Biochimica et Biophysica Acta, 941 (1988) 225–231
Elsevier

BBA 74027

Further studies on alterations in magnesium binding during cold storage of erythrocytes

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(Received 1 March 1988)

Key words: NMR; Magnesium binding; Blood storage; (Human erythrocyte)

Free intracellular Mg^{2+} concentration ($[Mg^{2+}]_i$) was measured in cold-stored human erythrocytes by the method of null-point titration with ionophore A23187. $[Mg^{2+}]_i$ was 311 ± 41 μM (mean ± S.D.) for cells stored 0–10 days, increasing to 458 ± 64 μM for cells stored 22–48 days. The values for stored cells were higher than those previously determined by a ^{31}P-NMR method (Bock et al. (1985) Blood 65, 1526–1530); however, the null-point method requires extensive washing of the cells, which we have found to increase NMR-measured $[Mg^{2+}]_i$. The null-point values still represent a small fraction of total cell Mg^{2+}, and confirm that binding of Mg^{2+} to ligands other than ATP and 2,3-bisphosphoglycerate must increase during storage. As an initial test of whether this may imply suboptimal availability of Mg^{2+} for cell preservation, we used A23187 to prepare erythrocytes with altered Mg^{2+} content, then removed ionophore and stored the cells in plasma-free medium for up to 2 weeks. Higher Mg^{2+} content had a very significant positive correlation ($P < 0.0001$) with higher cell ATP concentrations. Storage did not significantly affect basal or Na^+-stimulated efflux of Mg^{2+} from Mg^{2+}-loaded red cells.

Introduction

Cellular transport and regulation of Mg^{2+} remain poorly understood. The erythrocyte is a convenient model system because cells are readily available and hardy, and their interior is essentially a single compartment. Hemoglobin has a very low Mg^{2+} affinity, and it would seem that binding of Mg^{2+} within the erythrocyte should be mainly to ATP and 2,3-biphosphoglycerate (DPG) [1]. However, measurements of free Mg^{2+} ($[Mg^{2+}]_i$) in erythrocytes have disclosed surprisingly low values, indicating that an additional ligand or ligands must be binding substantial quantities of the ion [2,3]. Recently we have found that cold storage of erythrocytes, which results in depletion of ATP and DPG, actually causes a decrease in $[Mg^{2+}]_i$, as measured by ^{31}P-NMR [4,5].

If $[Mg^{2+}]_i$ really decreases during cold storage of erythrocytes, this might have important implications in explaining the binding of Mg^{2+} in erythrocytes and other cells. There may also be practical consequences for blood banking. Mg^{2+} ion is a cofactor for enzymatic synthesis of ATP, which is required for maintenance of cell integrity during cold storage [6,7]. Decreasing $[Mg^{2+}]_i$ levels during storage may imply that $[Mg^{2+}]_i$ is suboptimal for ATP maintenance, and that a

Abbreviations: $[Mg^{2+}]_i$, free intracellular magnesium ion concentration; DPG, 2,3-bisphosphoglycerate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CPDA-1, standard blood preservative containing citrate/phosphate/glucose/adenine.

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harmless means for increasing $[Mg^{2+}]_i$ could increase the shelf-life of stored erythrocytes. Darley [8] actually reported some beneficial effect of supplementing preservation media with high concentrations of Mg^{2+}, but this related to DPG maintenance rather than ATP or cell viability. In any case, red-cell uptake of Mg^{2+} from preservative medium would be extremely slow at 4° C [9].

Because of the importance of these issues, and because our NMR measurements of $[Mg^{2+}]_i$ showed storage changes that were contrary to what might be expected, we have endeavored in the present investigation to measure $[Mg^{2+}]_i$ in stored erythrocytes by an alternative method, namely the null-point titration technique of Flatman and Lew [2,10]. We have also examined the effect of altered Mg^{2+} levels on maintenance of erythrocyte ATP, and have examined Na^+-stimulated Mg^{2+} efflux in fresh and stored cells.

