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STUDIES ON THE FRAGMENTATION OF ERYTHROCYTE GHOST MEMBRANE WITH *p*-CHLOROMERCURIBENZOATE IN THE MICROMOLAR RANGE

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The effects of nonsaturating amounts (5–60 nmol/mg membrane protein) of *p*-chloromercuribenzoate on the stability of unsealed erythrocyte ghosts were studied by turbidimetric measurements and direct observation by phase contrast microscopy. The organic mercurial provokes drastic disorganization of the membrane involving vesicle formation by inter- and externalization of the bilayer. These effects are not associated with a release in solution of membrane proteins which was shown in previous studies to occur at higher *p*-chloromercuribenzoate concentration. Attempts have been made to identify the proteins involved in this phenomenon by the use of nonsaturating amounts of radioactively-labelled *p*-chloromercuribenzoate. Actin and band 3 protein which are the first to be labelled, represent plausible candidates as sensitive targets for the disrupting organic mercurial. Stroma obtained from spherocytes did not show significant differences with normocytes in their stability with regard to *p*-chloromercuribenzoate. Other reagents including *N*-ethylmaleimide, diamide and DNAase I were also studied. The results suggest strongly that the integrity of the sulfhydryl groups of actin, as well as those of band 3 protein, is essential for the stability of the erythrocyte membrane.

Introduction

One of the well established functions of the erythrocyte cytoskeleton is to provide resistance and deformability to the plasmic membrane. In the last years an increasing knowledge has emerged from many laboratories on the nature of the proteins involved in this essential structure and their interactions [1,2]. Purified proteins including spectrin, ankyrin, protein 4.1 *, actin and the cytoplasmic fragment of band 3 protein were generally used in these studies. A different approach was taken in experiments in which the structure of the cytoskeleton as a whole was selectively disturbed with specific chemicals. Sulfhydryl-binding reagents and particularly *p*-chloromercuribenzoate (PCMB) are potent effectors of protein-protein interactions. They have been largely used in the case of the red blood cell membrane [4–8]. Most of these studies however were related to an analysis of the proteins released from the erythrocyte membrane with high and saturating amounts of sulfhydryl reagents. Contrarily, in the experiments here reported PCMB was used at very low concentration. As shown by turbidimetric measurements and phase contrast microscopy it is able to deeply alter the structure of erythrocyte ghosts although negligible amounts of protein are released in solution.

Radioactively-labelled PCMB was employed in an attempt to identify the proteins of the membrane involved in this process. The effects of other

* Nomenclature of erythrocyte membrane proteins according to Fairbanks et al. [3].

Abbreviation: PCMB, *p*-chloromercuribenzoate.

reagents and stroma prepared from spherocytes were also studied.

Materials and Methods

Preparation of red blood cell membrane and treatment with sulfhydryl reagents. Preparation of human ghosts was achieved as previously described [9]. A final wash was made either in 5 mM or 10 mM sodium phosphate buffer, pH 8 and the experiments were performed on the same day. The amount of membrane proteins was assayed by the method of Lowry et al. [10] with bovine serum albumin as the standard. The reaction of membrane with sulfhydryl reagents was conducted at a final protein concentration of 0.4 mg/ml. Necessary quantities of the packed membranes were added to a solution of PCMB at the appropriate concentration. This procedure was essential when nonsaturating amounts of the highly reactive PCMB were used, in order to prevent the formation of local gradients of PCMB concentration in the test tube.

Spherocytes were obtained from patients with proven Minkowski-Chauffard hereditary spherocytosis and were processed as the normocytes.

Turbidimetric measurements and phase contrast microscopy. Changes in the light scattering properties of a suspension of membranes in presence of the reagents were studied in a Cary 118 spectrometer by measuring the changes in light transmission in a 1 cm pathlength cell at 700 nm and at a controlled temperature.

