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Changes in membrane polypeptides, polyphosphoinositides and phosphatidate in dense fractions of sickle cells

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When sickle erythrocytes were fractionated on discontinuous isotonic stractan gradients the denser fractions, which were rich in irreversibly sickled cells contained less polyphosphoinositides and more phosphatidate than either lighter sickle cell fractions or normal cells. These changes could be due to activation of a polyphosphoinositide phosphodiesterase in the denser cells. Membrane polypeptide analysis of the denser fractions also showed a marked depletion of band 4.1 and a protein of molecular mass about 110 kDa but an increased amount of a 180 kDa polypeptide which might be a breakdown product of ankyrin. These biochemical alterations could be consequences of Ca^{2+} accumulation in the denser sickle cells and may contribute to the structural alterations which give rise to irreversibly sickled cells.

Introduction

In sickle cell disease, erythrocytes are thought to undergo a progressive change in morphology from young discocytic cells which can sickle reversibly to old 'irreversibly sickled' cells (ISCs) which are ultimately removed from the circulation. This progression parallels the normal process of cell ageing but occurs about 10-times faster, causing excessive demands on the cellular mechanisms involved in erythrocyte disposal and formation and resulting in the characteristic anaemia associated with the disease. Despite the considerable efforts which have been expended over the last two decades to pinpoint the precise molecular changes which are involved in the creation of ISCs, it is still not generally agreed what are the crucial biochemical factors in the process. However, it does seem clear that accumulation of Ca^{2+} within sickle cells and the interaction of Ca^{2+} with the membrane have important roles since not only do ISCs contain relatively high amounts of Ca^{2+} [1–3], but they show certain changes which may be a consequence of elevated intracellular levels of calcium. For instance, ISCs are dehydrated relative to young sickle cells and normal cells and it has been suggested that this is a result of a Ca^{2+}-dependent increase in K^+ permeability (the Gardos effect) [4]. ISCs also appear to have lost up to 15% of their phospholipids [5] and it seems likely that this loss is induced not only by repeated oxygenation-deoxygenation cycles during the passage of sickle cells through the circulation [6], but also is due to vesicle release promoted by increased intracellular Ca^{2+} levels [7,8].

In normal erythrocytes, evidence has accumulated to show that Ca^{2+} entry into cells results in a number of biochemical alterations: these in-

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Abbreviations: PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate.

clude breakdown of polyphosphoinositides (phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2)) to diacyl glycerol [8–11], the aggregation of protein components catalysed by transamidase [12–15] and the apparent proteolysis of ankyrin and band 4.1 [8,12–16]. A similar correlation between Ca^{2+} accumulation and some of these protein alterations has also been demonstrated in sickle cells, especially in ISC-rich fractions [15,17].

The work we present here confirms these findings and further demonstrates that the denser fractions of normal and particularly sickle cells have reduced levels of polyphosphoinositides and raised concentrations of phosphatidate relative to other phospholipids and this observation is consistent with the idea that older cells may have suffered an irreversible Ca^{2+}-dependent breakdown of PIP and PIP_2 to diacylglycerol which is subsequently converted to phosphatidate. In addition, a loss of certain phosphoproteins which may be associated with cytoskeleton also seems to have occurred in the denser fractions of sickle cells.

Materials and Methods

Stractan (arabinogalactan polysaccharide from larch wood), 4-morpholinepropanesulphonic acid (Mops), Trizma base, glycine, EDTA and EGTA were from Sigma Chemical Co. (Poole, Dorset, U.K.). All other reagents were Analar or Electran grade from BDH (Poole, Dorset, U.K.).

