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# TEMPERATURE AND FREQUENCY DEPENDENCES OF THE RESISTANCE AND CAPACITANCE OF ERYTHROCYTE MEMBRANES AS A TOOL FOR DETECTING ANEMIA OF THE TYPE MEMBRANOPATHY

**Iv. T. Ivanov[1*], M. Karabaliev[1], P. Zagorchev[2]**

[1]Medical Faculty, Trakia University, Stara Zagora, Bulgaria
[2]Medical University, Plovdiv, Bulgaria

**ABSTRACT**
The passive electric parameters of suspensions containing intact erythrocytes and isolated erythrocyte membranes were measured over the frequency range of 50 kHz – 13 MHz as a function of the suspension temperature. Both the equivalent parallel resistance and capacitance of suspension underwent two threshold changes at 50°C and 65°C. The first change is attributed to the denaturation of the peripheral erythrocyte membrane protein spectrin resulting in a threshold change of membrane capacitance. The second change was related to increased ion permeability of erythrocyte membrane due to a processe involving the anion exchanger protein. It is demonstrated that the method of thermal analysis of the erythrocyte suspension could be used to detect hereditary changes in the erythrocyte membranes of patients with Minkowski-Chauffard anemia.

**Key words.** Hemolytic anemia, erythrocyte membrane, spectrin, band 3 protein, membranopathy.

## INTRODUCTION

Hereditary hemolytic anemias are rare but acute conditions that demonstrate themselves during the first months and years of childhood (1, 2). Most of them are due to congenital alterations in erythrocyte proteins including cytosolic protein hemoglobin and plasma membrane proteins. Hereditary alterations or deficiency of erythrocyte membrane proteins produce anemic conditions (3) that include hereditary spherocytosis (4), poikilocytosis and pyropoikilocytosis, stomatocytosis (5) and hereditary elliptocytosis. There are two main categories of hereditary stomatocytosis, the overhydrated (hydrocytosis) and dehydrated (xerocytosis) forms.

Hereditary spherocytosis originates from defects in some membrane proteins (i.e., deficiency of spectrin and band 3 protein known as the anion exchanger) (6). Besides in the erythrocytes membranes some of these proteins are also expressed in the cells of kidney, lung alveoli, and blood circulation system. As a consequence the anemic conditions of the membranopathy type are frequently combined, roughly in 60% of anemic patients, to chronic disorders of indicated systems (7, 8).

Most of the methods for investigation of the anemic membranopathic conditions are expensive, non-specific or time consuming (9-11). Another problem is the need to differentiate between hemolytic conditions due to membranopathies from these related to hemoglobinopathy and enzymopathy. In addition, the correct diagnosis and differentiation of the primary membranopathy from the secondary hemolytic conditions is important for the proper choice of therapy.

In previous study a rapid and specific method for registration of congenital alteration in the main proteins of erythrocyte membrane, spectrin and the anion exchanger, is proposed that allow differentiation between hemolytic conditions due to membranopathies from these related to hemoglobinopathy (12). For that

***Correspondence to:** *Ivan Tanev: Dept. Physics, Biophysics, Rentgenology and Radiology, Medical Faculty, Thracian University, Armeyska Str. 11, Stara Zagora 6000, Bulgaria. E-mail: ivanov_it@gbg.bg*

purpose two threshold changes of electric conductivity were established and used, one at 50°C (peak A), which is due to denaturation of the membrane peripheral protein spectrin, and another one at 65°C (peak G) in which the anion exchanger protein is assumed involved (13).

The aim of this study is to extend the range of this method by using more powerful and specific instruments for dielectroscopy of heated erythrocyte suspension. The main advantage of this kind of technical equipment is the possibility to record the changes in suspension impedance and its components during heating simultaneously at several frequencies of the applied electric field.

