

Received 4 February 2003
Accepted 16 May 2003

THE KINETICS OF HAEMOLYSIS OF SPHEROCYTIC ERYTHROCYTES

GRZEGORZ PAŹDZIOR¹, MAREK LANGNER^{1,2}, ANNA CHMURA¹,
DŹAMILA BOGUSŁAWSKA³, ELŹBIETA HEGER³, ANNA
CHORZALSKA⁴ and ALEKSANDER F. SIKORSKI^{2,4}

¹Institute of Physics, Wrocław University of Technology, Wrocław, Poland,
²Academic Centre for Biotechnology of Lipid Aggregates, Wrocław, Poland,
³Institute of Biotechnology and Environmental Sciences, University of Zielona
Góra, Poland, ⁴Institute of Biochemistry and Molecular Biology, University of
Wrocław, Poland

Abstract: Spherocytosis is a hereditary disease. It results from mutations in genes that encode proteins participating in the attachment of the membrane skeleton to the plasma membrane bilayer of the erythrocyte. In affected cells, interaction between the spectrin-actin meshwork and integral membrane proteins is altered. This results in the weakening of plasma membrane mechanical resistance and diminishing its elasticity. Since defective cells are prone to mechanical destruction and phagocytosis in the spleen, the fraction of morphologically-altered erythrocytes is rather small; this in turn means such an examination is prone to errors. In this paper, we describe a simple method which could be useful in the identification of red blood cells with altered osmotic properties. The method is based on the measurement of the amount of light scattered by a suspension of the red blood cells, during which cells are exposed to osmotic stress in the stopped-flow regime. The obtained plots are fitted to a mathematical formula, the parameters of which can be used as quantitative indicators of the changes in red blood cells' osmotic features. Two types of spherocytotic samples were examined: those with a proven deficiency in ankyrin and those with a decrease in the band 3 anion transporting protein. The presented data show that this method gives a reliable indication of altered osmotic properties of the spherocytic cells.

Key Words: Spherocytosis, Erythrocytes, Kinetics of Haemolysis

INTRODUCTION

The shape and mechanical stability of the mammalian erythrocyte derives from the equilibrium of the surface area to volume ratio and the lateral distribution of integral membrane proteins [1, 2]. The membrane skeleton is a spectrin-actin meshwork formation, which is regulated by several other well-known proteins such as: ankyrin, protein 4.2, protein 4.1, p55, adducin, tropomyosin, tropomodulin and calmodulin (for a review, see e.g. [3, 4]). Spectrin, which is the major component of the membrane skeleton, is a high molecular weight, 200 nm long tetrameric protein composed of two heterodimers (100 nm in length). Heterodimers are formed by the antiparallel interaction of α (280 kD) and β (247kD) subunits along their long axes [5-7]. Both the α and β subunits are the products of separate genes belonging to an at least seven-member family (two alpha and 5 beta) of spectrin genes. The primary structure of both α and β is characterised by the presence of the 106 amino acid residue, a triple α -helical motif which comprises most of their structure [8]. The actin-binding domain (tandem-repeated calponin homology domain), which is present at the amino terminal end of the β -chain, the structure of which was resolved at atomic resolution [9], is responsible for the side binding of a short, 37 nm actin protofilament occurring in the red cell membrane skeleton. Binding of spectrin tetramer to actin protofilament is activated by protein 4.1 and/or adducin. This interaction, together with a dimer-dimer interaction, is responsible for the horizontal stability of stability of the skeleton, and therefore the stability of the membrane. Mutations in spectrin genes affecting mainly the dimer-dimer association site lead to asymmetrical distortions in the shape and deformability of the red blood cell, resulting in various forms of hereditary elliptocytosis [review 10].

The spectrin-actin meshwork attaches to the lipid bilayer via "vertical" interactions, the major two being the ankyrin-anion exchanger protein which is stabilised by protein 4.2 and protein 4.1-p55-glycoprotein C(D) interactions [11, 12]. Mutations in genes which affect these interactions, in particular spectrin-ankyrin-anion exchanger protein interactions, lead to a rather common (1 in 2000 individuals in Caucasians) hereditary disease, called spherocytosis [10, 13]. Major mutations underlying this disorder are the defects in the *ANK1*, *SPTA*, *SPTB*, *EPB3* and *EPB42* genes coding for ankyrin, α -spectrin, β -spectrin, anion exchanger protein and protein 4.2. The most frequent is the mutation in the *ANK1* gene located in human chromosome 8. In many cases, the decrease in spectrin content in the membranes of HS patients was proved secondary to ankyrin deficiency [13]. About 20% of HS cases are connected to mutations in the anion exchanger gene. These occur not only in cytoplasmic domain of the expressed protein, but also in its intrabilayer parts. Mutations in the protein 4.2 coding gene are less common in the Caucasian population [14]. In some cases, a reduced level or absence of this protein is linked to the homozygous mutation in the anion exchanger protein [review 13]. A spheroid shape of red blood cells is

