Biochimica et Biophysica Acta, 512 (1978) 341—349

BBA 78143

THE USE OF PHOSPHOLIPASE C TO DETECT STRUCTURAL CHANGES IN THE MEMBRANES OF HUMAN ERYTHROCYTES AGED BY STORAGE

S.D. SHUKLA *, R. COLEMAN, J.B. FINEAN and R.H. MICHELL

Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT (U.K.)

(Received February 3rd, 1978)

Summary

Human blood was stored under blood transfusion conditions for up to 10 weeks. At various times samples were removed, erythrocytes isolated and the susceptibility of the erythrocyte membrane lipids to non-lytic concentrations of phospholipase C from either *Bacillus cereus* or *Clostridium perfringens* tested. The morphology of the cells at various times and the release of microvesicles from the erythrocytes were also assessed. Initially the cells were attacked very little by the phospholipases at the concentrations chosen, but their susceptibility increased markedly after about 2 weeks, stabilised until 5 weeks, and then increased again to approach a nearly stable value after 8—10 weeks. The first rise accompanied the conversion of most of the cells to crenated and echinocytic configurations and was reversed if cells were incubated in a 'rejuvenating' medium designed to restore their energy supplies. The second rise occurred during the period when the cells underwent extensive microvesiculation and eventually became spherocytes: this phase involved, in particular, an increase in availability of phosphatidylethanolamine for hydrolysis by phospholipase C and was not reversed by attempts at 'rejuvenation'. When microvesicles released from the cells were harvested and their phospholipase susceptibility compared with that of the residual cells it was found that the microvesicles were the more susceptible. These changes in phospholipase susceptibility presumably reflect subtle changes in membrane organization that occur during storage and vesiculation of erythrocytes; the possible nature of such changes is discussed.

Introduction

During their lifespan in the circulation human erythrocytes undergo characteristic changes in surface area, total lipid, cholesterol [1—3], sialoglycoprotein

* To whom correspondence should be addressed.
Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

[4], deformability [5] and osmotic fragility [6]. During this process they lose about one-fifth of their membrane but very little of their contents [7].

When human erythrocytes are stored for several weeks under standard transfusion service conditions they undergo an extensive series of morphological changes, from discocyte to echinocyte and finally to spherocyte [8–10], with this being accompanied by a marked decrease in deformability [11]. During this period there is a decline in the phospholipid content of the cells, indicating a loss of membrane material [12,13]. This lost material can be recovered in the form of small membrane vesicles filled with cytoplasm which are released by the cells [14]. Such microvesiculation can be accelerated by incubation of erythrocytes for several hours at 37°C in the absence of a metabolic substrate [15,16] or by using an ionophore (A23187) to induce a rapid rise in the intracellular Ca^{2+} concentration [17]. Up to about a fifth of the total membrane can be released in this way, leaving smooth spherocytes which are incapable of further vesiculation. The membranes of the released vesicles have a higher lipid: protein ratio than native erythrocyte membranes, mainly because they are markedly depleted of spectrin and membrane actin, the two major extrinsic protein components normally located at the cytoplasmic surface of the membrane [17].

We recently found that treatment of erythrocytes and erythrocyte ghosts with low, sublytic concentrations of phospholipase C can provide a sensitive method for detecting subtle changes in erythrocyte membrane organization which occur during lysis and resealing and which are partially reversible by intracellular ATP [18]. In the present paper we describe the use of a similar technique to detect changes in membrane organization in intact erythrocytes during the shape changes and microvesiculation which occur in cells stored for long periods. We have also investigated to what extent these changes could be reversed by procedures designed to 'rejuvenate' the 'aged' cells.

Materials and Methods

Storage conditions of erythrocytes

Blood (6 vols.) was collected aseptically into citrate/dextrose/phosphate (1 vol.) and stored at 4°C; some samples were donated by the Regional Blood Transfusion Centre, others from volunteers in our laboratory. Samples were withdrawn aseptically at different time intervals. No bacterial contamination was detected during 10 weeks of storage.

