldialdehyde, produce the cross-links through Schiff base formation [6]. More recently, attention has been drawn onto another type of cross-link, involving the formation of amide linkages catalyzed by a Ca^{2+}-dependent transglutaminase [7–11].

Each of these modifications brings about important changes of ghost properties. It should be pointed out, however, that ghost proteins can acquire alterations that do not apparently involve the establishment of covalent bonds and that, in any event, do not engender any high molecular weight polymer, thus remaining electrophoretically 'silent'. This is the case, for example, of white erythrocyte ghosts incubated under isotonic conditions and undergoing 'resealing [12,13]. By resealing, we shall refer in this paper to the rendering of white ghosts impermeable to lipid vesicles (and not to cations), in a way similar to that used by Johnson and Kirkwood for hemoglobin [12,13].

In an initial search of the effects of cholesterol depletion on various erythrocyte membrane proteins, we noted that incubation of human erythrocyte white ghosts with phosphatidylcholine (PC) or cholesterol/phosphatidylcholine (C/PC) vesicles lead to the formation of a high-molecular-weight polymer associated with dramatic changes of ghost characteristics. The polymer was hypothesized to result from membrane protein (mainly spectrin) cross-linking. Cross-linking was unexpected, since it did not appear when intact red cells were used instead of ghosts [14]. We describe a number of alterations associated with, if not resulting from, the high-molecular-weight polymer ghosts are incubated with vesicles: loss of spectrin extractibility, redistribution of intramembrane particles and bleb formation and, in hypotonic conditions, progressive impermeabilization of ghosts to vesicles. The cross-links are not disulfide bonds. They do not seem to arise from the transglutaminase-catalyzed synthesis of amide linkages. Nor do they seem to result from amino group bridges with such bifunctional reagents as malonyldialdehyde through Schiff base formation.

Material and Methods

Chemicals. L-α-Phosphatidylcholine from egg yolk (fraction V E) and cholesterol were purchased from Sigma; SDS, TEMED and ammonium persulfate from BioRad. Most of the other chemicals were obtained from Merck. Glycerol tri-[9,10(n)-^{3}H]oleate was obtained from Amersham International.

Preparation of erythrocyte ghosts. Blood was collected from healthy volunteers in heparin. Erythrocytes were washed three times with cold isotonic solutions. Ghosts were prepared essentially according to Dodge et al. [15] and stored at $-70°C$. They were thawed only once and washed with 5 mM Tris buffer (pH 7.5) at 4°C, before use.

Preparation of PC and C/PC vesicles. PC and C/PC vesicles refer to (i) vesicles containing PC and (ii) vesicles containing cholesterol and PC (in a molar ratio of 0.85), respectively. 100 mg PC (PC vesicles) or 33 mg cholesterol + 66 mg PC (C/PC vesicles) in organic solvents were mixed with glycerol tri-[^{3}H]oleate, a non-exchangeable marker which was used to detect the presence of vesicles associated with the ghosts after their co-incubation

and to calculate the vesicle recovery after sonication. The solvents were evaporated under nitrogen at 30°C. The lipid mixture was redissolved in 10 ml ethanol, evaporated again and then dispersed in 30 ml of either 5 mM Tris (pH 7.5) alone or 5 mM Tris (pH 7.5) and 140 mM NaCl, two media that defined the hypotonic or the isotonic conditions, respectively.

Dispersion was sonicated at 4°C for 15 min with a probe sonicator to form unilamellar vesicles. Sonication was performed under air or nitrogen and/or in the presence of butylated hydroxytoluene. Following sonication, centrifugation (100000 × *g*, 1 h, 4°C) pelleted undispersed lipids and titanium particles. The supernatant contained 60–80% of the initial amount of lipids in the form of vesicles. C/PC vesicles after sonication usually had a *C/P* ratio of about 0.85. Vesicles were prepared fresh before each incubation.

Standardized incubations were performed under agitation at 25°C for 15 h in hypotonic (5 mM Tris (pH 7.5)) or isotonic (5 mM Tris (pH 7.5)/140 mM NaCl) medium containing 0.5 mM NaN_3. Ghost protein concentration was approx. 0.1 mg/ml. The C/PC or PC vesicle/ghost ratio (mg PC/mg protein) was between 10 and 25. PC vesicles induced a 15–30% cholesterol depletion in erythrocyte ghosts. After incubation, suspensions were centrifuged (27000 × *g*, 4°C, 15 min) in order to pellet erythrocyte ghosts. Supernatants were concentrated 25-fold by ultrafiltration using Amicon PM-10 filters or Minicon B-15 cells. Membrane pellets were washed thrice with the hypotonic solution in all cases. Proper amounts of the supernatants and pellets were saved for analytical procedures (see below).

In typical experiments, two subsequent incubations (15 h, 25°C) were carried out. G1, G2 and G3 refer to ghosts pelleted following an initial incubation under hypotonic conditions with buffer alone (G1), C/PC vesicles (G2) or PC vesicles (G3). S1, S2 and S3 refer to the corresponding supernatants. G11, G12, and G13 designate the ghost pelleted after subsequent incubation of G1, G2 and G3, respectively in a still hypotonic, but now vesicle-free medium. S11, S12 and S13 designate the corresponding supernatants. G4, G5 and G6 refer to ghosts pelleted following initial incubation under isotonic conditions with buffer alone (G4), C/PC vesicles (G5) or PC vesicles (G6). S4, S5 and S6 refer to the corresponding supernatants. G14, G15 and G16 designate the ghosts pelleted after subsequent incubation in a hypotonic, vesicle-free medium of G4, G5 and G6, respectively. S14, S15 and S16 designate the corresponding supernatants.

In another type of experiment, ghosts were placed in a dialysis bag (Spectrapor: M_r cut-off 3500) in order to be physically separated from the vesicles during incubation. Otherwise, incubation was conducted as usual wit PC vesicles at 25°C, for 15 h. At the end, the content of the bag was centrifuged to separate the ghosts pellet and the supernatant. Malonyldialdehyde was assayed in the supernatant (see below).