Materials and Methods

Human Blood. Blood was obtained from Hudson Valley Blood Services. It was collected from donors meeting standard criteria and stored in CPDA-1 preservative at 4° C either as whole blood or red-cell concentrate. The storage bags were periodically sampled using aseptic technique.

Reagents. Ionophore A23187 (free acid), bovine serum albumin (98–99%, fraction V powder), and other reagents were obtained from Sigma.

Assays. Magnesium was measured by atomic absorption spectrophotometry at 285 nm using a Perkin-Elmer model 370 with air-acetylene flame. Samples and standards were diluted in 4% trichloroacetic acid containing 1% $La(NO_3)_3$. Chloride concentrations were measured using a Beckman ASTRA-8 analyzer. For analysis of cell pellets, the cells were lysed by addition of 2 vol. of H_2O and freezing, and correction was made for interference by hemoglobin. ATP and DPG concentrations in erythrocytes were measured enzymatically using commercial kits (Sigma). To express measurements on cell pellets in terms of cell water, the fraction of cell water was taken to be 0.65 throughout. While fluctuations in cell water content undoubtedly occur [2], small corrections for these would not significantly alter the conclusions of this study.

Null-point titration. Measurement of erythrocyte free magnesium by null-point titration was performed using a modification of the method described by Flatman and Lew [2,10]. Erythrocytes were first washed twice at 4° C in 10 vol. of medium containing (mM) 75 NaCl, 75 KCl, 10 Tris-HCl (pH 7.5), and 0.1 EGTA to chelate calcium. They were washed twice more in the same medium containing only 10 μM EGTA. 2-ml aliquots of cells were then placed in beakers, and 20 ml of the same medium, but containing varying amounts of $MgCl_2$, were added to each beaker. 100 μl of a 1 mg/ml suspension of A23187 in ethanol was then added, with mixing, to each beaker at time zero. At 10, 20 and 30 min, aliquots of each cell suspension were removed for analysis. The cells were immediately separated by microcentrifugation through dibutyl phthalate, then cell pellet and supernatant were analyzed for magnesium and chloride. For each titration point, $[Mg^{2+}]_i$ was calculated using the formula [2]:

$$[Mg^{2+}]_i = [Mg^{2+}]_o \cdot ([Cl]_o/[Cl]_i)^2 \quad (1)$$

where $[Mg^{2+}]_o$ is the extracellular Mg^{2+} concentration (assumed to be all "free"), and the last term is the squared ratio of extracellular to intracellular chloride. This latter term corrects for the Donnan equilibrium, resulting from non-diffusable charged species within the cell, based on the passive distribution of Cl^- [2]. The null-point, representing the unperturbed cell's $[Mg^{2+}]_i$ value, is obtained from the curve relating free and total intracellular Mg^{2+}, at the point corresponding to the total Mg^{2+} value of the unperturbed cells (see Fig. 1).

Manipulation of erythrocyte Mg^{2+}. The procedure described for avian erythrocytes by Günther et al. [11] was essentially followed. Cells were first washed at 4° C with medium containing (mM) 140 KCl, 10 glucose, 0.1 EGTA, and 20 Hepes (pH 7.4). They were then incubated for 20 min at 25° C in the same medium also containing varying concentrations of $MgCl_2$ and 5 μM A23187. Ionophore was then removed by three washes in 10 vol. of similar medium containing no ionophore and containing 1% bovine serum albumin. For each of

these washes the cells were incubated with the 1% albumin medium for 10 min at 37°C before centrifugation. The cells were then washed twice more in the same medium without the albumin.

Mg^{2+} efflux. Cells were loaded as described above, with 2 mM $MgCl_2$ in the loading medium. After the final wash step, aliquots of cells were added to 4 vol. of an efflux medium and incubated with occasional resuspension at 37°C. Efflux media typically used were 'Na medium', containing (mM) 135 NaCl, 5 KCl, 10 glucose, and 20 Hepes (pH 7.4), and 'K medium', identical except with KCl substituted for NaCl. At 1, 2 and 3 h, aliquots of cell suspension were removed, immediately centrifuged through dibutyl phthalate, and analyzed for intra- and extracellular Mg^{2+} by atomic absorption.