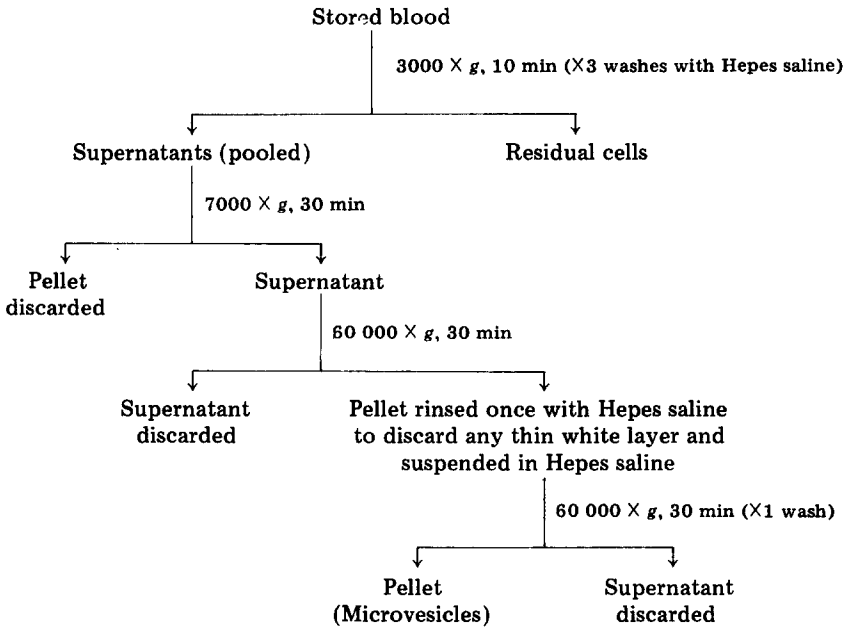
Scanning electron microscopy

To 1 vol. of washed cells one volume of 2% glutaraldehyde in Hepes saline solution (130 mM KCl, 10 mM NaCl, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (Hepes), pH 7.0) was added and the mixture allowed to stand at room temperature for 1 h. The cells were then sedimented at 200 × *g* for 2 min. The pellet was washed once with Hepes saline solution, resuspended in an equal volume of 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.5, and kept at 4°C for 2 h. The fixed suspension was centrifuged as above and washed with Hepes saline. The supernatant was discarded and the cells were dehydrated by resuspending successively in 40, 60 and 100% acetone. The sam-

ples were allowed to stand overnight in 100% acetone and small amounts were put on scanning electron microscope 'stubs' and allowed to dry. The stubs were kept in a vacuum desiccator until required. For examination, samples were coated with gold by sputtering in an argon atmosphere and viewed using a Cambridge Steroscan 600 Microscope.

Separation of microvesicles and residual cells

Blood at different intervals of storage was aseptically withdrawn and the microvesicles and residual cells were separated at 4°C as shown below: The protocol is slightly simplified as compared with ref. 14 but the vesicles isolated are indistinguishable.



'Rejuvenation' of aged cells

The method of 'rejuvenation' was based on a previous report [19]. Cells were isolated from stored blood and washed twice in Hepes saline (130 mM KCl, 10 mM NaCl, 10 mM Hepes, pH 7.0). The cells from one unit of blood (approx. 450 ml) were mixed with 50 ml of Hepes saline solution containing 50 mM pyruvate, 50 mM inosine, 100 mM glucose, 50 mM phosphate and 5 mM adenine. This suspension was incubated for an hour at 37°C. The cells were then sedimented at 3000 × *g* for 10 min and washed once with Hepes saline solution. The resuspended pellets ('rejuvenated' cells) were immediately treated with 8 U/ml assay, phospholipase C (*Bacillus cereus*).