For phase contrast microscopy the samples were fixed at given times after the beginning of the reaction with 1% glutaraldehyde. In some cases a direct observation was made as a function of time after the addition of the reagent to the ghosts. The shortest time interval necessary to prepare and set the sample under the microscope was about 15 s.

Measurements of protein released from the ghosts and use of [^{14}C]PCMB. Increasing amounts of PCMB were added to erythrocyte ghosts and the reaction was allowed to proceed at 35°C until no changes in light transmission were detected. The experiments were conducted in 10 mM sodium phosphate buffer, pH 8 since at a lower ionic strength in the absence of mercurials, the ghosts showed a slight unstability as a function of time. After centrifugation at 49000 × g for 20 min the absorbance of the supernatant was recorded at 280 nm.

Variable amounts (5–160 nmol/mg protein) of [^{14}C]PCMB were also reacted with erythrocyte membrane. Later the samples were centrifuged, washed once and the radioactivity present in the supernatant and the pellet was counted in an Intertechnique Scintillation counter. The pellets were also submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 5–15% gradients gels by the method of Laemmli [11] in the absence of β-mercaptoethanol. They were dissolved in 2% sodium dodecyl sulfate at room temperature since boiling the samples provokes some loss of the protein-bound radioactivity.

The labelled proteins were identified on the gel by autoradiography. Before drying, the gels were soaked one hour with EN^{3}HANCE solution from New England Nuclear in order to improve the sensitivity of the detection. A microdensitometer Joyce was used to read the exposed film.

Reagents. PCMB was from BDH, *N*-ethylmaleimide from Koch-Light, diamide from Sigma and DNAase I from Worthington (grade D). In order to eliminate residual proteases, DNAase I was chromatographed on hydroxyapatite according to Price et al. [12]. [^{14}C]PCMB radioactively-labelled on the carboxylic function was obtained from Commissariat à l'Energie Atomique at a specific activity of 15 mCi/mmol. Other reagents were of analytical grade.

Results

Turbidimetric studies and analysis of the release of proteins from erythrocyte ghosts in the presence of sulfhydryl reagents

The light scattering properties of a suspension of erythrocyte ghosts were measured in the presence of sulfhydryl reagents. The intensity of monochromatic light transmitted through a suspension of unsealed ghosts increased substantially on the addition of PCMB inferring the disruption of the membranes into smaller particles. This phenomenon was occurring slowly in a half-time of 3 min in 5 mM sodium phosphate buffer, pH 8, with 30 nmol/mg protein of PCMB and at 35°C. The rate of the reaction was further slowed down either by

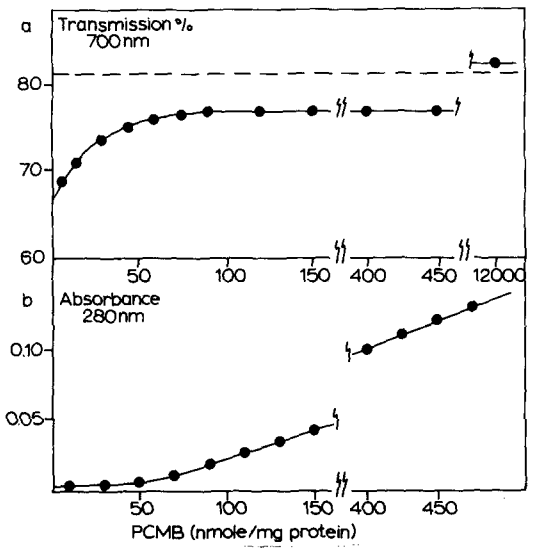

Fig. 1. (a) Variations in the turbidity of a suspension of erythrocyte ghosts incubated in the presence of PCMB or in water. Erythrocyte ghosts were diluted at a concentration of 0.4 mg membrane proteins/ml in 10 mM sodium phosphate buffer, pH 8 (●) or in water (— — —). The suspension of ghosts in sodium phosphate buffer was incubated at 35°C with increasing concentrations of PCMB and for each addition the total variation in light transmission was measured at 700 nm in 1-cm pathlength cuvettes. (b) Elution of proteins from the erythrocyte ghost membrane with increasing amounts of PCMB. The appearance of proteins in solution (●) was detected by the increase in the absorbance of the supernatant at 280 nm (see text). Experimental conditions are the same as in (a).