Preparation and fractionation of cells. 10-ml samples of blood was obtained in heparized tubes from either healthy normal human (HbAA) donors or from patients with sickle cell disease (HbSS) having 10–15% ISCs. A single experiment was carried out with cells from one patient (P.R.) with an unusually high content of ISCs (45%). Fresh blood samples were centrifuged at $1000 \times g_{av.}$ for 5 min, and erythrocytes were washed three times with 150 mM NaCl at 4°C, with removal of the buffy coat. The cells were given a final wash with 20 mM Mops/NaOH buffer (pH 7.1) containing 150 mM NaCl (Mops/saline). Washed normal or sickle erythrocytes were then fractionated on discontinuous stractan gradients [18] made up as follows: two standard stock stractan solutions of density 1.12 and 1.15 at 20°C were made by dissolving either 4 g or 6 g of stractan in 9 ml each of Mops/saline containing 0.5 mM phosphate, 1 mM Mg^{2+}, 0.1 mM EGTA and 10 mM glucose. Solutions of density 1.13, 1.135, 1.14, and 1.145 were prepared by mixing appropriate volumes of density 1.12 and density 1.15 solutions.

For separation of erythrocytes, 1.5 ml of stractan solution of density 1.145 was poured into polyallomer centrifuge tubes measuring 14 mm by 90 mm. 2 ml of stractan solutions of density 1.14, 1.135, 1.13 and 1.12 at 20°C were layered on top of each other and 2-ml samples of either normal or sickle erythrocytes at 50% haemocrit in Mops/saline were layered on top of the gradients which were centrifuged in a Beckman L2-65B ultracentrifuge using a SW41 Ti swing out rotor for 2 h at 20° at $156\,000 \times g_{av.}$. Individual gradients were fractionated by aspiration. Each fraction was made up to 40 ml with Mops/saline, the total number of cells and ISCs per fraction was counted and the cells were sedimented at $30\,000 \times g_{av.}$ for 5 min at 4°C. Cells were classified as ISCs if under oxygenated conditions, they retained one or more pointed projections or if their length was greater than twice their width [19].

Preparation of ghosts and analysis of membrane polypeptides and phospholipids. Ghosts were prepared from normal and sickle erythrocyte fractions by lysis at 4°C in 40 vols. of 20 mM Tris-HCl (pH 7.4), containing 1 mM EDTA and 0.1 mM phenylmethylsulphonyl fluoride, followed by three washes by centrifugation at $30\,000 \times g_{av.}$ for 10 min in the same medium. Analysis of membrane polypeptides was carried out by SDS-polyacrylamide gel electrophoresis on 6% (w/w) acrylamide gels by the procedure of Laemmli [20]. Rabbit muscle phosphorylase *b* (97 kDa), bovine serum albumin (68 kDa), and bovine muscle actin (43 kDa) were used as standard molecular mass markers. Polypeptides were quantified, after staining the gels with Coomassie brilliant blue R, by scanning with a Bio-Rad scanning densitometer (model 1650) linked to a Shimadzu C-RIB integrator.

Separation and quantification of phospholipids was carried out as described recently [21]. Briefly, ghosts were treated first with neutral chloroform/methanol to extract the major phospholipids to-

gether with phosphatidylinositol and phosphatidate and then with acidic chloroform/methanol to extract polyphosphoinositides. Phospholipids from the neutral extracts were separated on EDTA-impregnated TLC plates and phosphoinositides were separated on oxalate-impregnated plates. Iodine-staining spots were identified by comparison with standard phospholipids and after digestion with 70% $HClO_4$ at 180°C for 1 h were analysed for phosphorus by the method of Bartlett [22].

Results

When erythrocytes from individuals with sickle cell disease were fractionated using a five-step discontinuous stractan gradient, the cells showed a bimodal density distribution with about 50% of the total cell population in fraction S2 centred at

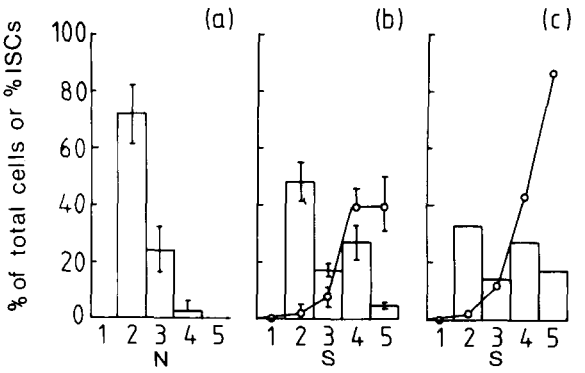

Fig. 1. Fractionation of normal and sickle erythrocytes on stractan density gradients. Cells from normal (a) and sickle donors (b, c) were fractionated on stractan gradients. The percentage of cells in each fraction is indicated in bar form and the percentage of ISCs in the sickle fractions is shown (open circles). Results are presented as the mean ± S.E. from five normal donors (a) and six sickle donors (b) with 10–15% ISCs. A single experiment is shown (c) with blood from a patient (P.R.) with an unusually high content of ISCs (45%). S1–S5 and N1–N5 refer to the fractions from sickle and normal cells, respectively, in order of increasing density.