Treatment with phospholipase C

Purified phospholipase C from *B. cereus* [20] was a gift from Dr. C. Little. Phospholipase C from *Clostridium perfringens* was purified on an immunosorbent column by the method of Bird et al. [21]. Activities of these prepara-

tions are given elsewhere [18]. Cells or microvesicles were treated with enzyme by incubating them in a mixture which contained 200—300 nmol membrane phospholipid and enzyme in a final volume of 0.5 ml of Hepes saline solution (130 mM KCl, 10 mM NaCl, 10 mM Hepes, pH 7.0). In the case of the *C. perfringens* enzyme, $CaCl_2$ was added to a final concentration of 1 mM. Incubation was for 30 min at 37°C and the reaction was stopped by the addition of 2 mM EDTA and 0.5 mM phenanthroline in 0.5 ml of Hepes saline solution.

Extraction and analysis of lipids

Lipids were extracted and analysed as described before [18].

Results

Phospholipase C susceptibility, shape changes and microvesiculation during storage

Throughout the experiments incubations with purified phospholipases C (*B. cereus* and *C. perfringens*) were under conditions in which there was negligible haemolysis (less than 3%) or phospholipid hydrolysis in fresh human erythrocytes. During the first week of storage, susceptibility to these enzymes showed little change, but after about 2 weeks their susceptibility to phospholipid hydrolysis by both enzymes had risen substantially. It then remained largely

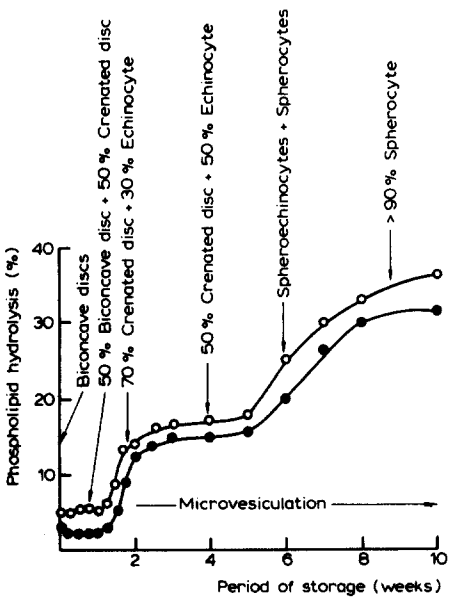


Fig. 1. Susceptibility of stored erythrocytes to phospholipase C. Cells at various intervals of storage were separated from microvesicles and washed with Hepes saline. These cells were treated with purified phospholipase C (*B. cereus*, 8 U/ml assay ●———●; *C. perfringens*, 20 mU/ml assay ○———○) and phospholipid hydrolysis determined from the decrease in organic phosphorus content of extracted lipids (see Materials and Methods). Shape changes (approx. % values) were monitored by phase contrast microscopy and scanning electron microscopy. The curves of this figure are composites based on 15—20 experiments. Points may represent single or multiple experiments.

unchanged until about the 5th week, when susceptibility once again increased relatively rapidly (Fig. 1). During the same period cells underwent a series of morphological changes (Fig. 2). Crenated discs and echinocytes were dominant between 2 and 5 weeks; spheroechinocytes and smooth spherocytes became the major cell configurations after about 6 weeks. Simultaneously, the cells microvesiculated, releasing 100—150 nm diameter vesicles filled with cytoplasm.

If cells were incubated in a physiologically favourable medium designed to restore their energy supplies and thus, it was hoped, to biochemically 'rejuvenate them, then their increase in phospholipase C susceptibility induced by storage could be substantially reversed for the first five weeks, but not thereafter (Fig. 3). It thus seems that the two phases of increase in susceptibility to phospholipase attack are distinct, with the first phase (up to 5—6 weeks) a reversible process and the second phase (after 6—7 weeks) essentially irrevers-