lowering the temperature of the experiment or increasing the ionic strength of the solution. The total changes in light transmission were dependent only on the concentration of PCMB. They reached a constant level when the amounts of PCMB were comprised between 60 and 500 nmol/mg protein and increased further for higher PCMB concentrations (Fig. 1a). A slight increase in turbidity was always noticed during the first minute following the addition of PCMB at concentrations below 60 nmol/mg of membrane protein. It could correspond to a transient swelling of the ghosts but no attempt has been made to study this phenomenon in detail. This side effect was also present when the ghosts were disrupted in the absence of PCMB by decreasing the ionic strength with water. The large transmission change observed in this case was quite comparable to the one obtained with the same membrane suspension in phosphate buffer and in the presence of a large excess of PCMB (Fig. 1a). Experiments were also performed with stroma prepared from spherocytes. The amplitude and the time course of the changes in turbidimetry were similar with those of normocytes.

Other reagents were studied. The membranes were incubated with the chemicals 1 h at 35°C. *N*-Ethylmaleimide induced effects quite similar to those of PCMB except that a 25-fold higher concentration of the reagent was necessary. Diamide, a bifunctional molecule, was found to modify very slightly the light scattering properties of a suspension of erythrocyte ghosts.

In view of the drastic effects that low concentrations of PCMB provoke on the turbidity of a suspension of red blood cell membrane we have examined the possibility that some membrane proteins could be released at the same time. For PCMB concentrations smaller than 50 nmol/mg protein no significant amounts of protein were released although it was shown in the light transmission experiments that PCMB had exerted a strong effect (Fig. 1). When the concentrations of PCMB were increased above 60 nmol/mg protein the amounts of protein released from the ghosts increased monotonously in agreement with the findings of other authors [4–7]. In some experiments we have identified actin as the first protein to be eluted from the membrane.

Phase contrast microscopy analysis of the erythrocyte ghost membrane in the presence of sulfhydryl reagents

Modifications in the light scattering properties of red blood cell ghosts on the addition of PCMB (Fig. 1) were suspected to be due to modifications in the size of the particles in suspension. In order to further characterize the morphological changes which were occurring in the presence of mercurials we have examined under phase-contrast microscopy all of the samples of the membranes which were previously studied in the light-transmission experiments. In the absence of reagents, unsealed ghosts showed their usual morphology (Fig. 2). One minute after the addition of PCMB the population of ghosts became heterogeneous showing a smaller average size and several characteristic features. Some of the ghosts were surrounded by small vesicles bound at the outside