Effect of Mg^{2+} on ATP maintenance in stored cells

Within 72 h of collection, red cells were manipulated to modify their Mg^{2+} content as described above. External Mg^{2+} concentrations were chosen to give final intracellular concentrations below, near and above those of unperturbed cells. Control cells were taken through identical washing steps except that they were never treated with ionophore. The cells were then stored at 4°C in sterile plastic test-tubes, after addition of 0.5 vol. of sterile-filtered preservation medium containing (mM) 120 glucose, 40 mannitol, 136 NaCl, 5 KCl, 2 adenine, 0.3 sodium benzylpenicillin, 0.3 streptomycin sulfate and 10 Hepes (pH 7.0). At varying times up to 2 weeks, the tubes were aseptically sampled and cell ATP was measured enzymatically.

Statistics. Statistical calculation were performed using SAS for Personal Computers, Version 6, on an IBM PC AT. Comparison of means was performed by two-tailed *t*-test and regarded as significant for $P < 0.05$.

Results

Null-point titration studies of fresh and stored erythrocytes

Fig. 1 shows a typical null-point titration curve of relatively fresh (3 days storage) human erythrocytes. The curve relates $[Mg^{2+}]_i$ values (abscissa), estimated using Eqn. 1, to total cellular

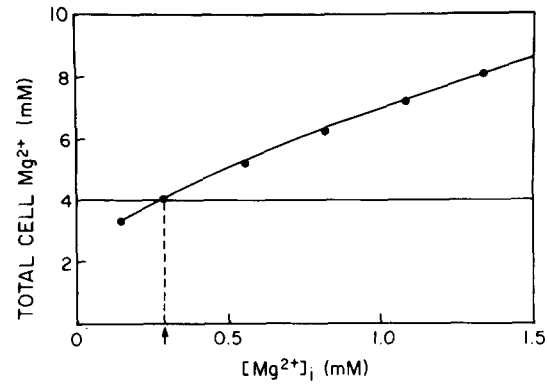

Fig. 1. Example of a null-point titration curve for measurement of $[Mg^{2+}]_i$. The red cells had been stored for 3 days at 4°C in CPDA-1. The horizontal line indicates the total Mg^{2+} content of unperturbed cells, and its intersection with the titration curve indicates an $[Mg^{2+}]_i$ value (arrow) of 284 μM.

Mg^{2+} concentrations (ordinate) measured by atomic absorption. The $[Mg^{2+}]_i$ value of unperturbed cells is then given by the point on the curve where total Mg^{2+} is equal to that of unperturbed cells. For this experiment the value is 284 μM.

Storage was associated with a small increase in $[Mg^{2+}]_i$ measured by the null-point technique. Overall, for seven samples of blood stored 0–10 days the null-point external Mg^{2+} concentration was 211 ± 49 μM (mean $\pm$ S.D.), and for 12 samples stored 22–48 days it was 390 ± 65 μM. The chloride ratio (see Eqn. 1) declined with storage, however, so that the final calculated $[Mg^{2+}]_i$ values were 311 ± 41 μM for 'fresh' blood and 458 ± 64 μM for 'old' blood. The decrease in chloride ratio implies a decrease in the Donnan potential during storage, possibly related to decreases in the negatively charged ATP and DPG molecules. Such a change in Donnan potential was also inferred from our earlier NMR experiments [4].