the hallmark of hereditary spherocytosis, although the presence of spherocytes is a rather uncertain diagnostic criterion. Several haemolysis tests, e.g. estimation of the osmotic fragility with and without preincubation at 37°C [15, 16] and the autohaemolysis test, have been used as the diagnostic standard for many years. Another approach called hypertonic cryohaemolysis, which is the lysis in hypertonic solution when the temperature is lowered from above 15-18°C to below that temperature range [17, 18].

Here, another approach to analyse the osmotic behaviour of red blood cells is presented. We follow the changes in transmittance at 700 nm of erythrocyte suspension in hypotonic conditions in a stopped-flow regime. Our results [25] indicate qualitative and quantitative differences in the haemolysis kinetics between the erythrocytes of healthy individuals and the erythrocytes of patients diagnosed with hereditary spherocytosis. The results presented below come from the quantitative analysis of experimental data presented elsewhere [25].

MATERIALS AND METHODS

Erythrocytes of healthy individuals and patients diagnosed with HS were isolated as described elsewhere by Boguslawska *et al.* [25]. Most of the patients were characterised by a 10-55% decrease in ankyrin content (had a characterised 10-55% decrease in ankyrin content), and one family had an over 30% anion exchanger protein deficiency, as compared to the red cell membrane preparations of healthy individuals, which was estimated by densitometric scanning of Coomassie blue stained SDS-polyacrylamide gel electrophoretograms.

The kinetics of haemolysis

Erythrocytes were washed three times in an isotonic phosphate buffer (pH 7.4) and diluted to obtain a cell suspension with a hematocrit of 0.1 %. The kinetics of erythrocyte lysis was measured in a home-made stopped-flow device (with death time around 1 ms). The cell suspension was mixed with an equal amount of distilled water, and light transmittance at 700 nm was continuously monitored. Each experiment was repeated at least five times. The initially scattering cell suspension became more transparent as haemolysis progressed, as illustrated in Fig. 1.

The intensity of transmitted light depends on the amount of light scattered and absorbed by the cell suspension. We assumed that absorption results exclusively from the presence of haemoglobin, the concentration of which remains constant during each experiment. Therefore, the amount of absorbed light is also constant. Consequently, the contribution of absorbance can be safely subtracted during data analysis. The effect of light scattering on the transmittance is a complex function of cell shape and size and the geometry of the sample and detector [19]. We simplified our analysis by disregarding these factors. The simplification is based on the following assumptions: after an initial swelling

period (a few seconds) all erythrocytes are spherical; therefore, there are no shape differences during the lysis period, which is used for the subsequent analysis. Differences in cell sizes alter the overall scattering process, but in a way that is proportional to the amount of cells present in the sample. Hence, its effect on haemolysis kinetics can be safely omitted. In addition, we have independently measured the level of haemolysis (data not shown), revealing that there is a good correlation between the final transmittance and the quantity of surviving cells. Consequently, the final value of transmittance (after 45 sec, diluted twice) indicates the extent of sample haemolysis, and can be used to evaluate size in the erythrocyte population that remained intact. The transmittance of the scattered sample depends on the experimental setup configuration, i.e. the angular acceptance of the detector. This in turn is a function of sample heterogeneity and the distance between the scattering medium and the detector. We assume that cells are uniformly distributed and that the solutions are well mixed in the chamber, ensuring constant geometry for the system. Therefore, the quantity of transmitted light depends solely on the number of intact cells present in the chamber during the progress of haemolysis.