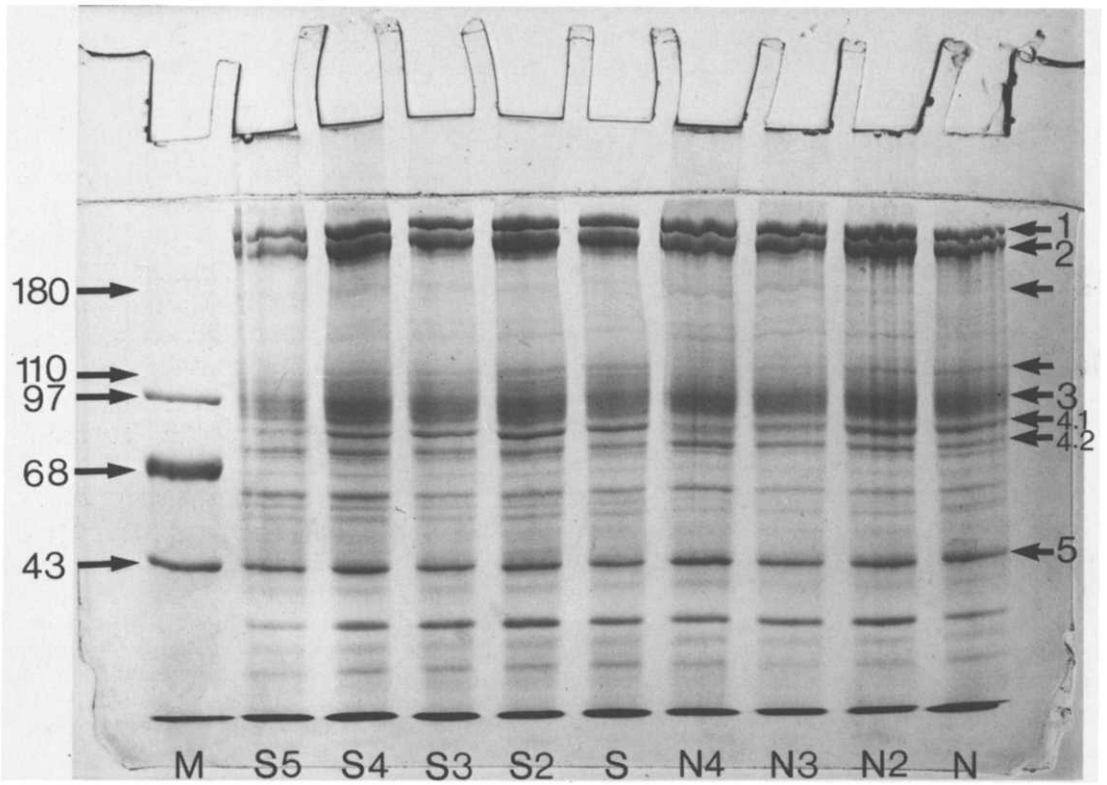

Fig. 2. SDS-polyacrylamide gel electrophoresis of membranes from normal and sickle erythrocytes fractionated on stractan gradients. Numbering of fractions is as in Fig. 1 but samples of unfractionated cells (S and N) are also included. Numbers on the left refer to the molecular masses (kDa) of protein markers (lane M) and of minor polypeptides (180, 110). Polypeptides are numbered on the right according to the nomenclature of Steck [33].