Analytical procedures. Protein determination was performed according to Lowry et al. [16], using bovine serum albumin as standard. SDS-polyacrylamide gel electrophoresis of membrane protein was carried out essentially according to Fairbanks et al. [17]. β-Mercaptoethanol was used at the basal concentration of 6 mM, in order to suppress background aggregates that unavoidably develop and impede the reproducibility of the electrophoretic profiles. 100 mM β-mercaptoethanol was used to tentatively reduce more specific aggregates due to other disulfide bonding. Protein bands were numbered according to Fairbanks et al. [17]. Membrane lipid were extracted with isopropanol/chloroform [18]. Cholesterol content was measured either chemically with *o*-phthalaldehyde [19] or enzymatically with cholesterol oxidase (Boehringer-Mannheim). Both techniques yielded the same results. Phospholipids were determined according to Bartlett [20]. The amount of exogenous lipids reflecting vesicle attachment was assayed by measuring the radioactivity of the lipid extract, or was derived from phospholipid or cholesterol measurements. Oxidation products arising from the breakdown of polyunsaturated long-chain fatty acids (assumed to be mainly malonyldialdehyde) were determined with the thiobarbituric procedure [21,22]. The purity of the PC used, as well as that of the sonicated vesicles, was checked for the absence of lysoPC by TLC with chloroform/methanol/H_2O (65 : 35 : 8, v/v) Since no lysophosphatidylcholine was detectable, its amount, if any, was less than 1%, of the phos-

phatidylcholine, given the sensitivity of the technique.

Freeze-fracture electron microscopy. Samples of ghost suspensions were fixed at 37°C for 30 min with 2.5% glutaraldehyde in 5 mM Tris (pH 7.5). They were washed once with 5 mM Tris (pH 7.5) and permeated with 30% glycerol. They were then frozen into copper discs by immersion into liquid Freon 22 cooled by liquid nitrogen. The freeze-fractures were carried out in a Balzers BA-360M apparatus at -118°C under $2 \cdot 10^{-6}$ torr vacuum. Replicas consisted of platinum carbon shadowing and a carbon coating. They were cleaned overnight with sodium hypochloride and washed in distilled water. They were observed with a Philips EM 300 electron microscope. The shadowing direction is indicated by an arrow on each micrograph.

Results

Protein changes

After hypotonic incubation (Figs. 1 and 2), spectrin was partially absent from G1. As is classical [17], the missing fraction was recovered in S1. Most unexpectedly, in the presence of C/PC or PC vesicles, spectrin was lacking completely in S2 and S3, and at the same time a high-molecular-weight polymer appeared in G2 and G3. The polymer was not reduced by 100 mM β-mercaptoethanol. It became apparent within 15 min of incubation, but increased over a period of