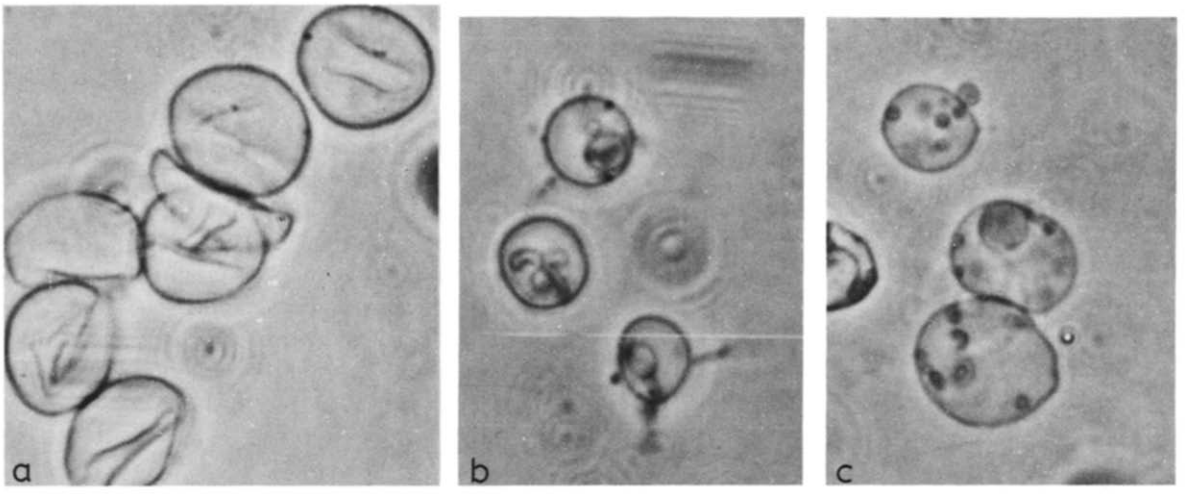

Fig. 2. Phase-contrast microscopy of a suspension of ghosts alone (a) and in the presence of PCMB (b, c). The ghosts at a protein concentration of 0.4 mg/ml were reacted in 5 mM sodium phosphate buffer, pH 8, at 35°C, with PCMB (50 nmol/mg protein) and observed 3 min (b) and 60 min (c) after the addition of the mercurial. The magnification was 2400. In (c) the bigger size in the ghosts as compared to the case in (b) is only apparent and reflects stronger flattening of the sample on the plate of the microscope. Other conditions are given in Materials and Methods. In (b) and (c) the vesicles present inside the ghosts are not clearly discernable since they were subjected to Brownian movements.

surface of the membrane. Others showed one or two bigger vesicles located inside the envelope of the ghosts. After 3 min most of the vesicles previously detected were separated from the membrane and the ones which had been trapped inside the ghosts were subjected to Brownian movements (Fig. 2). After 30 min the pattern was the same except that all of the ghost population was modified.

In order to describe further the details of the changes which were occurring in the first minutes following the addition of PCMB a continuous observation was performed under the microscope. It was then possible to follow the process of vesicles formation by endo- or exocytosis and to detect other intermediate features than the one found in the static experiment with glutaraldehyde-fixed samples (Fig. 3).

Ghosts prepared from spherocytes gave similar results. When they were incubated 1 h at 35°C with 500 nmol/mg protein of *N*-ethylmaleimide identical modifications of the membrane were obtained. Diamide (1000 nmol/mg protein) and DNAase I (0.1 mg/ml) were without effect even after one hour at 35°C. When erythrocytes ghosts preincubated with diamide were reacted with PCMB the expected morphological changes in the membrane were observed.

Attempts to identify the proteins involved in the disorganization of the erythrocyte ghost membrane at low PCMB concentrations

Erythrocyte ghosts were also reacted with variable amounts of [^{14}C]PCMB (Fig. 4). At the lower

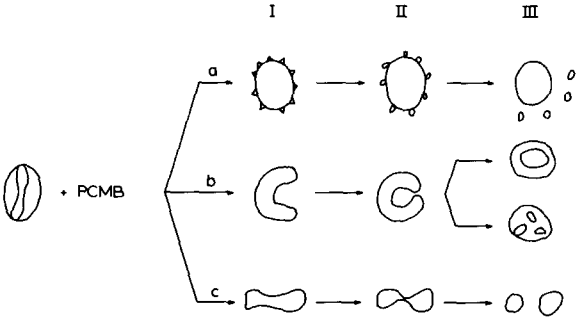

Fig. 3. Scheme of the different transformations occurring in erythrocyte ghosts on the action of PCMB as detected by phase-contrast microscopy. Patterns I are present in the minute following the addition of PCMB. The transformation of I into II and III occurs in a time range of several minutes. The experimental conditions are those of Fig. 2.