the 1.12/1.13 density interface (Fig. 1b) and 25–30% in fraction S4 centred at the 1.135/1.14 density interface. A small number of cells (about 5%) sedimented at the 1.14/1.145 density interface (S5). A single experiment with a patient (P.R.) with an abnormally high ISC count (45%) showed a similar bimodal distribution but with a greatly increased number of cells in the densest fraction (Fig. 1c). In contrast, normal cells (Fig. 1a) showed one major peak of distribution, with about two thirds of the cells at the 1.12/1.13 density interface (N2) whilst the majority of the remaining cells collected at the 1.13/1.135 density interface (N3). Less than 5% of the total cell population migrated through 1.14 density stractan. The densest of the sickle cell fractions (S4 and S5) showed the highest content of ISCs, generally 3–4 times that of the unfractionated cells, although fraction S5 from patient P.R. was almost 90% ISCs.

Fig. 2 illustrates the polypeptide composition of membranes prepared from the fractionated sickle and normal erythrocytes. Essentially, the Coomassie blue-stained polypeptide pattern of unfractionated HbSS cells was similar to that of unfractionated HbAA cells. Nevertheless, significant decreases in the amount of band 4.1 and of a polypeptide of about 110 kDa were seen with increasing cell density (Figs. 2 and 3). Fraction S5, which contained the highest number of ISCs, showed a decrement of more than 50% in these polypeptides compared with S2. In addition, consistent increases with cell density were seen in the amount of a polypeptide of about 180 kDa. These polypeptide changes were not restricted to sickle cells since they were also observed (albeit to a smaller extent) in fractionated normal cells (Figs. 2 and 3a) and it was noteworthy that the single sickle sample (P.R.) with the most dense cells and ISCs (Fig. 1c) showed the least-marked decrease

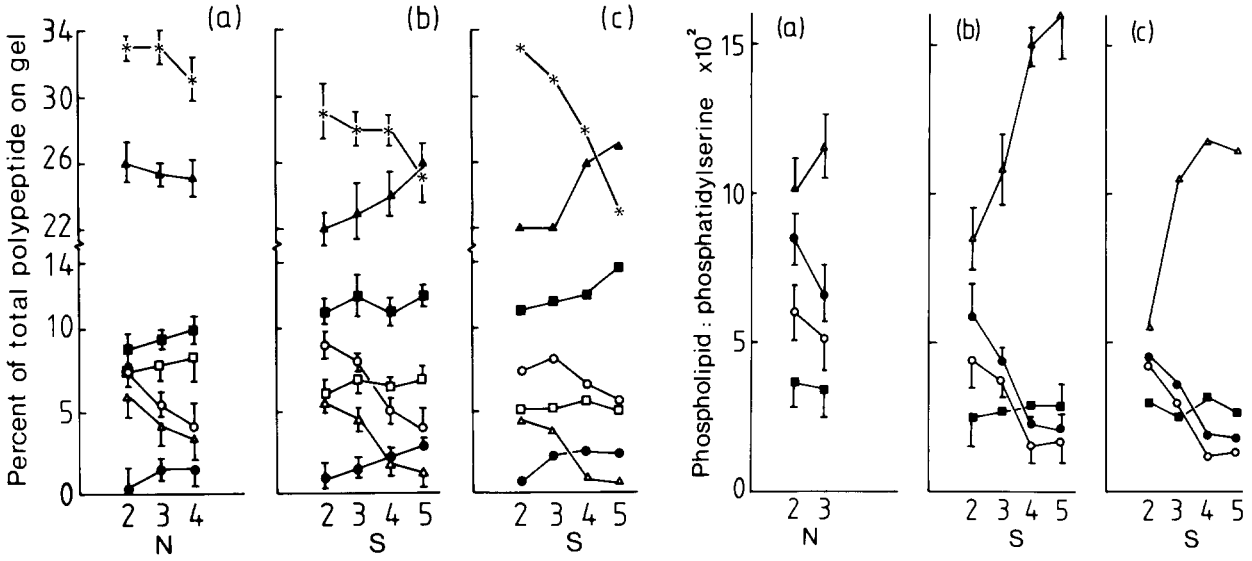

Fig. 3. Changes in polypeptide composition in fractions of sickle and normal cells. Gels similar to that shown in Fig. 2 were analysed densitometrically and the results are expressed as the percentage of the total density on each track (not including the front) which appeared in certain bands. Individual points represent means ± S.E. from five experiments for (a) (normal cells) and (b) (sickle cells) but (c) is based on a single experiment with a sample containing an unusually high number of ISCs. ★———★, band 1+2 (spectrin); ▲———▲, band 3; ■———■, actin; ○———○, band 4.1; □———□, band 4.2; △———△, 110 kDa band; ●———●, 180 kDa band.