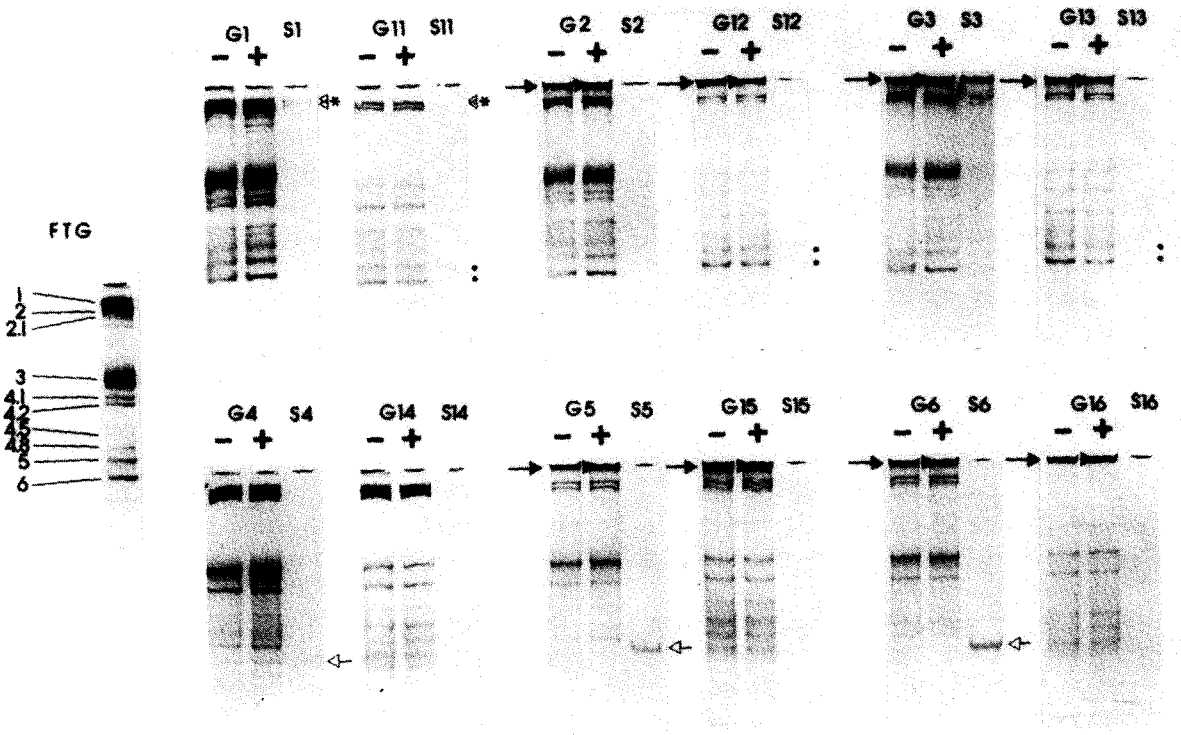

Fig. 1. SDS-polyacrylamide gel electrophoresis of ghost membrane proteins. Ghosts were incubated (0.1 mg protein/ml) as described in 'Material and Methods' either in hypotonic (G1–G3) or in isotonic (G4–G6) medium, without (G1 and G4), or with C/PC vesicles (G2 and G5) ($C/P = 0.85$, 17 mg PC/mg protein) or PC vesicles (G3 and G6) (24 mg PC/mg protein). After three washes in hypotonic medium they were reincubated (0.1 mg/ml) in the same vesicle-free hypotonic medium (G11–G16). G refer to ghost pellets and S to the corresponding supernatants. −, 6 mM β-mercaptoethanol; +, 100 mM β-mercaptoethanol. FTG: freshly thawed ghosts. For ghost pellets, 15 μg protein were applied. For supernatant, 20 μl after 25-fold concentration, were applied. In S3, a visible artefact reproducibly occurred at the level of spectrin. Major protein bands were numbered according to Fairbanks et al. [17]. ⋛*, spectrin bands 1 and 2 in S1 and S11; :, occurrence of bands between bands 5 and 6 in S11–S13; ◁–, band 6 (glyceraldehyde-3-phosphate dehydrogenase) in S4–S6.

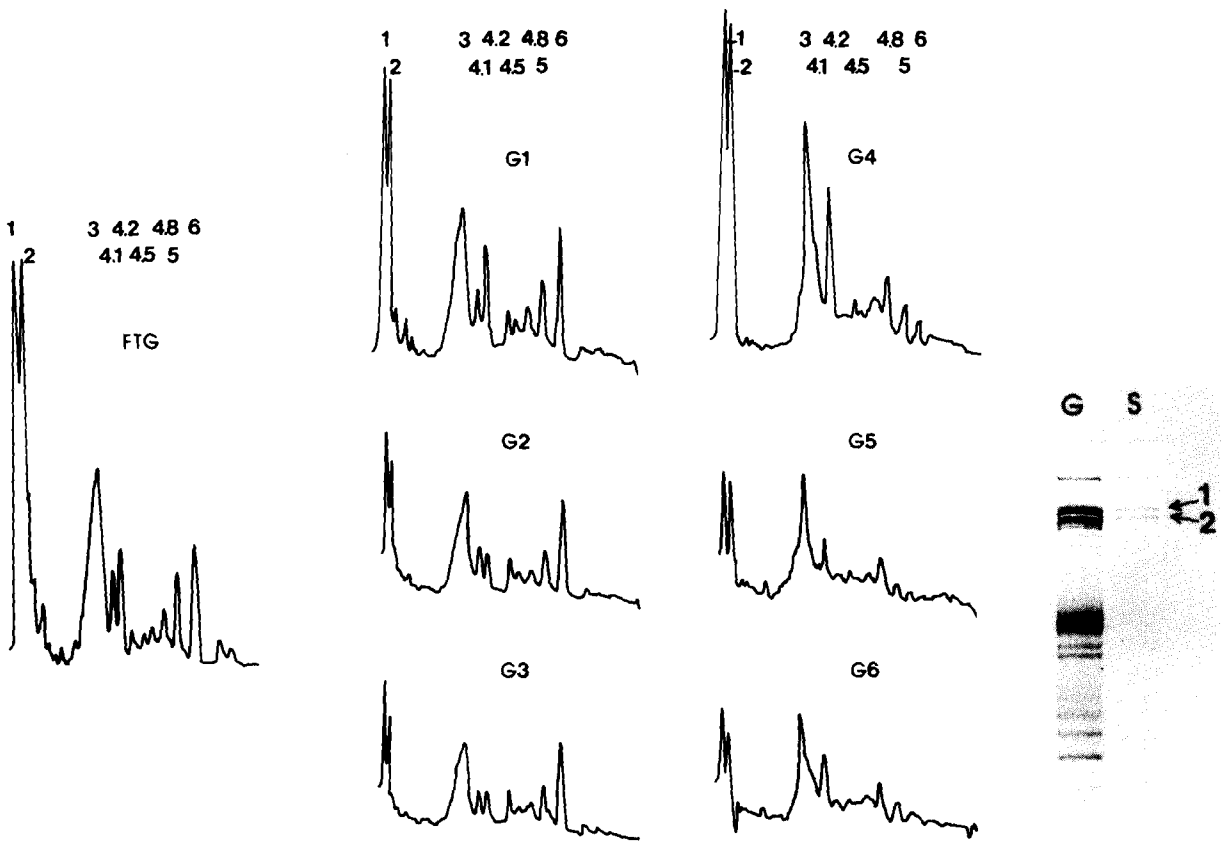

Fig. 2. Scanning of the protein electrophoretic profiles. Gels of Fig. 1. were scanned at 530 nm. The high-molecular-weight polymer could not be recorded because it lies at the edge of gel. For experimental details, see Fig. 1.

Fig. 3. (Gel on right.) SDS-polyacrylamide gel electrophoresis of membrane proteins of ghosts placed in a dialysis bag and incubated in hypotonic medium with PC-vesicles. Ghosts were placed in a dialysis bag (2 mg protein/6 ml) with the vesicles outside (33 mg PC in 18 ml of hypotonic buffer). G, ghost pellet; S, supernatant. No high-molecular-weight polymer appeared in G (100 mM β-mercaptoethanol) and spectrin was normally extracted in S. In addition, no lipid change occurred in G (not shown). In the dialysis bag the malonyldialdehyde concentration was 0.12 μM (control without vesicles, 0.05 μM).

24 h (not shown). Although a reduction of the whole electrophoretic profile was observed, the most affected components were, by far, components 1 and 2. The same changes were observed irrespective of whether PC or C/PC vesicles were used, a fact indicating that cholesterol depletion, induced by PC vesicles, brought about no further effect, at least none detectable (Figs. 1 and 2). Therefore, incubation with PC or C/PC vesicles induces partial cross-linking of spectrin that leads, among other consequences, to loss of spectrin extractibility and to what we will call the slow resealing of the ghosts (see below). A subsequent incubation in a still hypotonic, but vesicle-free medium caused a minute amount of spectrin to be further extracted into S11 (Fig. 1). Nevertheless, there still appeared no spectrin in S12 and S13 and the high-molecular-weight polymer appeared unchanged in G12 and G13. These results show that the alterations due to the first incubation are irreversible.