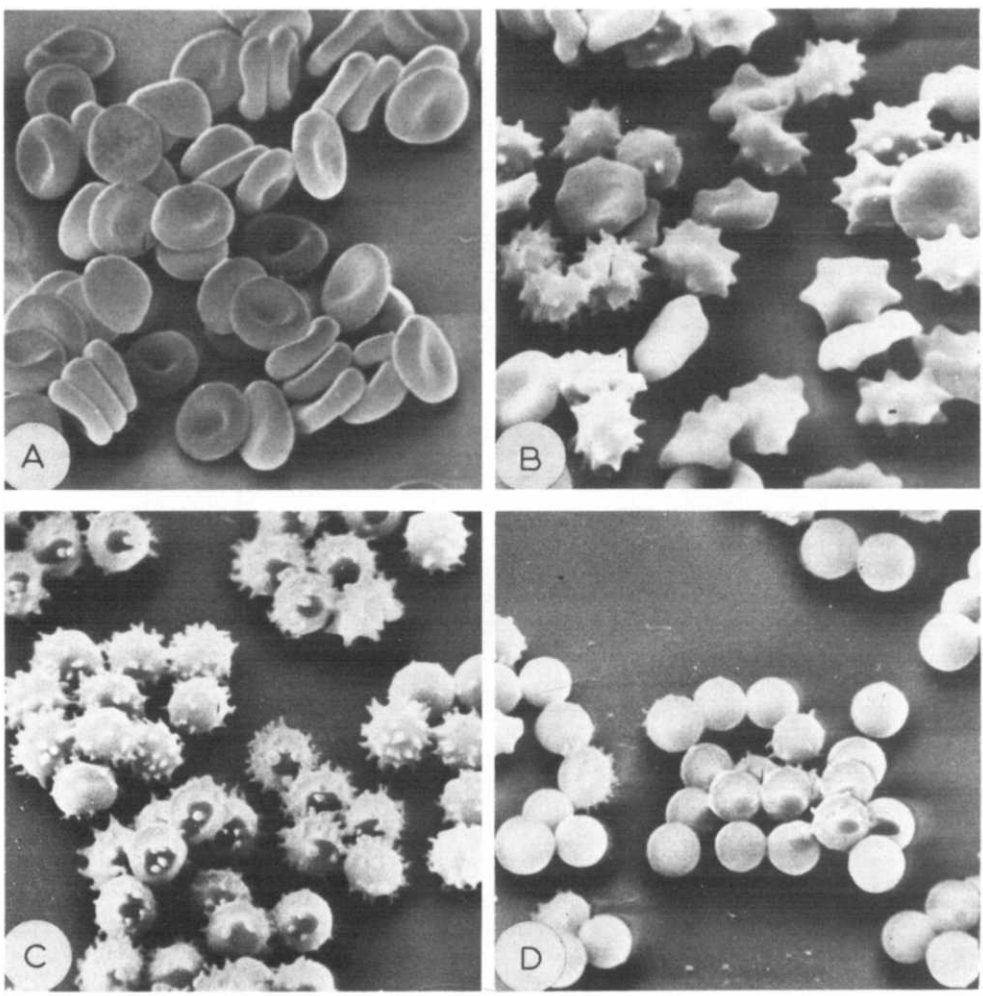


Fig. 2. Scanning electron micrographs of cells (residual cells) at different periods of storage. (A) fresh intact cells, (B) 3rd week cells, (C) 6th week cells, (D) 10th week cells. Magnification X 1500.

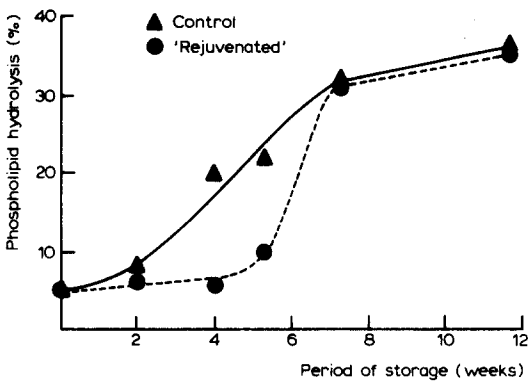


Fig. 3. Susceptibility of 'rejuvenated' erythrocytes to phospholipase C. At various periods of storage the residual cells were washed free of microvesicles. Cells were 'rejuvenated' by incubating with a solution of Hepes saline containing pyruvate (5 mM), inosine (5 mM), glucose (10 mM), phosphate (5 mM) and adenine (0.5 mM) at 37°C for 1 h and were washed once with Hepes saline (see Materials and Methods). 'Rejuvenated' cells were treated with phospholipase C (*B. cereus*), 8 units/ml assay, and the percentage phospholipid hydrolysis determined as described in Fig. 1.

ible. We therefore selected erythrocytes and microvesicles from blood stored for 5 and 9 weeks for detailed study.