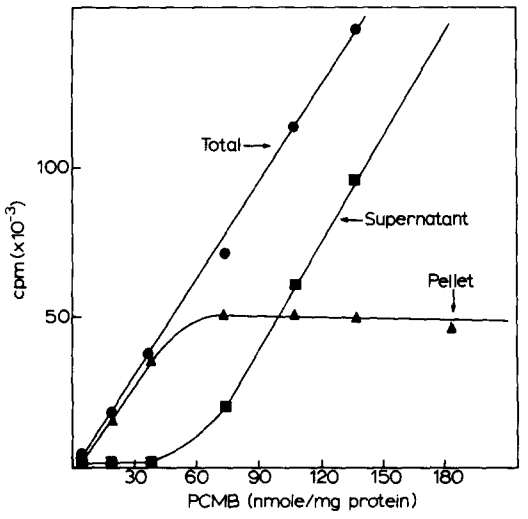

Fig. 4. Distribution in the radioactivity of a suspension of erythrocyte ghosts reacted with increasing amounts of [^{14}C]PCMB. The radioactivity was measured in the total suspension of the ghosts (●), in the pellet (▲), and the supernatant (■). The sharp increase in the radioactivity in the supernatant fraction above 60 nmol/mg protein of [^{14}C]PCMB corresponds to the sudden solubilization of membrane proteins. The plateau found in the radioactivity measured on the pellet fraction does not mean that the membrane is saturated with respect to PCMB since in this range of PCMB concentration radioactively-labelled membrane proteins are continuously released in solution. The plateau is only apparent and results from compensatory effects between protein solubilization and titration with [^{14}C]PCMB of the residual membrane proteins.

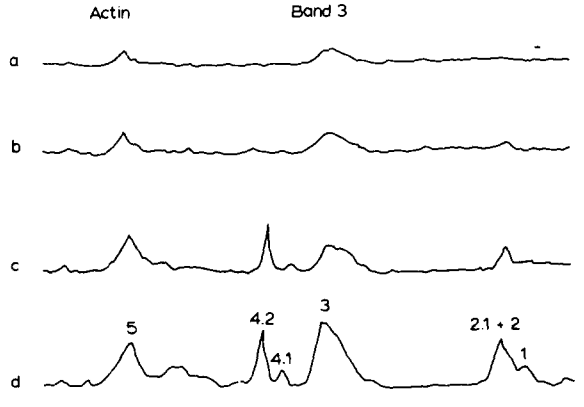

Fig. 5. Autoradiography of sodium dodecyl sulfate gels obtained after electrophoresis of the pellet fraction of the ghosts incubated with increasing amounts of [^{14}C]PCMB. The quantities of [^{14}C]PCMB were: (a) 7 nmol/mg protein; (b) 15 nmol/mg protein; (c) 30 nmol/mg protein and (d) 65 nmol/mg protein. The attribution of bands is made according to Fairbanks et al. [3]. Other experimental conditions are given in Materials and Methods.

PCMB concentrations (below 60 nmol/mg protein) there is a progressive increase in the radioactivity measured in the pellet fraction indicating that PCMB is not saturating with respect to the available binding sites present on the membrane. Experiments of Fig. 4 also confirm the absence of proteins released in the supernatant. The pellets obtained after reaction of the ghosts with increasing and nonsaturating amounts of [^{14}C]PCMB were submitted to sodium dodecyl sulfate gel electrophoresis and analyzed by autoradiography. Actin and band 3 protein were the first proteins to be labelled (Fig. 5).

Discussion

The action on the erythrocyte membrane of several sulfhydryl-binding reagents including the highly reactive PCMB was studied in this work. Several reports on this question have already appeared but with the difference that much higher concentrations of the reagents were used [4–8]. It was shown that erythrocyte ghosts were broken down into small vesicles and that large amounts of membrane proteins were released in solution. Since in these studies the concentrations of PCMB used were saturating with regard to the number of available reactive groups on the membrane, identification of the proteins playing a prominent role on membrane stability was not straightforward.