When the first incubation with vesicles was carried out under isotonic conditions, a similar β-mercaptoethanol-resistant polymer became apparent (G5 and G6) and no spectrin was extracted in S5 and S6. As is classical [17], spectrin was not extracted in S4 either (vesicle-free medium). In this case, however, no high-molecular-weight polymer

was visible in G4. Therefore, the molecular alteration that is responsible for spectrin inextractibility from G4 into S4 and for what we will call fast resealing of the ghosts (see below), did not generate any high molecular weight polymer and thus was electrophoretically silent. As is also classical [17], band 6 (glyceraldehyde-3-phosphate dehydrogenase) was partially extracted from G4 into S4. It was present in S5 and S6 as well, despite the formation of the polymer in G5 and G6. Incubation under hypotonic conditions, but in the presence of 2.5 mM Mg^{2+} that is known to also induce fast resealing [13], resulted in the abolition of spectrin extractibility, but not in polymer formation; nor was band 6 extracted (not shown). Subsequent incubation in a hypotonic, vesicle-free medium left the polymer unchanged (G15 and G16). It restored spectrin extractibility neither in S15 or S16, nor in S14.

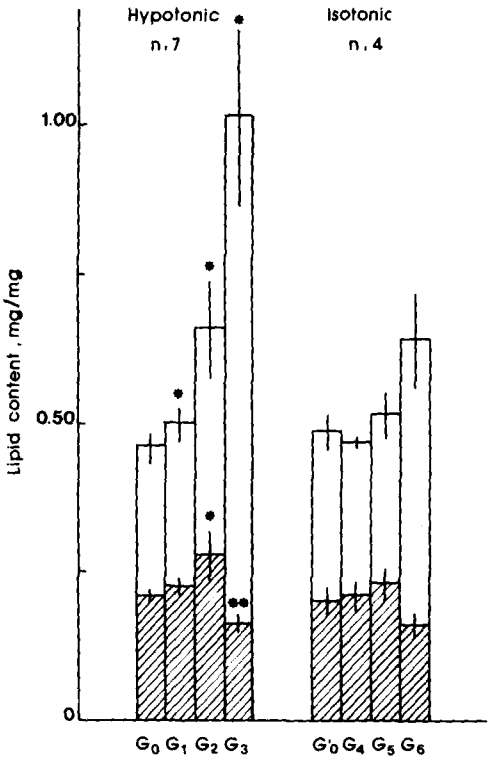

Fig. 4. Lipid content of ghosts. Ghosts were incubated as described in 'Material and Methods' either in hypotonic (G0, G1–G3) or in isotonic (G'0, G4–G6) medium, at 4°C (G0 and G'0) or 25°C (G1–G6), without (G1 and G4), or with C/PC vesicles (G2 and G5) or PC vesicles (G3 and G6). Cholesterol (hatched area), phospholipid (white area) and protein contents were measured after three washes in hypotonic medium to remove the vesicles. Increases of the phospholipid contents were measured both by phosphorus assay and radioactive counting of a non-exchangeable marker ([^{3}H]glycerol trioleate) incorporated in the vesicles during their sonication. Both methods gave the same values. Values are mean ± S.E. of seven experiments (hypotonic) or four experiments (isotonic). *, $P < 0.05$; **, $P < 0.01$.

A number of other electrophoretic changes were visible: (i) further reduction of the profile after the second incubation (G11–G16), (ii) narrowing of band 3 and decrease in band 4.1 in G4–G6, G11–G16 and (iii) occurrence of bands between bands 5 and 6 in S11–S13. Although these changes were in general reproducible, we do not know their meaning. The unusually long and stressing treatments applied to ghosts presumably account for part of them.

In order to test the hypothesis that the formation of the high-molecular-weight polymer could be mediated by some low-molecular-weight compound such as malonyldialdehyde, ghosts were incubated under hypotonic conditions with PC vesicles after having been placed in a dialysis bag (Fig. 3). After incubation, the concentration of thiobarbituric acid-reactive material (assuming that this material was entirely accounted for by malonyldialdehyde) was 0.1–0.2 μM in the supernatant of the dialysis bag content. All the vesicle-induced effects, i.e., polymer formation and spectrin inextractibility, as well as vesicle uptake and

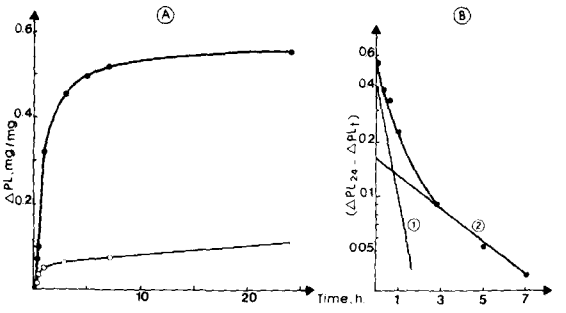

Fig. 5. Kinetics of the uptake of vesicles by the ghosts. Ghosts were incubated (0.3 mg protein/ml) with PC (●) vesicles (12.2 mg PC/mg protein) or C/PC (○) vesicles (10.4 mg PC/mg protein) in hypotonic medium for various times. A: vesicle uptake is expressed as the increase in the phospholipid content (ΔPL) in mg/mg protein. B: semi-logarithmic plot of the difference between the vesicle uptake at 24 h (ΔPL_{24}) and the vesicle uptake at each time (ΔPL_t); in this experiment the time constants were $\tau_1 = 44$ min and $\tau_2 = 6.6$ h.

cholesterol depletion (see below) were missing. This critical experiment showed that direct contact of the vesicles with the ghosts is required for the high-molecular-weight polymer to appear. In another experiment, PC vesicles were sonicated under N_2 in a medium containing butylated hydroxytoluene (100 μg/ml). Although the presence of the antioxidant reduced slightly the amount of thiobarbituric acid-reactive material in the vesicle suspension (3 μM instead of 6 μM without butylated hydroxytoluene and assuming that this was malonyldialdehyde alone), it was unable to prevent the generation of the high-molecular-weight polymer when these vesicles were incubated with the ghosts.