Fig. 2 shows estimated changes in various intracellular Mg^{2+} pools during the course of storage of a unit of red cells. Total Mg^{2+} was measured by atomic absorption, and $[Mg^{2+}]_i$ by the null-point titration technique. Concentrations of the MgATP and MgDPG complexes were calculated using the equation:

$$MgL = [Mg^{2+}]_i L_{tot} / ([Mg^{2+}]_i + K_{app}) \quad (2)$$

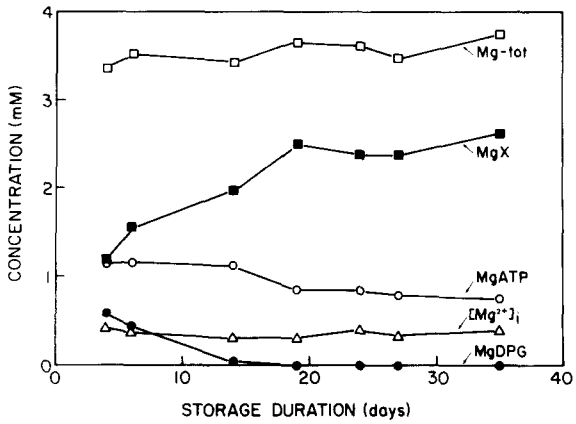

Fig. 2. Changes in various Mg^{2+} fractions during storage of a unit of red cells. Mg_{tot} = total intracellular Mg^{2+}, measured by atomic absorption. $[Mg^{2+}]_i$ = free intracellular Mg^{2+}, measured by null-point titration. MgATP and MgDPG are the respective complexes of ATP and DPG, whose concentrations are calculated from Eqn. 2. MgX is the remaining fraction of Mg^{2+}, calculated as $Mg_{tot} - [Mg^{2+}]_i$ – MgDPG – MgATP.

where L_{tot} represents the total ligand concentration (measured enzymatically), and K_{app} represents an apparent dissociation constant under the ionic conditions of the red cell. We used published K_{app} values of 50 μM for MgATP [12] and 3 mM for MgDPG [3], and did not attempt to make small corrections for pH or hemoglobin binding. Because ATP and DPG decline with storage, their Mg^{2+} complexes must also decrease substantially in concentration despite the small increase in $[Mg^{2+}]_i$ measured by null-point. Since total Mg^{2+} remains approximately constant, it is apparent that the concentration of another complex or complexes of Mg^{2+} must increase with storage. This fraction of bound Mg^{2+}, whose chemical nature is unknown at present, is denoted MgX in Fig. 2. Overall, MgX averaged 1.36 mM, or 38% of total Mg^{2+}, in 'fresh' cells and 2.37 mM, or 67% of total, in 'old' cells.

Effect of Mg^{2+} on ATP maintenance in stored erythrocytes

To provide some indication of an actual relationship between Mg^{2+} and storage damage to red cells, we measured ATP levels in stored cells whose Mg^{2+} content had been modified. Fig. 3, summarizing ATP measurements during the first week of storage, illustrates a trend toward higher ATP concentration with higher Mg^{2+} concentration. A multiple regression calculation relating ATP concentration to both storage duration (up to 14 days) and initial total cell Mg^{2+} gave

$$\text{ATP (mM)} = 1.16 - 0.016 \times \text{days} + 0.13 \times Mg_{tot} \quad (3)$$

with a correlation coefficient $r = 0.70$. The positive dependence on cell Mg^{2+} was highly significant statistically ($P < 0.0001$). As can be seen in Fig. 3, ionophore-treated cells with near-normal Mg^{2+} content had ATP levels slightly lower than the untreated control cells, suggesting that exposure to ionophore may itself cause some metabolic impairment of the cells. However, the treated cells with the highest Mg^{2+} content (group 5) had slightly more ATP than the control cells, though the difference was not statistically significant. We have not yet studied cells with an even higher degree of Mg^{2+} loading to determine whether ATP can be increased any further.