Data analysis

Each haemolytic curve was smoothed before fitting was performed. Lysis kinetics can be satisfactorily fitted with a sum of exponentials (equation 1) [20]. The number of exponentials needed to fit the experimental data can be correlated with the number of distinctly different cell populations [20]. The least number of exponentials that ensure correlation parameters better than 0.999 was considered to be satisfactory for the fitting procedure. The dashed line in Fig. 1A represents the best single exponential fit. In this case, correlation with the experimental points is very unsatisfactory. Fitting was performed with standard libraries from the MathCad Professional 2001 software package. An assumption of three cell populations in each sample yielded a satisfactory fitting of the experimental data. The function used for the subsequent analysis of transmittance as a function of time has the following form:

$$T = P_0 + P_1 e^{P_2 t} + P_3 e^{P_4 t} \quad (1)$$

There are two components responsible for hemolytic kinetics: fast (parameters P_1 and P_2) and slow ones (parameters P_3 and P_4). Parameter P_0 can be correlated with the extent of final haemolysis, since not all erythrocytes are lysed in the experimental conditions. The statistical significance of the difference between the sample populations was evaluated with the Fisher test [21].

RESULTS AND DISCUSSION

Haemolytic kinetics was measured for fresh erythrocyte samples from patients with diagnosed spherocytosis and compared with a representative number of samples obtained from a blood bank. Spherocytic erythrocytes were characterised only using the above-mentioned membrane protein deficiencies

(ankyrin and band 3), as estimated by densitometric scanning analyses. The exact molecular defects characterising HS families are under investigation. Selected examples of the experimentally-derived dependence of transmittance on time, along with the calculated fits, are presented in Fig. 1.

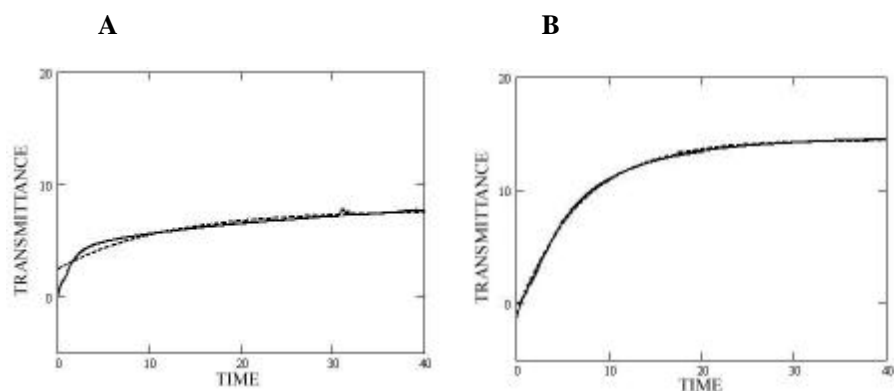


Fig. 1. A comparison between the measured dependencies of transmittance on time for the control sample (A) and a selected spherocytic sample (B). Both the experimental results (thin continuous line) and numerically-derived fits (dotted line) are shown. Agreement of the experimental and calculated curves is very strong. The single-exponential fitting (dashed line) for the control sample is also indicated to show the need for two exponentials in the fitting procedure.

Erythrocyte samples from patients with diagnosed spherocytosis were divided into two groups, according to previously determined membrane defects. The first group was comprised of these with ankyrin deficiency and the second one of those with a band 3, anion-transporting protein deficiency.

The value of parameter P_0 can be correlated with the overall blood sample propensity to hemolysis. The fractions of haemolysed erythrocytes are: control sample – 17%, ankyrin deficiency – 35%, band-3 deficiency – 49%. The difference between the control samples and those with diagnosed spherocytosis are statistically relevant, hence the parameter P_0 alone is an indicator of the disease. This result concurs with expectations, since spherocytic cells are prone to hemolysis even under mild osmotic stress [22]. The value of P_0 for the erythrocytes with the band 3 deficiency (49% haemolysed cells) is greater than that for the cells with the ankyrin defect (35% hemolysed cells). However, the present number of tested samples is too small to show that this difference is statistically significant. It should be mentioned that the heterogeneity of lysis in HS cell populations is a rather well-known phenomenon [23].

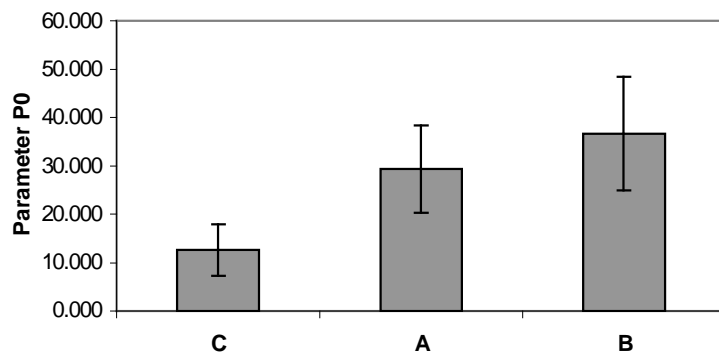


Fig. 2. The average values of parameter P_0 (Equation 1) for the control blood samples (C), and those with diagnosed spherocytosis with ankyrin (A) and band 3 protein (B) defects.