## MATERIALS AND METHODS

### Erythrocytes and isolated erythrocyte membranes

Citrated blood was taken through venipuncture from healthy donors (control) and anemic patients in the pediatric hospital of the Medical faculty of Thracian University, Stara Zagora, Bulgaria. Each anemic disorder was established by classical procedure. In several hours the erythrocytes were isolated, twice washed in excess volume of NaCl saline and used. White erythrocyte membranes were isolated from the erythrocytes of healthy donors, and resealed with 150 mM NaCl and 5 mM $PO_4$ buffer, pH 7.4 as described earlier (14). Briefly, packed erythrocytes were diluted 20 times with a 4°C-cold hypotonic solution (1 mM $MgCl_2$, 0.5 mM EGTA, 5 mM phosphate buffer, pH 8.0). The membranes were isolated by centrifugation at 15000 ×g, washed thrice in the same medium to white colour, resealed with 150 mM NaCl for 15 min at 37 °C, isolated and used immediately.

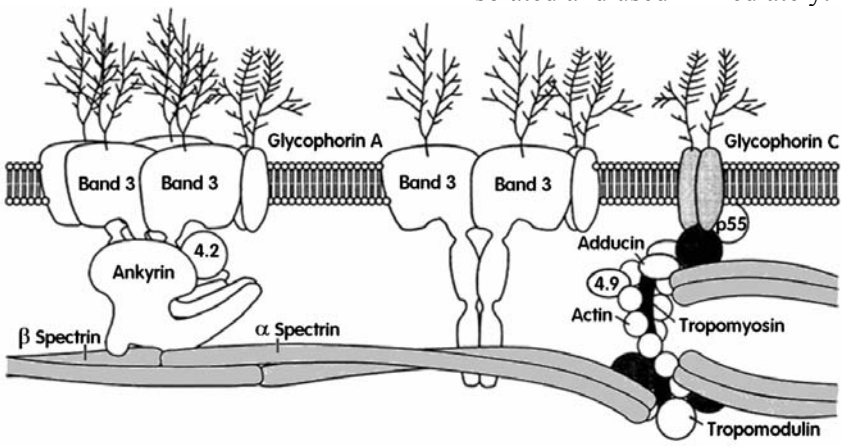


**Figure 1**. A model of the membrane of human erythrocytes. Shown are the major membrane proteins; band 3 protein, spectrin and glycophorin (10) each comprising about 25 % (w/w) of the membrane protein content.

### Spectroscopy of the impedance of heated suspensions

Deviations in the structure and/or quantity of the main proteins of erythrocyte plasma membranes, spectrin and the anion exchanger **(Figure 1)**, were detected through thermal analysis of the of electric impedance of erythrocyte suspension at different frequencies. The method was based on determining the denaturation temperatures of both group membrane proteins, peripheral and intrinsic, in erythrocyte membrane as explained earlier (13). Briefly: to impose an outward transmembrane gradient of ion concentration, the erythrocytes and resealed erythrocyte membranes were once washed in isotonic 30 mM NaCl / sucrose solution and immediately used. The suspension, usually 75 μl packed erythrocytes or resealed erythrocyte membranes, was heated at 2.0 °C/min and its impedance was continuously measured at the indicated frequencies from 50 kHz to 13 MHz. The temperature was measured through thermocouple. The impedance of the suspensions was measured with Solartron 1260A Impedance/Gain-phase analyzer, England, connected to computer. The conductometric cuvette contained two electrodes of platinum wire under 100 mv voltage of alternating current. From the measured impedance the equivalent parallel resistance R and capacitance C were calculated for each frequency. The final result was

exhibited in integral and derivative forms. The former one represented the temperature dependence of the suspension resistance and capacitance while the latter one used the temperature dependence of the temperature derivative of suspension resistance and capacitance. When the resistance or capacitance sustained a threshold change at a given temperature a sharp peak appeared on the derivative thermogram of the respective parameter, centered at this temperature.

## RESULTS AND DISCUSSION

**Figure 2** shows the temperature dependence of the resistance of erythrocyte suspension simultaneously followed at 8 different frequencies from 50 kHz to 13 MHz as indicated. For each frequency the resistance is normalized to its initial value in order to demonstrate the temperature profile of this electrical parameter.