Lipid changes

Ghosts incubated in hypotonic, vesicle-free medium at 25°C (G1) exhibited a parallel increase of phospholipids and cholesterol (9%) relative to control ghosts incubated at 4°C (G0) (Fig. 4). These increases were accounted for by the decrease of membrane proteins extracted from G1 into S1. In G2 and G3 incubated with C/PC and PC vesicles, respectively, since no protein was extracted into S2 and S3 (see Fig. 1), the increases of cholesterol and phospholipids (G2) and of phospholipids (G3) corresponded to a net uptake of vesicles (Fig. 4). At comparable vesicle concentrations (expressed as mg PC/mg membrane protein) during the incubation, the phospholipid uptake was always smaller with C/PC vesicles (G2) than with PC vesicles (G3) (Fig. 4, see also Fig. 5 and 6). Cholesterol depletion was observed in G3 resulting from a net movement of cholesterol towards the cholesterol-free phospholipid vesicles as classically described [23]. The time-course of the uptake (Fig. 5) was biphasic, displaying a rapid phase for the first 2 h (time constant $\tau_1 = 30$–50 min) followed by a slow phase ($\tau_2 = 5$–10 h). Reincubation of G2 and G3 for 15 h at 25°C in a vesicle-free, hypotonic medium as well as mild trypsinization (10 min, 25°C, 0.01%) did not release any substantial amount of vesicles (Table I). These results indicated that the uptake was irreversible and was not an adsorption. The uptake was dependent on the temperature and on the vesicle amount added per mg ghost protein with

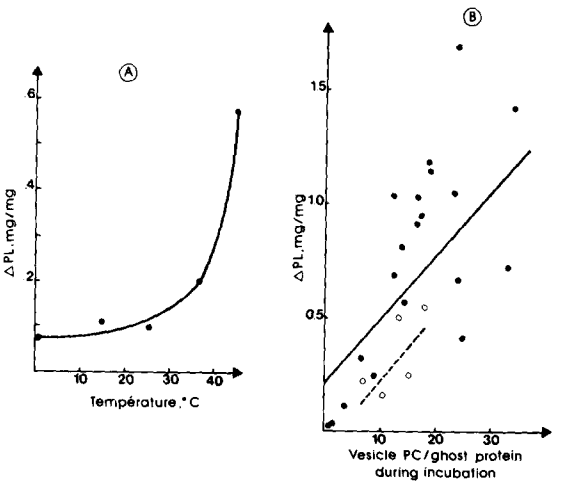

Fig. 6. Temperature and vesicle concentration dependence of the vesicle uptake by the ghosts. Ghosts were incubated in hypotonic medium (0.1 mg/ml) at various temperatures with PC vesicles (41 mg PC/mg protein) or with different amounts of PC (●) vesicles or of C/PC (○) vesicles. Vesicle uptake is expressed as the increase in the phospholipid content (ΔPL) in mg/mg protein. Regression lines: PC vesicles (———), $y = 0.03\ x + 0.21$ ($n = 20$), $r = 0.6867$, $P < 0.01$; C/PC vesicles (- - - - -), $y = 0.03\ x + 0.05$ ($n = 5$), $r = 0.6966$, n.s.

TABLE I

EFFECT OF REINCUBATION IN VESICLE-FREE HYPOTONIC MEDIUM AND TRYPSINIZATION ON THE VESICLE RELEASED FROM ERYTHROCYTE GHOSTS

Erythrocyte ghosts were incubated in hypotonic medium containing C/PC (G2) or PC vesicles (G3) as described in Material and Methods. After three washes, aliquots were taken for measurement of the phospholipid uptake and the remaining membranes were reincubated for 15 h at 25°C in the same medium alone or for 10 min at 25°C with this medium containing 0.01% trypsin (+T) or no trypsin (−T). The percentage of the initially associated vesicles which were released by each treatment is shown (mean value ± S.E. when n, the number of experiments was 4, or mean value when n was 2). The low percentage of vesicles released in these conditions could correspond to the removal of externally attached vesicles and thus indicated that the majority of the vesicle were irreversibly sequestrated inside the ghosts.