Comparison of residual cells and microvesicles

Detailed analysis indicated that at both 5 and 9 weeks the lipid compositions of residual cells and of microvesicles were very similar, with the 9 week micro-

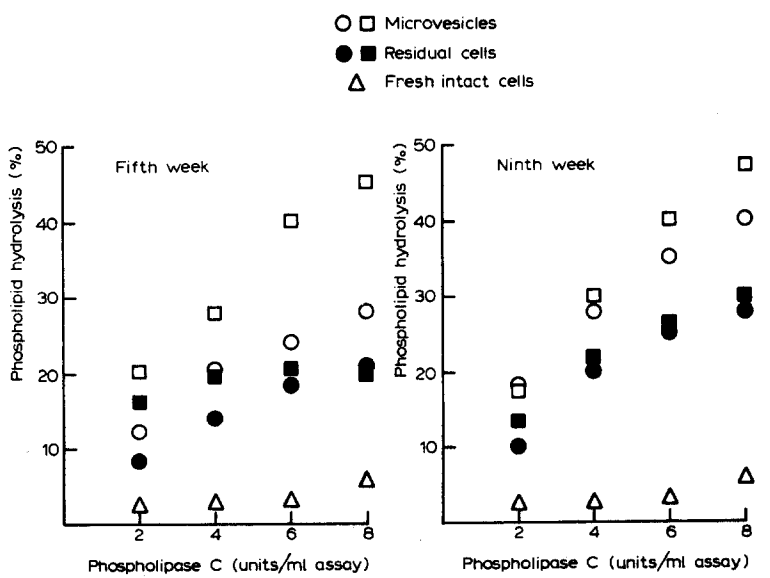


Fig. 4. Susceptibilities of residual cells and microvesicles to phospholipase C. Residual cells and microvesicles were isolated from blood stored for 5 and 9 weeks and were treated with different concentrations of phospholipase C (*B. cereus*) as described in Materials and Methods. Phospholipid hydrolysis was determined by estimating organic phosphorus in the extracted lipid. Open symbols represent microvesicles and filled symbols represent residual cells. Circles and squares represent experimental points from two different experiments.

TABLE I

PHOSPHOLIPID PROFILES OF PHOSPHOLIPASE C-TREATED RESIDUAL CELLS

Washed residual cells from blood stored for 5 and 9 weeks were treated with purified phospholipase C (*B. cereus*), 8 units/ml assay, as described in Materials and Methods. The reaction was stopped and the cells were sedimented at 20 000 × *g* for 15 min at 4°C. A control incubation of cells without enzyme was run parallel in each case. Lipids were extracted from the pellet and analysed by TLC. Values are expressed as percentage hydrolysis of individual phospholipids ± S.D. with the number of experiments in parentheses.

	Phospholipid hydrolysis (%)		
	Fresh intact cells	5th week	9th week
Total phospholipid hydrolysis (%)	2—4	15—18	30—35
Sphingomyelin	Not determined	12 ± 3 (4)	11 ± 4 (4)
Phosphatidylcholine	Not determined	19 ± 3 (4)	47 ± 12 (4)
Phosphatidylserine + phosphatidylinositol	Not determined	18 ± 7 (4)	32 ± 15 (4)
Phosphatidylethanolamine	Not determined	3 ± 2 (4)	51 ± 19 (4)

vesicles slightly richer in sphingomyelin than 9-week residual cells (data not shown). When residual cells and the microvesicles they had released were both recovered and exposed to phospholipase C (*B. cereus*) it was found that microvesicles were more susceptible to phospholipase attack than were the cells: this applied to cells and microvesicles isolated after either 5 or 9 weeks of storage (Fig. 4).