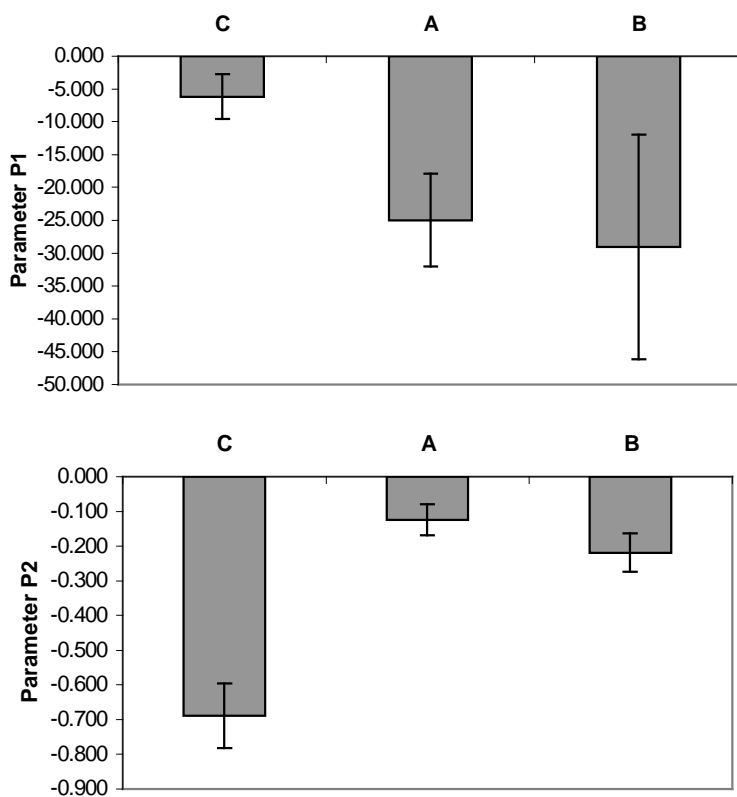


Fig. 3. The fast kinetics of erythrocyte hemolysis as described by the exponential function $P_1 \exp(P_2 t)$. P_1 is related to the size of the fast breaking (fast-breaking) cell population, whereas P_2 is a measure of the progress of its lysis.

The fast kinetic component described by the exponential $P_1 \exp(P_2 t)$, of spherocytic erythrocytes is distinctly different from the control samples (Fig. 3). Parameter P_1 reflects the amount of cells that undergo fast lysis, whereas parameter P_2 can be associated with plasma membrane properties responsible for the progress of hemolysis. The average values of these two parameters and their standard deviations are presented on Fig. 3. Control blood samples have a relatively small volume of parameter P_1 , showing that only about 50 % of lysed cells are contributing to this fraction. The same parameter for the ankyrin and band 3 defects are 85% and 80%, respectively. The difference is even larger considering that a large fraction of the control sample is not lysed at all.