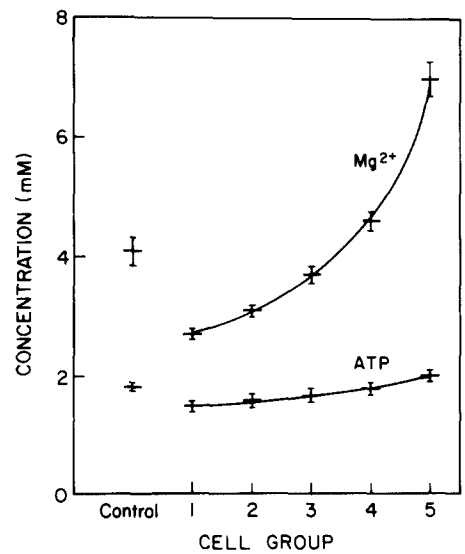

Fig. 3. Relationship of Mg^{2+} and ATP concentrations in ionophore-treated erythrocytes. Cell groups 1–5 had their Mg^{2+} content altered by treatment with ionophore A23187 in the presence of the following concentrations of external Mg^{2+} (mM): 0, 0.1, 0.2, 0.4 and 1.0. Control cells were taken through identical wash steps but never treated with ionophore. Total cell Mg^{2+} measurements were made right after the ionophore treatment; ATP measurements were made after 1–7 days of storage in plasma-free medium. All measurements shown as mean ± S.E. ($n = 6$).

Effect of storage on Mg^{2+} efflux from erythrocytes

Erythrocytes were loaded with Mg^{2+} using ionophore A23187, and ionophore was removed as described in Materials and Methods. Complete removal of ionophore was documented as described by Günther et al. [11]. In agreement with their results on avian erythrocytes, we found that if Mg^{2+}-depleted cells were placed in Mg^{2+}-containing medium, and removal of ionophore was incomplete, there was rapid uptake of Mg^{2+} from the medium. When exhaustive washing with albumin was used as described, however, there was no measurable uptake of Mg^{2+} by depleted cells.

Mg^{2+}-loaded cells placed in K medium exhibited a measurable rate of Mg^{2+} efflux, which always increased in Na medium. The rates were similar whether measured as a decrease in intracellular Mg^{2+} or as an increase in extracellular Mg^{2+}. For purposes of comparison, average efflux per hour was calculated by subtracting the 3 h intracellular Mg^{2+} from the zero-time value and dividing by 3. For 'fresh' blood (under 48 h storage), efflux in K medium was 124 ± 60 μmol Mg^{2+}/l per h (mean $\pm$ S.D., $N = 7$), and the increment in Na medium was 115 ± 34. For 'old' blood (over 28 days) the rate in K medium was 130 ± 56 ($N = 5$), and the increment in Na medium was 81 ± 31. Differences between 'fresh' and 'old' blood were not statistically significant.

Discussion

The studies reported here were motivated by our earlier observation of marked chemical shifts in the ^{31}P-NMR spectrum of cold-stored erythrocytes, which implied decreased Mg^{2+}-complexation of ATP and therefore decreased $[Mg^{2+}]_i$ [4]. This finding has been difficult to explain, since total Mg^{2+} remains fairly constant during storage, while the phosphorylated metabolites that bind the ion deteriorate. The NMR changes occurred in a variety of storage media, could not be induced in fresh cells by a variety of ionic manipulations, and could be reversed in stored cells by re-incubation in fresh plasma [5].

Unfortunately, few other techniques for measurement of red-cell $[Mg^{2+}]_i$ are available, making corroboration of the NMR findings difficult. Rose [13] proposed a measuremed based on the adenylate kinase equilibrium, but aside from being technically demanding, this method is difficult to calibrate properly to the binding of nucleoside phosphates to hemoglobin. Flatman and Lew [2] later described a null-point titration method, using the divalent cation ionophore A23187, which appears highly suitable for the measurement of erythrocyte $[Mg^{2+}]_i$ under varying conditions. However, for studies of stored cells the method has a significant limitation in requiring that the cells be equilibrated with buffers (in fact the cells are washed extensively before the actual titration in order to remove contaminant calcium). During equilibration with buffers, metabolites that have accumulated may diffuse out and other storage changes may be affected. To minimize this problem, we modified their published method slightly by reducing the number of wash steps, and by eliminating inosine, which might act to 'rejuvenate' stored cells.