The curves obtained for different frequencies are not identical suggesting a frequency dependence of the resistance. The initial segment of curves between 20°C and 50°C corresponds to the well-known Bolzman dependence of the resistance on temperature. Above this range two threshold changes are observed at all frequencies corresponding to two sharp peaks on the derivative thermograms (for example see the Figure 4, curve C). The first one takes place at the temperature of 50°C and is indicated as A peak. The second one is centered at 65°C and is named G peak. Both peaks were irreversible and did not show up on the thermogram obtained during the repeated heating of the same suspension (not shown). Either of the peaks A and G exhibited frequency dependence although in a different ways. The A peak changed its amplitude as well as its direction with the frequency increased. On the other hand the G peak changes only its amplitude but not its direction that was in compliance to the direction of the imposed transmembrane ion gradient of ion concentration.

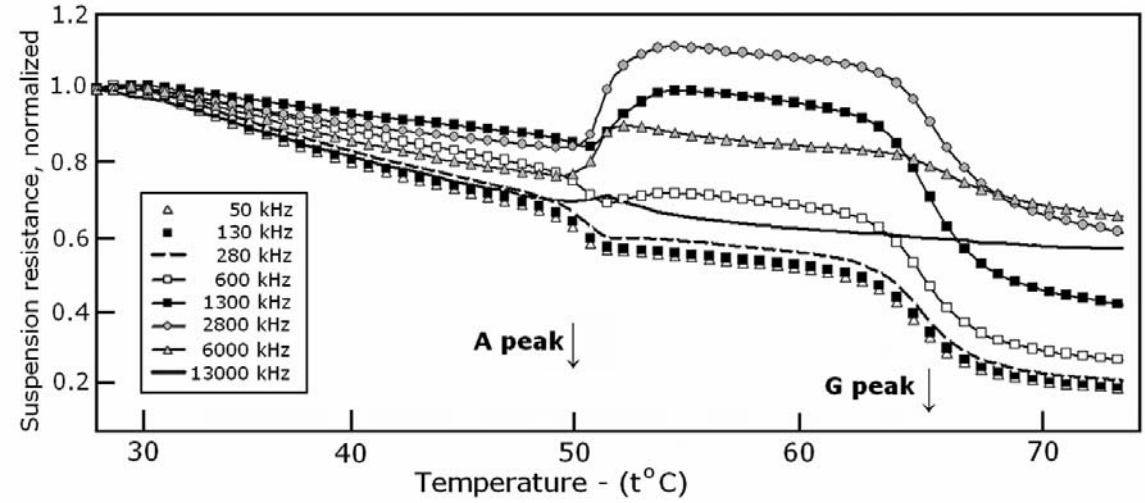


**Figure 2**. Frequency dependence of the temperature profile of suspension resistance, normalized to its initial value. The suspension contained isolated erythrocyte membranes, suspended in isotonic 30 mM NaCl / sucrose solution. The hematocrit was 0.95 and the frequency is indicated within the insert.

Similar curves as those shown in **Fugure 2** were obtained using packed erythrocytes instead of resealed erythrocyte membranes (not shown). This result suggests that the irreversible threshold changes at 50°C and 65°C were due to processes that take place in the membrane but not in the erythrocyte cytosole. The decrease in suspension resistance at 65°C is due to the activation of membrane ion permeability at hyperthermia. The amplitude of the G peak demonstrated frequency dependence. Above the critical frequency of the Maxwell-Vagner superfacial polarization (about 0.5 MHz at this NaCl concentration), the amplitude of the G peak tended to zero as expected. The A-peak showed more complex behaviour. It varied by amplitude and direction with the frequency; at lower frequencies the resistance changes were negative, while above the critical frequency of the Maxwell-Vagner superfacial polarization the changes became positive. A possible

reason for this behaviour is that the overall changes of the suspension impedance at this temperature were mainly due to the changes in its capacitive part.

As for suspension resistance similar irreversible changes at 50°C and 65°C were detected in the equivalent parallel capacitance of packed erythrocytes **(Fig. 3)** and isolated erythrocyte membranes (not shown). This result supports the above conclusion that the threshold changes at 50°C and 65°C both are thermally-induced membrane transitions that involved major membrane proteins.

Another way to express the changes in suspension electrical parameters, resistance and capacitance, is to use the temperature derivative of these parameters. In such a derivative form each threshold change in the parameter appears as a peak. **Fig. 4** shows the temperature derivative of the resistance, dR/dt, of suspension containing packed control erythrocytes of healthy donor. The threshold changes in the resistance at 50°C and 65°C are clearly identified as peaks on the derivative thermogram. The top temperature, shape, amplitude and half-width of each peak could be conveniently measured using control erythrocytes and compared to these obtained with anemic erythrocytes.