	G2	G3	n
Hypotonic medium	14.2 ± 6.2	17.2 ± 6.0	4
−T	6.8	2.8	2
+T	18.9	8.2	2

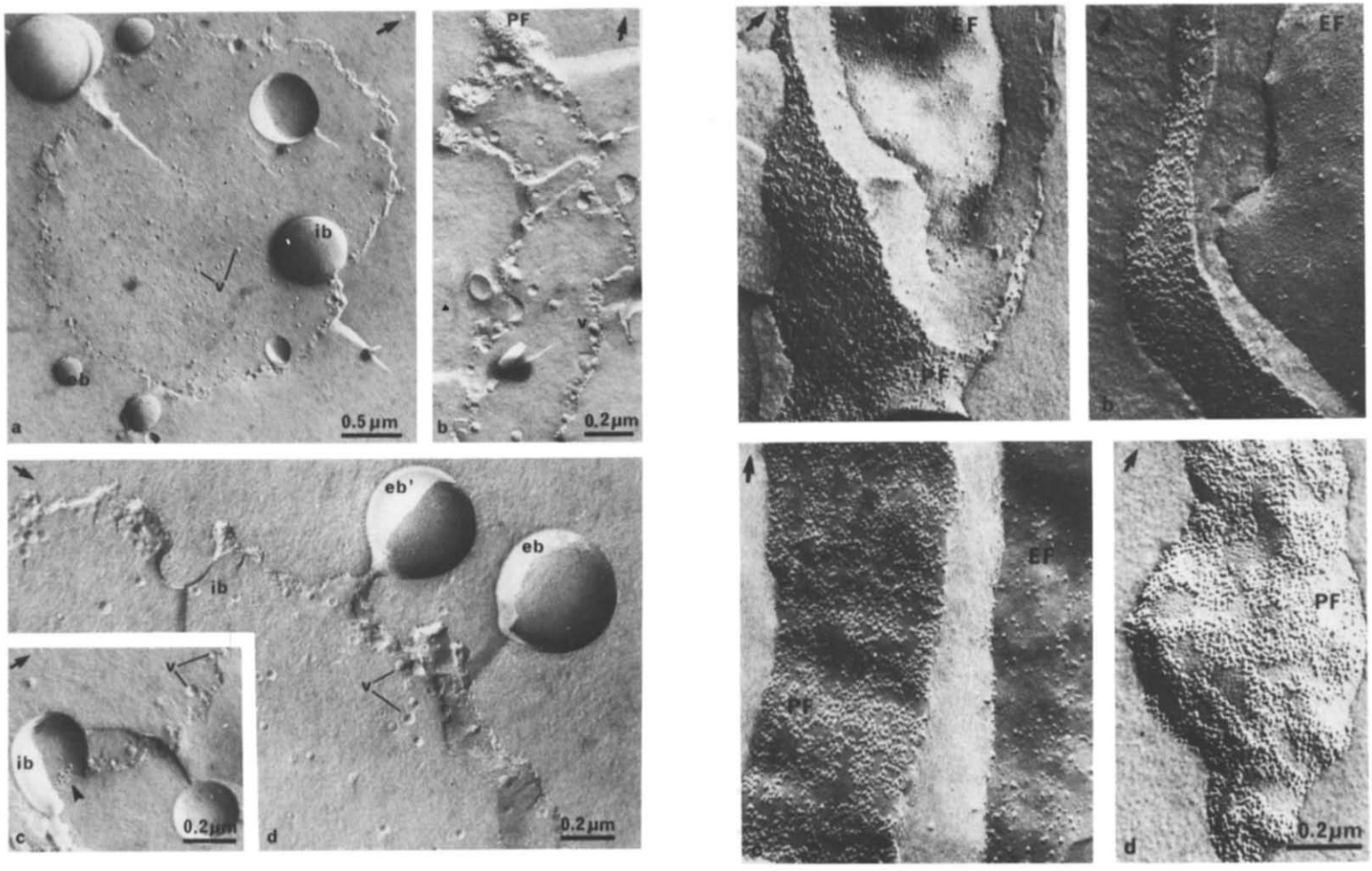

Fig. 7. (Left set) Freeze-fracture electron microscopy of ghosts after incubation with PC vesicles, in hypotonic medium. Ghosts were incubated (0.22 mg protein/ml) with PC vesicles (23 mg PC/mg protein). (a) Numerous vesicles (V) are seen entrapped within a closed ghost. Note the presence of internal (ib) and external (eb) blebs. (b) Some vesicles (V) are close to the membrane. Only small portions of the membrane were fractured as compared with control ghosts; in some instances protoplasmic face (PF) is seen. (c) An internal bleb (ib) in formation is seen together with an aggregation of intramembrane particules (arrow head). (d) Detached external bleb (eb), formation of an external bleb (eb') and cross-section of an internal bleb in formation (ib) are shown. (V), entrapped vesicles.

Fig. 8. (Right set) Freeze-fracture electron microscopy of ghost membranes after incubation in hypotonic medium without or with PC vesicles. Ghosts were incubated (0.1 mg protein/ml) at 4°C (a), at 25°C (b) at 25°C with PC vesicles (7.6 mg PC/mg protein) (c and d). Aggregation of intramembrane particles is generally seen after incubation with vesicles, although the intensity of the process varied from one sample to the other (compare c to d).

no apparent saturation over the range tested (Fig. 6).

Under isotonic conditions, no lipid change was visible in G4. The small increase of phospholipids noticed in G5 and G6 (incubated with C/PC and PC vesicles, respectively) was not statistically significant, indicating the virtual absence of vesicle uptake. Incubation in hypotonic medium containing 2.5 mM Mg^{2+} greatly reduced the vesicle uptake (not shown), in a way similar to incubation in the isotonic medium.

Morphological changes

On the freeze-fracture electron micrograph of G3 (Fig. 7) and in G2 (not shown), numerous lipid vesicles (20–30 nm) could be seen entrapped within the ghosts, some of them being very close to the internal face of the membrane. This result associated with the irreversibility of the uptake (see Table I) indicates that the ghost were irreversibly resealed on the vesicles, i.e., that the vesicle uptake was a sequestration. In addition, some blebs (150–250 nm) were always observed in G3, some of them pinching off from the ghost surface towards the outside or the inside (Fig. 7c and d). Blebs were nearly devoid of intramembrane particles. In G5 and G6 (not shown), although extensive membrane vesiculation occurred, some blebs were also detected (not shown). When the fracture went through the plane of the membrane, which occurred less frequently than in control ghosts, slight aggregation of intramembrane particles was seen in G3 (Figs. 8c and d) and to a lesser extent in G1 (Fig. 8b). In control ghost (G0) the intramembrane particles were evenly distributed, as is classical (Fig. 8a).