Effects of storage upon the susceptibility to hydrolysis of individual lipid classes

Fresh cells and cells stored for 5 and 9 weeks were separated from the released microvesicles and exposed to phospholipase C (*B. cereus*). They were then analysed to determine which lipids had been hydrolysed by such non-lytic treatment with phospholipase (Table I). Fresh cells were only attacked to a very slight extent, and it was therefore not feasible to determine which lipids were hydrolysed. After 5 weeks there was appreciable hydrolysis of phosphatidylcholine, sphingomyelin and phosphatidylserine + phosphatidylinositol. By 9 weeks, however, the susceptibility of phosphatidylethanolamine to phospholipase C hydrolysis had risen dramatically. Smaller increases were seen in the susceptibilities of phosphatidylcholine and phosphatidylserine plus phosphatidylinositol but sphingomyelin showed no further change.

Discussion

The changes of shape, loss of phospholipid and microvesiculation that we have observed during the storage of erythrocytes are similar to those reported by previous observers but our studies have covered a longer period of storage and have included a parameter, namely susceptibility of membrane phospholipids to hydrolysis by phospholipase C, which probably relates directly to perturbation of membrane structure.

The sharp rise in susceptibility to phospholipid hydrolysis during the second week occurred at about the same time as the majority of cells changed shape

from biconcave disc to crenated discs and echinocytes and this was followed by a plateau in the hydrolysis curve coincident with the commencement of microvesiculation. The second steep rise in extent of phospholipid hydrolysis started approximately at the point where the majority of cells changed in morphology 'from echinocyte to spheroechinocyte and spherocyte': at the same time the increased susceptibility to phospholipase C became irreversible.

Changes in susceptibility to phospholipases C are probably a result of changes in the molecular organization of the membrane which make phospholipids more accessible to the enzymes. The reversible first stage of this change in molecular accessibility and the accompanying changes in morphology may be caused at least in part by a fall in cytoplasmic ATP concentrations [22]. However, it is uncertain whether or not these changes are interdependent [23]. The irreversibility of the second stage of change in phospholipid susceptibility beyond the fifth week of storage seems likely to be a direct consequence of a substantial loss of membrane by vesiculation.

The pattern of phospholipid hydrolysis during the reversible first stage showed no striking features, but during the second, irreversible stage (after the fifth week) there was a dramatic increase in susceptibility of phosphatidylethanolamine to phospholipase C. Although there was still only a very low level of haemolysis (less than 3%) the amount of this phospholipid that was hydrolysed was substantially greater than has been calculated to be present in the outer leaflet of the membrane bilayer in the fresh, intact erythrocyte [24–26]. Perhaps there have been changes in membrane organisation which allow the phospholipase to penetrate into the membrane so as to interact with phospholipid in the inner leaflet, although this would imply penetration of the enzyme into non-polar regions of the membrane. Alternatively there may be substantial lipid exchange between inner and outer leaflets during prolonged storage of cells.

Microvesicles, produced either by storage or by treatment of erythrocytes with Ca^{2+} plus ionophore A23187 (Shukla, S.D., unpublished), were more susceptible to phospholipase C than were residual cells. In both cases the most striking difference in composition between cells and vesicles lies in the virtual absence of spectrin and depletion of membrane actin in the vesicles [14,17]. Although these proteins are located at the cytoplasmic surface of the membrane whereas the phospholipase was presented only to the outer surface, it may be relevant to recall that internal ATP can protect erythrocyte ghosts against attack by external phospholipase C [18].

These results provide confirmation that purified phospholipases C can be used as sensitive tools with which to detect slight changes in the organization of the erythrocyte membrane (and maybe of other membranes), but a lack of detailed knowledge of the interactions between these enzymes and membranes means that we cannot yet define the nature of these changes. In the experiments on storage of cells that are reported here this difficulty of detailed interpretion may arise partly from the sequential occurrence of several distinct types of change in membrane organization in the cells and microvesicles. It may, however, be significant that in at least three different situations, namely erythrocyte storage in vitro, the incubation of energy-depleted erythrocytes at 37° C for a few hours [27,28] and the comparison of erythrocyte ghosts with and with-

out internal ATP [18], the largest changes in behaviour are shown by phosphatidylethanolamine. This may mean that the structural interrelationships between this lipid and other membrane components are particularly susceptible to perturbations of membrane organization.