By null-point titration we measured an average $[Mg^{2+}]_i$ of 311 μM for cells stored 0–10 days, as compared to Flatman's value of 390 μM for fresh, oxygemated cells [10], and NMR measurements of about 200–250 μM [3–5]. For stored cells there was a much greater discrepancy between the null-point measurements and our earlier NMR measurements. The average null-point measurement for cells stored 22–48 days was 458 μM, as compared to about 81 μM by NMR [4]. This discrepancy does not indicate a true disagreement between the two measurement techniques, since they are carried out under different conditions. The washing procedures in the null-point titration procedure may alter $[Mg^{2+}]_i$, and indeed we have recently shown this to be the case: washing stored red cells in buffer, like incubation in plasma [5], causes their NMR-measured $[Mg^{2+}]_i$ to increase to values near those of fresh cells (Bock, J.L. and Gupta, R.K., unpublished data).

In any case, the null-point value of $[Mg^{2+}]_i$ in washed, stored cells is still remarkably low, given the deterioration in phosphometabolites that occurs with storage. ATP and DPG are the only molecules at substantial concentration in the red cell known to have high affinity for Mg^{2+} [1]. From their published affinity constants, their measured concentrations, and the measured $[Mg^{2+}]_i$

values, it is straightforward to calculate the amount of bound Mg^{2+} that they can account for (Eqn. 2). Even for fresh cells, as has been previously noted [2,3], total Mg^{2+} appears to be greater than the sum of $[Mg^{2+}]_i$, MgATP, and MgDPG. The discrepant amount, labeled 'MgX' in Fig. 2, increases during storage by about 1 mM, using the null-point $[Mg^{2+}]_i$ values. Thus, MgX accounts for about one-third of total Mg^{2+} in fresh cells, but increases to about two-thirds of the total in stored cells. If one instead used NMR-measured $[Mg^{2+}]_i$ to estimate MgX, it would comprise about 80% of Mg^{2+} in stored cells.

Thus, measurements of $[Mg^{2+}]_i$ by null-point titration, despite quantitative differences from the NMR technique that may be due largely to the washing steps, support the conclusions that: (1) only a small portion of total Mg^{2+} in either fresh or stored erythrocytes is unbound; and (2) during storage the degree of Mg^{2+} binding attributable to ATP and DPG declines. The latter conclusion is based on published dissociation constants [3] that agree fairly well with other published measurements [14]; we doubt that any inaccuracy would be so great as to invalidate this conclusion. However, we can still only speculate as to what additional ligands may be involved. Inorganic phosphate accumulates during storage, but its published affinity constant [14], as well as our own calculations from ^{31}P-NMR spectra of model solutions, indicate that it could account for at most only 0.2–0.5 mM bound Mg^{2+}. Inorganic pyrophosphate is another candidate [5], but its concentration in stored cells is not in the millimolar range. As proposed earlier [5], it seems plausible that the cell membrane may bind increasing amounts of Mg^{2+} during storage. Storage is known to be associated with large changes in the chemistry of the erythrocyte membrane [7].

In view of the membrane alterations that occur during storage, it is also of interest to know how transport of Mg^{2+} may be affected by storage. Mg^{2+} transport has been relatively little studied due to absence of a highly practical isotope tracer, but transport across the erythrocyte membrane has recently been convincingly demonstrated. Günther et al. [11,15] used A23187 to load chicken erythrocytes with Mg^{2+}, and after removal of the ionophore measured a slow rate of Mg^{2+} efflux, which was stimulated by Na^+ and inhibited by amiloride. The rate of efflux slowed markedly as intracellular Mg^{2+} approached its basal value, suggesting that efflux is under the control of a gating mechanism. More recently Féray and Garay [16] demonstrated a similar Na^+-stimulated efflux for human erythrocytes, but they used *p*-chloromercuribenzenesulphonate (PCMBS) rather than A23187 to accomplish the Mg^{2+} loading. In the present investigation we applied the A23187 method [11] to human erythrocytes, and again confirmed the presence of Na^+-stimulated Mg^{2+} efflux, at rates comparable to those previously reported. No significant storage effect, either on basal or Na^+-stimulated efflux, could be demonstrated. Wiley et al. [9] previously reported that Ca^{2+} but not Mg^{2+} permeability of human red cells increases during cold storage. These investigators, however, only measured influx into Mg^{2+}-depleted cells, which is not known to involve any mechanism other than passive diffusion. The present results indicate that a specific mechanism of Mg^{2+} efflux, which may be physiologically significant, is also unaffected by storage.