Fig. 4. Inositide and phosphatidate content of normal and sickle cells fractionated on stractan gradients. Fractions are numbered as in Fig. 1 and quantities are expressed as the molar ratio of each lipid to phosphatidylserine (15% of the total lipid in each case). Results are based on means ± S.E. for five samples of normal cells (a) and five samples of sickle cells (b) together with a single high ISC sample (c) ●———●, PIP_2; ○———○, PIP; △———△, phosphatidate; ■———■, phosphatidylinositol.

in band 4.1, suggesting that cell density and 4.1 content are not always closely associated (Fig. 3c).

The amounts of actin, band 4.2 and band 3 in sickle or normal cells showed no significant change with cell density (the apparent increase in band 3 in Figs. 3b and 3c is largely a reflection of relative decreases in other components) but there was a significant decrease of material in the spectrin region with increase in cell density. This could have been partly due to the increased protein polymer formation which was repeatedly observed as Coomassie-staining material which remained at the top of the stacking gel particularly in tracks derived from high-density cells. Additionally, loss from the spectrin region could have been due to breakdown of ankyrin (which did not separate from the lighter component of spectrin) and this interpretation would be consistent with the observed increase in the 180 kDa band, since breakdown of ankyrin often gives a fragment of this size (band 2.3?) [8,12,13].

In sickle cells (Figs. 4b and 4c) there were clear indications of greater than 50% decreases of polyphosphoinositides in cells of higher density. These changes were reflected not as an increase in cell content of phosphatidylinositol as might have been expected if polyphosphoinositides were progressively degraded by a phosphomonoesterase, but instead by an increased content of phosphatidate, which approximately doubled with increasingly cell density. The total decrease of PIP_2 + PIP was somewhat less than the increment in phosphatidate but these can probably be explained by relative losses in the extraction of the polyphosphoinositides. The simplest interpretation of this observation was that in the denser cells polyphosphoinositides were degraded by the phosphodiesterase known to exist in human erythrocytes [8–11], to give diacylglycerol which is then converted to phosphatidate by diacylglycerol kinase. Normal cells (Fig. 4a) showed some indication of changes similar to those seen in sickle cells, but shortage of material precluded reliable observations on the highest density fraction (N4) usually observed.

Discussion

There have been a number of reports in which sickle cells have been fractionated by density [5,15,17,23] and in some cases alterations in polypeptide composition have been noted [15,17], especially in cells of higher density. The stractan gradient conditions used in our work are similar to those employed by previous investigators and gave bands at comparable densities [15]. It is noteworthy that under our conditions, the density distribution of sickle cells was bimodal, with a discrete secondary distribution peak centred at the 1.135/1.14 density interface (S4) in addition to the major peak at the 1.12/1.13 density interface (S2) (Figs. 1b and 1c). Such a density distribution has been reported previously for sickle cells separated on Percoll/stractan [23] or Percoll/Renografin [24] gradients and contrasts with the unimodal distribution of normal cells under the same conditions (Fig. 1a). If, as seems likely, the increasing density of cells is a reflection of an ageing process, then the results suggest that the cells spend a relatively short period of time in the developmental phase corresponding to the density 1.13/1.135 interface (S3) and tend to accumulate in the phase corresponding to the density 1.135/1.14 interface (S4). This seems to indicate a relatively rapid transition from cells of 'normal' density (S2) to cells of high density (S4) which are rich in ISCs.