Discussion

Whenever erythrocyte ghosts have been exposed to lipid vesicles, a high-molecular-weight polymer resistant to β-mercaptoethanol, has developed regardless of the tonicity of the medium and of the vesicle composition. This polymer, not dissociated by SDS, reflects the formation of covalent linkages, since the detergent breaks all classes of non-covalent bonds. It arose mainly, but not exclusively, from spectrin that appeared the most altered protein on the electrophoretic profiles. Although no accurate determination of the polymer formation kinetics was feasible, it can be said that it started to occur within 15 min of incubation and required 24 h or more to be completed. Protein aggregates in erythrocyte membranes have been reported in various conditions. Disulfide bond formation may occur after ATP depletion [24] or treatment of cells with oxidative agents: Cu-*o*-phenanthroline, tetrathionate or diamide [1]. The irreducible nature of the polymer proves that disulfide bonds, if any, are not its sole cause. A second possibility is the synthesis of amide linkages between glutamic acid δ-carboxyl groups and lysine ϵ-amino groups, a reaction catalysed by a Ca^{2+}-stimulated transglutaminase [7–11]. But relatively high Ca^{2+} concentrations (over 300 μM) which were never reached in our preparations (no Ca^{2+} added), are required, and also the bulk of the enzyme appears to be cytosolic [25]. Thus the occurrence of amide linkages seems very unlikely.

Under hypotonic conditions, the formation of the high-molecular-weight polymer was associated with a progressive vesicle uptake by the ghost that obeyed a biphasic kinetics. The first phase was presumably accounted for by the simple equilibration of the vesicles on both sides of the membrane. After a few hours, it was overtaken by a second phase that is likely to reflect the gradual tightening of the polymer meshwork, i.e., a slow resealing process of the ghosts. This was demonstrated by the presence of entrapped vesicles within the ghosts on the electron micrographs (Fig. 7) and also by the virtual absence of vesicle release upon further incubation in a vesicle-free medium. Although the intensity of the polymer apparently did not differ accordingly whether PC/ or C/PC vesicles were used, more of the former than of the latter vesicles were always sequestrated. PC vesicles have a diameter of about 22–25 nm [26,27] whereas C/PC vesicles a diameter of about 36 nm [28]. We can conclude that the ghosts initially possess pores of about 40 nm, a figure in agreement with that proposed by Seeman [29] for the size of the transient holes of the erythrocyte membrane during osmotic hemolysis (20–50 nm).

The polymer-associated, vesicles-induced, slow resealing contrasts with the polymer-free salt-induced fast resealing when ghosts are incubated in isotonic media (or in hypotonic media containing

Mg^{2+}). Such resealing was fast enough to prevent the entry of the vesicles into the ghosts from the beginning of the incubation. This is in agreement with the results of Jonnson and Kirwood [12,13] who observed a complete resealing to hemoglobin in about 15 min, after a 10 min pre-equilibration period at 0°C. However, the slow, polymer-associated resealing gradually developed even though the vesicles were kept outside the ghosts. For this reason, we though that some diffusible, low-molecular-weight compound such as malonyldialdehyde might be responsible for the protein cross-links. A minute amount of thiobarbituric acid reactive material (approx. 3–6 μM, assuming that malonyldialdehyde only was involved) was found in our vesicle preparations. This material remains constant all over the experimental period, in particular during the sonication (whether it was conducted under air or nitrogen, in the absence or in the presence of butylated hydroxytoluene) and during the incubation with ghost. However, these malonyldialdehyde concentrations were lower than those usually required to achieve protein cross-links in vitro (over 15 μM) [5]. They were also lower than those (15 μM) resulting from white ghost treatment with 5 mM hydrogen peroxide in the presence of catalytic amount of reintroduced hemoglobin and associated with, but not necessarily responsible for, protein cross-linking [30]. In any case, they must be considered with caution because they largely reflect the generation of peroxidative products in the course of the thiobarbituric acid reaction itself. When ghosts were separated from the vesicles by a dialysis membrane during the incubation, and then centrifuged, the concentration of thiobarbituric acid-reactive material in the vesicle-free supernatant dropped to 0.1–0.2 μM, values similar to those observed in the control without vesicles. In this experiment, the absence of the high-molecular-weight polymer in the ghost pellet and the presence of spectrin in the supernatant (Fig. 3) clearly indicated that the thiobarbituric acid-reactive compounds, if any, were unable to generate the changes observed under standard conditions. Thus, direct contact of vesicles, at least with the outer surface of the ghosts, is required to produce the high-molecular-weight polymer.

A common consequence of both types of resealing was the loss of spectrin extractibility. Remarkably, neither type of resealing affected the extractibility of band 6, a fact assigning some degree of specificity to the molecular changes involved. In contrast, however, Mg^{2+}-induced, polymer-free fast resealing prevented band 6 from being extracted (not shown), clearly indicating that isotonicity is required in order to release glyceraldehyde-3-phosphate dehydrogenase.

Beside resealing and spectrin inextractibility, two other possible consequences of the formation of the high-molecular-weight polymer were detected. In hypotonic medium, intramembrane particles slightly aggregated, resulting in the formation of smooth lipid areas from which membrane blebs, almost devoid of intramembrane particles, were pinched off. In isotonic medium, although ghosts were extensively fragmented into smaller membrane vesicles, the same effects were also detected. Aggregation of intramembrane particles and bleb formation have been described previously by Elgsaeter et al. [31] in the same membranes treated with protamine sulphate. The effect of this basic protein was interpreted as a result of contraction of the spectrin meshwork because it induced spectrin precipitation in solution and was throught to produce its aggregation in the membrane. These authors also reported that divalent cations ([Ca^{2+}] over 5 mM) had the same effects on morphology and might cause the closing of the pores. Thus, the presence of lipid vesicles led to the same morphological rearrangements as those provoked by a basic protein or divalent cations. Spectrin reorganization resulting (or not) in the formation of stable cross-links could be at the origin of all the other process including resealing, spectrin inextractibility, intramembrane particle redistribution and bleb formation.