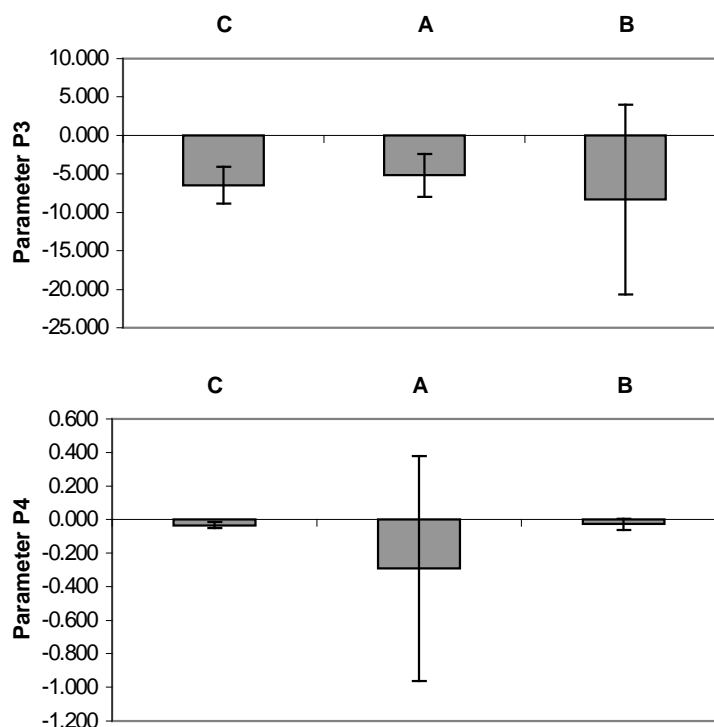


Fig. 4. The slow component for the cell kinetics of control (C), ankyrin defect (A) and band 3 defect (B) samples. Slow kinetics is described by the function $P_3 \exp(P_4 t)$.

Parameter P_2 is higher for control samples than for defected cells. These seemingly contradictory result can be explained using cell population analysis. In control samples, the number of cells lysed under particular conditions is small (as described by parameter P_0) reaching only 17 %. With sick people, a substantially larger erythrocyte population is lysed (35 % for ankyrin and 49%

for the band-3 defect). This result shows that a significant fraction of the erythrocytes from spherocytotic blood samples have their plasma membrane properties altered, albeit not to the extent of the fast hemolysing population in the control samples (as quantified by parameters P_1 and P_0).

When the parameters describing the slow kinetics are compared (Fig. 4) there are no obvious differences between the three sample groups (parameters P_3 and P_4 are statistically indistinguishable).

In summary, the kinetics of haemolysis proved to be a promising method for detecting erythrocytes with defective plasma membrane components and/or associated structures, i.e. the cytoskeleton. A comparison of the erythrocyte haemolytic kinetics of blood samples from patients with diagnosed spherocytosis and healthy people shows distinct differences, which can be parametrised and quantitatively evaluated. Erythrocytes from normal blood samples consist of two populations: the small one (17% – which lyse in the 75 mM salt solution) and the dominant one, which sustain such conditions intact, whereas almost 50% of erythrocytes from patients with spherocytosis are prone to hemolysis in the applied osmotic stress. The procedure presented in this paper and the underlying assumptions provide results that are in good agreement with data available from the literature [24]. They show that spherocytic cell plasma membranes are distinctly less resistant to osmotic stress. There are also differences between erythrocytes with various defects, i.e. between ankyrin and band-3 defects, which are possibly due to reduced plasma membrane strength. At present it is difficult, based on the available data, to differentiate between cells with these two defects. However, additional experimentally-derived values of some parameters (different P_0 values) may serve as an indicator to/for particular deficiencies. To explore this possibility further, studies on larger populations of patients are needed. Nevertheless, the method presented in this paper allows for a fast and efficient identification of erythrocyte samples with spherocytosis by measuring the fraction of cells that undergo lysis (parameters P_0 and P_1) and/or the properties of the plasma membrane (parameter P_2).

Acknowledgements. The authors thank Drs Jadwiga Hołojda, Alina Świdarska and Anna Straburzyńska for referring HS patients to them, and are indebted to the HS patients and their families for their permission to perform this study. This work was supported by: Institute of Physics, Wrocław University of Technology, University of Wrocław and University of Zielona Góra.

REFERENCES

1. Mohandas, N., and Evans, E. Mechanochemical properties of the red cell membrane in relation to molecular structure and genetic defects. **Annu. Rev. Biophys. Biomol. Str.** 23 (1994) 787-818.
2. Svetina, S., Iglic, A., Kraij-Iglic, V. and Zeks B. Cytoskeleton and red cell shape. **Cell. Mol. Biol. Lett.** 1 (1996) 67-75.