The apparently high degree of sequestration of Mg^{2+} in the erythrocyte may have important biochemical consequences, i.e., $[Mg^{2+}]_i$ may be more rate-limiting in certain enzymic reactions than would otherwise be thought [3]. In particular, sequestration of Mg^{2+} might be expected to diminish cellular ATP levels, since synthesis of ATP absolutely requires Mg^{2+}, whereas degradation might occur via nonspecific Mg^{2+}-independent phosphatases. Flatman and Lew [17] previously reported that using ionophore A23187 to increase red-cell Mg^{2+} caused a small increase in total cell ATP. We have confirmed this result and further shown that the increase persists over at least 2 weeks of cold storage. This finding is the first direct indication that limited availability of Mg^{2+} may play a role in the erythrocyte storage lesion. It may also relate to the hemolytic anemia, associated with decreased erythrocyte ATP, that occurs in animals fed Mg^{2+}-deficient diets [18,19]. If further studies suggest that Mg^{2+} loading can enhance viability as well as ATP content of stored erythrocytes, than a loading method more convenient and less toxic than ionophore treatment may be sought for blood bank application.

Acknowledgements

This work was supported in part by a Grant-in-Aid to J.L.B. from the American Heart Association, Westchester/Putnam Chapter.

References

1 Achilles, W., Cumme, G.A., Winnefeld, K. and Frunder, H. (1981) J. Biochem. 120, 571–576.
2 Flatman, P. and Lew, V.L. (1977) Nature 267, 360–362.
3 Gupta, R.K., Benovic, J.L. and Rose, Z.B. (1978) J. Biol. Chem. 253, 6172–6176.
4 Bock, J.L., Wenz, B. and Gupta, R.K. (1985) Blood 65, 1526–1530.
5 Bock, J.L., Wenz, B. and Gupta, R.K. (1987) Biochim. Biophys. Acta 928, 8–12.
6 Nakao, K., Wada, T., Kamiyama, T., Nakao, M. and Nagano, K. (1962) Nature 194, 877–879.
7 Mollison, P.L. (1983) Blood Transfusion in Clinical Medicine, 7th Edn., Blackwell Scientific, Oxford.
8 Darley, J.H. (1979) Med. Lab. Sci. 36, 121–140.
9 Wiley, J.S., McCulloch, K.E. and Bowden, D.S. (1982) Blood 60, 92–98.
10 Flatman, P. (1980) J. Physiol. 300, 19–30.
11 Günther, T., Vormann, J. and Förster, R. (1984) Biochem. Biophys. Res. Commun. 119, 124–131.
12 Gupta, R.K., Gupta, P., Yushok, W.D. and Rose, Z.B. (1980) Biochem. Biophys. Res. Commun. 117, 210–216.
13 Rose, I.A. (1968) Proc. Natl. Acad. Sci. USA 61, 1079–1086.
14 Martell, A.E. and Smith, R.M. (1977) Critical Stability Constants, Plenum, New York.
15 Günther, T. and Vormann, J. (1985) Biochem. Biophys. Res. Commun. 130, 540–545.
16 Féray, J.-C. and Garay, R. (1986) Biochim. Biophys. Acta 856, 76–84.
17 Flatman, P.W. and Lew, V.L. (1981) J. Physiol. 315, 421–446.
18 Oken, M.M., Lichtman, M.A., Miller, D.R. and Leblond, P. (1971) Blood 38, 468–478.
19 Elin, R.J., Armstrong, W.D. and Singer, L. (1971) Proc. Soc. Exp. Biol. Med. 137, 635–640.