Acknowledgements

We are grateful to the Science Research Council for financial support. We are also grateful to Mr. J. Berriman and to the Department of Materials Science for scanning electron microscopy.

References

1 Westerman, M.P., Pierce, L.E. and Jensen, W.N. (1963) J. Lab. Clin. Med. 62, 394—400
2 Van Gastel, C., Van Den Berg, D., De Gier, J. and Van Deenen, L.L.M. (1965) Br. J. Haematol. 11, 193—199
3 Winterbourn, C.C. and Batt, R.D. (1970) Biochim. Biophys. Acta 202, 1—8
4 Balduini, C., Balduini, C.L. and Ascari, E. (1974) Biochem. J. 140, 557—560
5 La Celle, P.L. and Arkin, B. (1970) Blood 36, 837
6 Danon, D. and Marikovsky, Y. (1964) J. Lab. Clin. Med. 64, 668—674
7 Cohen, N.S., Ekholm, J.E., Luthra, M.G. and Hanahan, D.J. (1976) Biochim. Biophys. Acta 419, 229—242
8 Ponder, E. (1955) Red Cell Structure and its Breakdown, Springer-Verlag, Vienna
9 Nakao, M., Nakao, T., Tatibana, M., Yoshikawa, H. and Abe, T. (1959) Biochim. Biophys. Acta 32, 564—565
10 Bessis, M. and Brecher, G. (1971) Nouv. Rev. Fr. Hematol. 11, 305—307
11 Weed, R.I., La Celle, P.L. and Merrill, E.W. (1969) J. Clin. Invest. 48, 795—809
12 Haradin, A.R., Weed, R.I. and Reed, C.F. (1969) Transfusion 9, 229—237
13 Weed, R.I., La Celle, P.L. and Udkow, M. (1974) in The Human Red Cell in vitro (Greenwalt, T.J. and Jamieson, G.A., eds.), pp. 65—89 Grune and Stratton, New York
14 Rumsby, M.G., Trotter, J., Allan, D. and Michell, R.H. (1977) Biochem. Soc. Trans. 5, 126—128
15 Lutz, H.U., Liu, S. and Palek, J. (1977) J. Cell Biol. 73, 548—560
16 Lutz, H.U., Lomant, A.J., McMillan, P. and Wehrli, E. (1977) J. Cell Biol. 74, 389—398
17 Allan, D., Billah, M.M., Finean, J.B. and Michell, R.H. (1976) Nature 261, 58—60
18 Shukla, S.D., Billah, M.M., Coleman, R., Finean, J.B. and Michell, R.H. (1978) Biochim. Biophys. Acta 509, 48—57
19 Valeri, C. (1974) in The Human Red Cell in vitro (Greenwalt, T.J. and Jamieson, G.A., eds.), pp. 281—321, Grune and Stratton, New York
20 Little, C., Aurebekk, B. and Otnaess, A.B. (1975) FEBS Lett. 52, 175—179
21 Bird, R.A., Low, M.G. and Stephen, J. (1974) FEBS Lett. 44, 279—281
22 Nakao, M. (1974) in Cellular and Molecular Biology of Erythrocytes (Yoshikawa, H. and Rapoport, S.M. eds.), pp. 35—54, University Park Press, Baltimore
23 Feo, C. and Mohandas, N. (1977) Nature 265, 166—168
24 Zwaal, R.F.A., Roelofsen, B. and Colley, C.M. (1973) Biochim. Biophys. Acta 300, 159—182
25 Bretscher, M. (1973) Science 181, 622—629
26 Renooij, W., Van Golde, L.M.G., Zwaal, R.F.A. and Van Deenen, L.L.M. (1976) Eur. J. Biochem. 61, 53—58
27 Gazitt, Y., Ohad, I. and Loyter, A. (1976) Biochem. Biophys. Res. Commun. 72, 1359—1366
28 Haest, C.W.M. and Deuticke, B. (1976) Biochim. Biophys. Acta 436, 353—365