3. Fowler, V.M. Regulation of actin filament length in erythrocytes and striated muscle. **Curr. Opin. Cell Biol.** 8(1996) 86-96.
4. Bennett, V. Ankyrins. Adaptors between diverse plasma membrane proteins and the cytoplasm. **J. Biol. Chem.** 267 (1992) 8703-8706.
5. Sahr, K.E., Laurila, P., Kotula, L., Scarpa, A.L., Coupal, E., Leto, T.L., Linnenbach, A.J., Winkelmann J.C., Speicher, D.W. and Marchesi, V.T. The complete cDNA and polypeptide sequences of human erythroid alpha-spectrin. **J. Biol. Chem.** 265 (1990) 4434-4443.
6. Winkelman, J., Chang, J., Tse, W., Scarpa, A., Marchesi, V. and Forget, B. Full-length sequence of the cDNA for human erythroid beta spectrin. **J. Biol. Chem.** 265 (1990) 11827-11832.
7. Hartwig, J. Actin-binding proteins 1: Spectrin superfamily. **Protein Profile** 2 (1995) 703-800.
8. Djinovic-Carugo, K., Gautel, M., Ylanne, Y. and Young, P. Te spectrin repeat: a structural platform for cytoskeletal protein assemblies. **FEBS Lett.** 513 (2002) 119-123.
9. Moores, N.H., Keep, C.A. and Kendrick-Jones, J. Structure of the utrophin actin-binding domain bound to f-actin reveals binding by an induced fit mechanism. **J. Mol. Biol.** 297 (2000) 465-480.
10. Tse, W.T. and Lux S.E. Red blood cell membrane disorders. **Brit. J. Haematol.** 104(1999) 2-13.
11. Bennett, V. and Baines, A.J. Spectrin and ankyrin-based pathways: Metazoan inventions for integrating cells into tissues. **Physiol. Rev.** 81 (2001) 1353-1392.
12. Hemming, N.J., Anstee, D.J, Staricoff, M.A., Tanner, M.J. and Mohandas, N. Identificaton of the the membrane attachment sites for protein 4.1 in the hman erythrocyte. **J. Biol. Chem.** 270 (1995) 5360-5366.
13. Delaunay, J., Aloisio, N. and Morle, L. Molecular genetics of hereditary spherocytosis. **Cell. Mol. Biol. Lett.** 1 (1996) 49-65.
14. Takaoka, Y., Ideguchi, H., Matsuda, M., Sakamoto, N., Takeuchi, T. and Fukumaki, Y. A novel mutation in the erythrocyte protein gene of Japanese patients with hereditary spherocytosis (protein 4.1 Fukuoka). **Brit. J. Haematol.** 88 527-533.
15. Godal, H.C., Elde, A.T., Nyborg, N. and Brosstad, F. The normal range of osmotic fragility of red blood cells. **Scand. J. Haematol.** 25 (1980) 107-112.
16. Parpart, A.K., Lorenz, P.B., Parpart, E.R., Gregg, J.R. and Chase, A.M. The osmotic resistance (fragility) of human red cells. **J. Clin. Invest.** 26 (1947) 636-640.
17. Green, F.A., Jung, C.Y., Cuppuletti, J. and Owens, N. Hyertonic cryohemolysis and the cytoskeletal system. **Biochim. Biophys. Acta** 648 (1981) 225-230.

18. Streichman, S. and Gesheidt, Y. Cryohemolysis for the detection of hereditary spherocytosis: correlation studies with osmotic fragility and autohemolysis. **Am. J. Hematol.** 58 (1998) 206-212.
19. Fujii, T., Tamura, A., Tanaka, T., Nagasawa-Fujimori, H. and Hiromi, K. Light scattering stopped-flow studies on the time course of shape changes induced in human erythrocytes by several amphiphilic lipids. **Biochem. Int.** 8 (1984) 83-88.
20. Didelon, P.M., Muller, S. and Stoltz, J.F. Osmotic fragility of the erythrocyte membrane: characterization by modeling of the transmittance curve as a function of the NaCl Concentration. **Biorheology** 37 (2000) 409-416.
21. Zar, J.H. Biostatistical analysis (1974) Prentice-Hall Int. Inc, London.
22. Iglauer, D.R., Schroter, W. and Pekrun, S. Cryohemolysis test as a diagnostic tool for hereditary spherocytosis. **Ann. Hematol.** 78 (1999) 555-557.
23. Lux, S.E. and Palek, J. Disorders of the red cell membrane. **Blood. Principles and Practice of Hematology**, 1995, (Handlin, R.I., Lux, S.E. and Stossel, T.P. Eds) Lippincott-Raven.
24. Steinke, A.P.S. Comparison of Mie theory and the light scattering of red blood cells. **Appl. Opt.** 27 (1988) 4027-4033.
25. Bogusławska, D.M., Heger, E., Chorzalska, A., Nierzwicka, M., Hołojda, J., Świdorska, A., Straburzyńska, A., Paździor, G., Langner, M. and Sikorski, A.F. Hereditary spherocytosis: identification of several HS families with ankyrin and band 3 deficiency in a population of Southwestern Poland. **Ann. Hematol.** (2003), in press.