In the present study we have found that in the micromolar range PCMB provokes vesiculation of erythrocyte ghosts although no significant protein release was observed (Fig. 1). Using [^{14}C]PCMB we have shown that in this range of concentrations PCMB is not saturating with regard to the membrane binding sites since increasing the amounts of PCMB is concomitant with a rise in the radioactivity found in the membrane pellet (Fig. 4). This condition was necessary to identify the proteins involved in the first steps of the chemically induced fragmentation of the erythrocyte membrane.

Phase-contrast microscopy has shown that very small amounts of PCMB provoke strong modifications of the membrane of erythrocyte ghosts. There is a decrease in the size of the ghosts and formation of vesicles (Fig. 2). When a continuous observation is made in the first minutes which follow

the addition of the organic mercurial three different processes are observed. Some of the ghosts are very quickly covered by small and numerous protrusions. Later in this process small vesicles are formed at the surface of the ghosts and then released in solution (Fig. 3a). In another case invaginations of the membrane occur. They are followed by the formation of one or several larger vesicles which are located inside the ghosts (Fig. 3b). Finally some of the ghosts are fractionated into two equal-sized particles (Fig. 3c). In the first process the burstings which are seen at the surface of the ghosts occur rapidly in an explosive process, apparently as the result of slackening a potentially contractible structure.

It is not clearly understood why the PCMB-induced inter- or externalization of the membrane prevails preferentially in different ghosts. Lange et al. [13] have presented evidences showing that the erythrocyte cytoskeleton is involved in membrane stability whereas the bilayer is more responsible for the final contour of the membrane. Erythrocytes have also been considered by Coakley and Deeley [14] as a model to study the interfacial stability of membranes. They have shown that inter- or externalization of the erythrocyte membrane may occur in different membrane bending conditions. The appearance of vesicles by heating erythrocytes mimicks very well what we have seen with PCMB. In our experimental conditions, once specific interactions between proteins inside the erythrocyte cytoskeleton or at the junction between the cytoskeleton and the membrane have been modified with PCMB, an exploding unstability of the bilayer happens with formation of vesicles by inter- or externalization of the membrane. The two processes may reflect pre-existing states of the bilayer or may occur by chance on the microscope slide depending on the shearing forces the PCMB-modified ghosts have been submitted to.

High sensitivity spectrophotometry has been used to study the amplitude and the time course of the structural changes induced by the action of several reagents on erythrocyte ghosts. For PCMB concentrations below 60 nmol/mg protein the total changes in light transmission increase with PCMB concentration (Fig. 1a). A decrease in ionic strength notably increases the velocity of the phenomenon. No further changes in light transmisson were noticed at higher PCMB concentrations between 65 and 500 nmol/mg protein. This indicates that once the very specific and limited modifications of one or several membrane proteins had occurred vesiculation of the ghost membrane is completely achieved. It is only for PCMB above 3000 nmol/mg protein that a new process appears with a large decrease in the turbidity of the membrane suspension (Fig. 1a).

N-Ethylmaleimide is much less reactive than PCMB since we have shown that it should be used at an higher concentration in order to produce the same effects. Ralston and Crisp [8] and Carter [5] have demonstrated that contrarily to PCMB, *N*-ethylmaleimide does not extract significant amounts of protein from the ghost, thus confirming that protein release and vesiculation of the ghost membrane are independent processes.

Diamide, a bifunctional reagent of thiols which produces intermolecular cross-links in the spectrin network, is known to decrease the shear elasticity of the erythrocyte membrane [15]. As shown in the present work diamide addition does not induce fragmentation of the ghosts. Either the bifunctional character of diamide prevents the disorganization of the membrane by linking some of its components or diamide does not react with the sulfhydryl groups of the proteins which are essential for the stability of the ghosts. This last hypothesis seems to prevail since we have found that PCMB exerts a full disturbing effect on diamide-reacted erythrocyte membrane.