The nature of this density transition is still a subject for speculation but some investigators now favour the hypothesis that a failure of Ca^{2+} homeostasis allows accumulation of Ca^{2+} in the cells and that this triggers the Gardos effect, with a loss of KCl and water which consequently increases cell density [2,4,5,24]. Such an explanation would be quite consistent with the further observation of changes in the polypeptide pattern of the cell membranes which also appear to depend on raised intracellular Ca^{2+}, such as loss of polypeptide 4.1 and ankyrin and the increase in cross-linked protein which does not migrate during gel electrophoresis [8,12–16]. The Gardos effect is particularly sensitive to cytoplasmic Ca^{2+} concentrations since half-maximal K^+ release (and the resulting cellular dehydration) occur at submicromolar levels of Ca^{2+} [25,26]. The polypeptide changes seem to require higher (10 μM) concentrations of intracellular Ca^{2+} [26]. The apparent loss of the 110 kDa polypeptide from denser cells reported here does not seem to have been

observed before, probably because it does not separate easily from band 3. However this observation does raise the possibility that there exists a family of proteins including ankyrin, 110 kDa protein and 4.1, the members of which are each subject to degradation in the presence of raised intracellular Ca^{2+}. Preliminary evidence (unpublished results) shows that the 110 kDa polypeptide is, like 4.1 and ankyrin, a phosphoprotein although it is not known if it also forms part of the membrane skeleton [27,28].

Relative decreases in overall lipid content of the denser fractions of sickle cells have been reported [5] and could in part also be a consequence of raised intracellular Ca^{2+}, which is known to promote the release of lipid-rich vesicles from erythrocytes [8]. Ca^{2+} also catalyses the breakdown of polyphosphoinositides by an endogenous phosphodiesterase in normal human erythrocytes [8–11,25] and this process offers a simple explanation for the present observations relating to inositide and phosphatidate content in the denser sickle fractions (Fig. 4). The results suggest that progressive Ca^{2+} accumulation in the sickle cells, in addition to promoting increases in cell density, loss of total membrane lipid and decreases in certain membrane polypeptides, also causes the breakdown of polyphosphoinositides to diacylglycerol which is then phosphorylated to give phosphatidate. It is interesting to note that there have been previous suggestions that the level of polyphosphoinositides in erythrocytes could influence cell shape [8,29] and a recent publication [30] has provided evidence that these lipids mediate the interaction of polypeptide 4.1 with the transmembrane glycoprotein, glycophorin. Thus a decrease in polyphosphoinositides might well affect the cytoskeletal structure which controls cell morphology.

The role of the above changes in the development of the ISC are unclear. Indeed, most of the changes seem to occur in the higher density fractions of normal cells, suggesting that the observed alterations could be a normal accompaniment to the process of erythrocyte ageing but are seen much more prominently in sickle cells because the production of 'aged' (i.e. dense) sickle cells exceeds the capacity of the system to eliminate them. Although it is possible that rigidification of the cell membrane produced by alterations in phospholipid content and polypeptide breakdown or aggregation could contribute to the inelastic properties of the denser cells which are recognised as ISCs, it seems more likely to us that dehydration induced by Ca^{2+} entry, leading to gelation of the already highly concentrated cellular haemoglobin must be a major factor in rigidifying ISCs [5,31]. It is difficult to see how membrane rigidity could be the largest influence on the rheological properties of ISCs when the membrane accounts for only about 1% of the erythrocyte mass.

The present data supports the suggestion that the primary lesion in the process leading to ISC formation is an increase in cytosolic Ca^{2+} since all the biochemical changes observed seemed to depend on a prior rise in Ca^{2+}. Although ISCs do have much higher overall Ca^{2+} contents than either non-ISC sickle fractions or normal erythrocytes, it is unclear whether ISCs actually have higher cytoplasmic Ca^{2+} levels. Recent evidence from Lew and his co-workers suggests that much of the ISC Ca^{2+} is locked up inside intracellular vesicles and is not available for activation of K^+ channels [25,32], but as these authors note, "cytoplasmic Ca^{2+} must be increased transiently at least, to lead to accumulation within endocytic vesicles" and even a transient increase in cytoplasmic Ca^{2+} may be sufficient to reach the micromolar levels of free Ca^{2+} which are required to activate the various Ca^{2+}-sensitive biochemical changes observed in ISCs.