Numerous previous studies have shown the existence of specific interactions between integral and peripheral membrane proteins [32–35]. More recently, the occurrence of a spectrin-ankyrin-band 3 complex has been demonstrated [35–37], and protein 4.1 is thought to interact with PAS protein (glycoconnectin) [38]. We have assumed that spectrin cross-linking was the cause of the intramembrane particle aggregation. However, as these cross-links can occur from the outside of the membrane (isotonic medium), the possibility also

exists that the intramembrane particle aggregation was the first event leading to the formation of the high-molecular-weight polymer. In one case only, where membrane protein cross-links develop (ATP depletion in rat erythrocytes) intramembrane particle aggregation has been observed [39]. It is thus not possible to decide whether or not the high-molecular-weight polymer is the cause or the consequence of intramembrane particle aggregation. In a different perspective, one might bring together the impairment of spectrin extractibility artificially generated in the present work, and that naturally occurring in hereditary spherocytosis [40,41], a condition in which the cells are prone to spontaneous vesiculation. Interestingly, no polymer appears when intact erythrocytes are incubated with vesicles [14]. One may assume that osmotic hemolysis induces preliminary membrane alterations permiting the cell membrane-vesicles interactions.

In this paper, we have described the development of membrane protein cross-links associated with other membrane property changes when erythrocyte ghosts are incubated with lipid vesicles. To our knowledge, no such phenomenon has been described before. Just as for salt-induced changes of peripheral proteins, the elucidation of the genesis and of the chemical nature of the cross-links has been only partially characterized.

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References

1 Haest, C.W.M., Kamp, D., Plasa, G. and Deuticke, B. (1977) Biochim. Biophys. Acta 469, 226–230
2 Palek, J., Liu, S.C. and Snyder, L.M. (1978) Blood 51, 385–395
3 Johnson, G.J., Allen, D.W., Cadman, S., Fairbanks, V.F., White, J.G., Lampkin, B.C. and Kaplan, M.E. (1979) N. Engl. J. Med. 301, 522–527
4 Kahane, I., Shifter, A. and Rachmilewitz, E.A. (1978) FEBS Lett. 85, 267–271
5 Snyder, L.M., Sauberman, N., Condara, H., Dolan, J., Jacobs, J., Szymanski, I. and Fortier, N.L. (1981) Br. J. Haematol. 48, 435–444
6 Chio, K.S. and Tappel, A.L. (1969) Biochemistry 8, 2821–2827
7 Anderson, D.R., Davis, J.L. and Carraway, K.L. (1977) J. Biol. Chem. 252, 6617–6623
8 King, L.E., Jr. and Morrison, M. (1977) Biochim. Biophys. Acta 471, 162–168
9 Lorand, L., Siefring, G.E. and Lowe-Krentz, L. (1978) J. Supramol. Struct. 9, 424–440
10 Lorand, D., Siefring, G.E., Jr. and Lowe-Krentz, L. (1979) Semin. Hematol. 16, 65–74
11 Liu, S.C. and Palek, J. (1979) Blood 54, 1117–1130
12 Johnson, R.M. (1975) J. Membrane Biol. 22, 231–253
13 Johnson, R.M. and Kirkwood, D.H. (1978) Biochim. Biophys. Acta 509, 58–66
14 Chailley, B., Giraud, F. and Claret, M. (1981) Biochim. Biophys. Acta 643, 636–641
15 Dodge, J.T., Mitchell, C.D. and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119–130
16 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
17 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606–2617
18 Rose, H.G. and Ocklander, M. (1965) J. Lipid Res. 6, 428–431
19 Rudel, L.L. and Morris, M.D. (1973) J. Lipid Res. 14, 364–366
20 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466–468
21 Stocks, J. and Dormandy, T.L. (1971) Br. J. Haematol. 20, 95–111
22 Stocks, J., Offerman, E.L., Modell, C.B. and Dormandy, T.L. (1972) Br. J. Haematol. 23, 713–724
23 Bruckdorfer, K.R., Edwards, P.A. and Green, C. (1968) Eur. J. Biochem. 4, 506–511
24 Palek, J. and Liu, S.C. (1979) J. Supramol. Struct. 10. 79–96
25 Triplett, R.B., Wingate, J.M. and Carraway, K.L. (1972) Biochim. Biophys. Res. Commun. 49, 1014–1020
26 Huang, C.H. (1969) Biochemistry 8, 344–352
27 De Kruijff, B., Cullis, P.R. and Radda, G.K. (1975) Biochim. Biophys. Acta 406, 6–20
28 Johnson, S.M. (1973) Biochim. Biophys. Acta 307, 27–41
29 Seeman, P. (1967) J. Cell. Biol. 32, 55–70
30 Alloisio, N., Michelon, D., Bannier, E., Revol, A. Beuzard, Y. and Delaunay, J. (1982) Biochim. Biophys. Acta 691, 300–308
31 Elgsaeter, A., Shotton, D.M. and Branton, D. (1976) Biochim. Biophys. Acta 426, 101–122
32 Nicolson, G.L. and Painter, R.G. (1973) J. Cell. Biol. 59, 395–406
33 Ji, T.H. and Nicolson, G.L. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 2212–2216
34 Yu, J. and Branton, D. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 3891–3895
35 Shotton, D., Thompson, K., Wofsy, L. and Branton, D. (1978) J. Cell. Biol. 76, 512–531
36 Bennett, V. and Stenbuck, P.J. (1980) J. Biol. Chem. 255, 6424–6432
37 Hargreaves, W.R., Giedd, K.N., Verkleij, A. and Branton, D. (1980) J. Biol. Chem. 255, 11965–11972
38 Mueller, T.M. and Morrisson, M. (1981) in Erythrocyte Membranes Vol. 2, Recent Clinical and Experimental Advances (Kruckenberg, W.C., Eaton, J.W. and Brewer, G.J., eds.), pp. 95–112, Alan P. Liss, New York
39 Gazitt, Y., Loyter, A., Reichler, Y. and Ohad, I. (1976) Biochim. Biophys. Acta 419, 479–492
40 Sheehy, R. and Ralston, G.B. (1978) Blut 36, 145–148
41 Hill, J.S., Sawyer, W.H., Howlett, G.J. and Wiley, J.S. (1981) Biochem. J. 201, 259–266