Finally, the turbidimetric method used in this work which allowed to estimate the degree and the time course of vesiculation of the ghosts, although very sensitive, has not allowed to show significant differences between ghosts preparated from spherocytes and normocytes in their stability toward PCMB.

The experiments made with [^{14}C]PCMB in an attempt to identify the proteins involved in the stability of the erythrocyte membrane should fulfil two necessary conditions for success. First, the amounts of the mercurial should be non-saturating for the membrane proteins and we have shown this to be the case (Fig. 4). Secondly, the proteins to be identified should be preferentially reactive toward PCMB otherwise the PCMB-binding sites on the membrane will be statistically modified.

Favorable conditions seem to prevail as the results show that [^{14}C]PCMB first labels actin and band 3 protein suggesting that these two proteins are concerned in the vesiculation of the ghosts (Fig. 5). Radioactivity was not found on protein 4.9 which is known to be labelled when higher saturating concentrations of the organic mercurial are used [8]. Since actin is one of the plausible responsible proteins we have also tried to evaluate the effects of the actin binding protein, DNAase I on ghosts. We have used DNAase I at a concentration which is know to permit in certain conditions the solubilization of the cytoskeleton extracted from erythrocytes [16]. We have not observed by phase contrast microscopy significant perturbations of the membrane as it is the case with PCMB or *N*-ethylmaleimide. Similarly Sheetz [16] could not detect the dissociation of actin from unsealed ghosts in the presence of DNAase I. The drastic effects produced by micromolar concentrations of PCMB on the morphology of the red blood cell membrane cannot be due to the depolymerization of membrane-bound erythrocyte actin. Although Katz and Mommaerts [17] have shown that PCMB can mediate depolymerization of muscle F-actin, the rate of titrating the two reactive thiol groups responsible for this transformation is too slow (reaction completed in 1 h) to account for the time course of the PCMB-induced fragmentation of the ghost-membrane (half-time of 3 min). More likely the action of PCMB on ghosts consists in weakening some of the links between oligomeric actin, band 3 protein and other components of the red blood cell cytoskeleton. Unequivocal demonstration must await quantitative studies on the interactions between the PCMB-modified proteins purified from the erythrocyte membrane.

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References

1 Branton, D., Cohen, C.M. and Tyler, J. (1981) Cell 24, 24–32
2 Gratzer, W.B. (1981) Biochem. J. 198, 1–8
3 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606–2616
4 Godin, D.V. and Schrier, S.L. (1972) J. Membrane Biol. 7, 285–311
5 Carter, J.R. (1973) Biochemistry 12, 171–176
6 Steck, T.L. and Yu, J. (1973) J. Supramol. Struct. 1, 220–232
7 Holzwarth, G., Yu, J. and Steck, T.L. (1976) J. Supramol. Struct. 4, 161–168
8 Ralston, G.B. and Crisp, E.A. (1981) Biochim. Biophys. Acta 649, 98–104
9 Cassoly, R. (1982) Biochim. Biophys. Acta 689, 203–209
10 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
11 Laemmli, U.K. (1970) Nature 227, 680–685
12 Price, P.A., Liu, T.-Y., Stein, W.H. and Moore, S. (1969) J. Biol. Chem. 244, 917–923
13 Lange, Y., Gough, A. and Steck, T.L. (1982) J. Membrane Biol. 69, 113–123
14 Coakley, W.T. and Deeley, J.O.T. (1980) Biochim. Biophys. Acta 602, 355–375
15 Fischer, T.M., Haest, C.W.M., Stöhr, M., Kamp, D. and Deuticke, B. (1978) Biochim. Biophys. Acta 510, 270–282
16 Scheetz, M.P. (1979) J. Cell. Biol. 81, 266–270
17 Katz, A.M. and Mommaerts, W.F.H.M. (1962) Biochim. Biophys. Acta 65, 82–92