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References

1 Eaton, J.W., Skelton, T.D., Swafford, H.S., Kolin, C.E. and Jacob, H.S. (1973) Nature 246, 105–106
2 Palek, J. (1977) J. Lab. Clin. Med. 89, 1365–1374

3 Bookchin, R.M., Raventos, C. and Lew, V.L. (1981) in Proceedings of the Fifth Annual Conference on Red Cell Metabolism and Function (Brewer, G.J., ed.), Alan Liss, New York
4 Glader, B.E. and Nathan, D.G. (1978) Blood 51, 983–989
5 Westerman, M.P. and Allan, D. (1983) Br. J. Haematol. 53, 399–409
6 Allan, D., Limbrick, A.R., Thomas, P. and Westerman, M.P. (1982) Nature 295, 612–613
7 Allan, D., Limbrick, A.R., Thomas, P. and Westerman, M.P. (1981) Br. J. Haematol. 47, 383–390
8 Allan, D. and Thomas, P. (1981) Biochem. J. 198, 433–440
9 Allan, D. and Michell, R.H. (1978) Biochim. Biophys. Acta 508, 277–286
10 Downes, C.P. and Michell, R.H. (1981) Biochem. J. 198, 133–140
11 Moore, R.B. and Appel, S.H. (1984) Can. J. Biochem. Cell Biol. 62, 363–368
12 Triplett, R.B., Wingate, J.M. and Carraway, K.L. (1972) Biochem. Biophys. Res. Commun. 49, 1014–1020
13 Anderson, D.R., Davis, J.L. and Carraway, K.L. (1977) J. Biol. Chem. 252, 6617–6623
14 Lorand, L., Siefring, G.E., Jr. and Lowe-Krentz, L. (1978) J. Supramol. Struct. 9, 427–440
15 Palek, J. and Lui, S.C. (1979) J. Supramol. Struct. 10, 79–96
16 Allen, D.W. and Cadman, S. (1979) Biochim. Biophys. Acta 551, 1–9
17 Lessin, L.S., Kurantsin-Mills, J., Wallas, C. and Weems, H. (1978) J. Supramol. Struct. 9, 537–554
18 Corash, L.M., Piomelli, S., Chen, H.C., Seaman, C. and Gross, E. (1974) J. Lab. Clin. Med. 84, 147–151
19 Jensen, W.N., Ruchnagel, D.L. and Taylor, W.J. (1960) J. Lab. Clin. Med. 56, 854–865
20 Laemmli, U.K. (1970) Nature 227, 680–685
21 Raval, P.J. and Allan, D. (1985) Biochem. J. 231, 179–183
22 Bartlett, G. (1959) J. Biol. Chem. 234, 466–468
23 Fabry, M.E., Mears, J.G., Patel, P., Shaefer-Rego, K., Carmichael, L.D., Martinez, G. and Nagel, R.L. (1984) Blood 64, 1042–1046
24 Ohnishi, S.T., Horiuchi, Y.K. and Horiuchi, K. (1986) Biochim. Biophys. Acta 886, 119–129
25 Lew, V.L. and Bookchin, R.M. (1980) Biochim. Biophys. Acta 602, 196–200
26 Allan, D. and Thomas, P. (1981) Biochem. J. 198, 441–445
27 Gratzer, W.B. (1981) Biochem. J. 198, 1–8
28 Goodman, S.R. and Shiffer, K. (1983) Am. J. Physiol. 244, C121–C141
29 Ferrell, J.E. and Huestis, W.H. (1984) J. Cell Biol. 98, 1992–1998
30 Anderson, R.A. and Marchesi, V.T. (1985) Nature 318, 295–298
31 Clark, M.R., Guatelly, J.C., Mohandas, N. and Shohet, S.B. (1980) Blood 55, 823–830
32 Lew, V.L., Hockaday, A., Sepulveda, M.I., Somlyo, A., Somlyo, A.V., Ortiz, O.E. and Bookchin, R.M. (1985) Nature 315, 586–589
33 Steck, T.L. (1974) J. Cell Biol. 62, 1–19