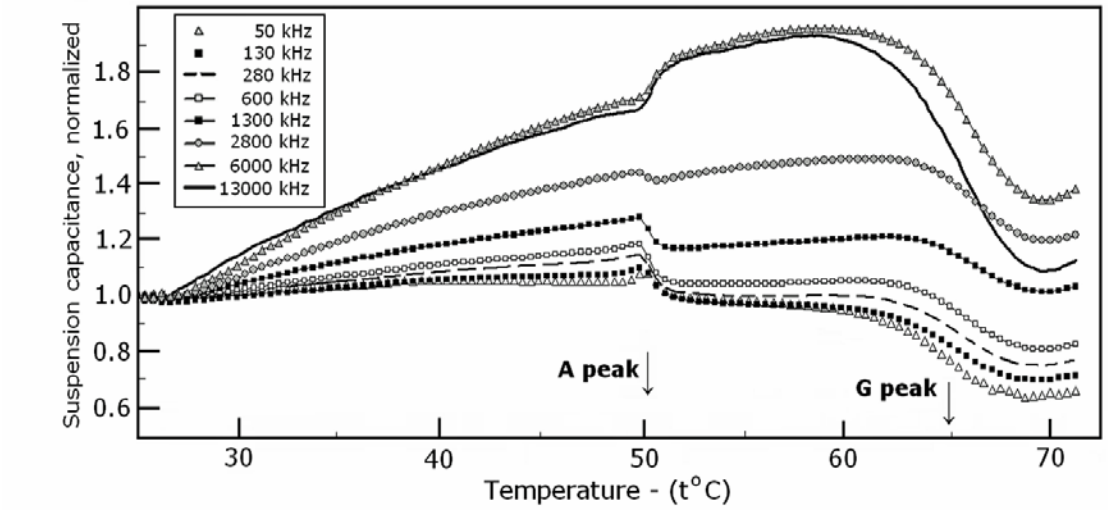


**Figure 3**. Frequency dependence of the temperature profile of suspension capacitance, normalized to its initial value. The suspension contained erythrocytes, washed and suspended in isotonic 30 mM NaCl / sucrose solution. The hematocrit was 0.95 and the frequency is indicated within the insert.

**Figure 4** compares the derivative resistance thermogram of control erythrocytes with that of erythrocytes taken from a patient with Minkowski-Chauffard anemia, microspherocytosis. Both the A peak and G peak are clearly outlined on thermograms. In both cases the A-peak has the same position and height possibly indicating preserved structure of spectrin. Beyond the A peak the membranes of control erythrocytes preserved its barrier function till about 62°C. With the anemic erythrocytes, however, the membranes became leaky immediately above the spectrin denaturation temperature, possibly indicating that the anemic erythrocyte membranes possessed defect in the proteins that connect the membrane skeleton to the lipid bilayer. This conclusion is in line with previous report that hereditary spherocytosis could be associated with defects in proteins linking the lipid bilayer to undermembrane spectrin network (15), mainly the band 3 protein.

Another important deviation is the new peak that appeared in front of the A peak, indicating leak of ions that begins at about 37°C and is maximal at 45°C. This is characteristic of erythrocytes with hereditary microspherocytosis and stomatocytosis which describe a group of hemolytic anemias where the red cells leak monovalent cations due to increased residual permeability of the red cell membrane to cations (16). Such erythrocytes have a mild cation leak at 37 °C but become

extremely leaky at low temperature (cryohydrocytosis) or at higher temperatures (as in this case). The temperature dependence of the cation leak is important characteristics of this membrane pathology and could be rapidly detected by the proposed method.

## CONCLUSIONS

The usage of impedance analyser instead of simple ordinary conductometer expands the opportunities for studying changes in erythrocyte membrane that involve major membrane proteins – spectrin and the anion exchanger. Further and more detailed studies on the subject will allow establishing of secure features for diagnostics of genetically determined alterations in these proteins, accompanied by anemia, by the aid of combined application of thermal analysis and impedance spectroscopy on the blood probes of anemic patients.

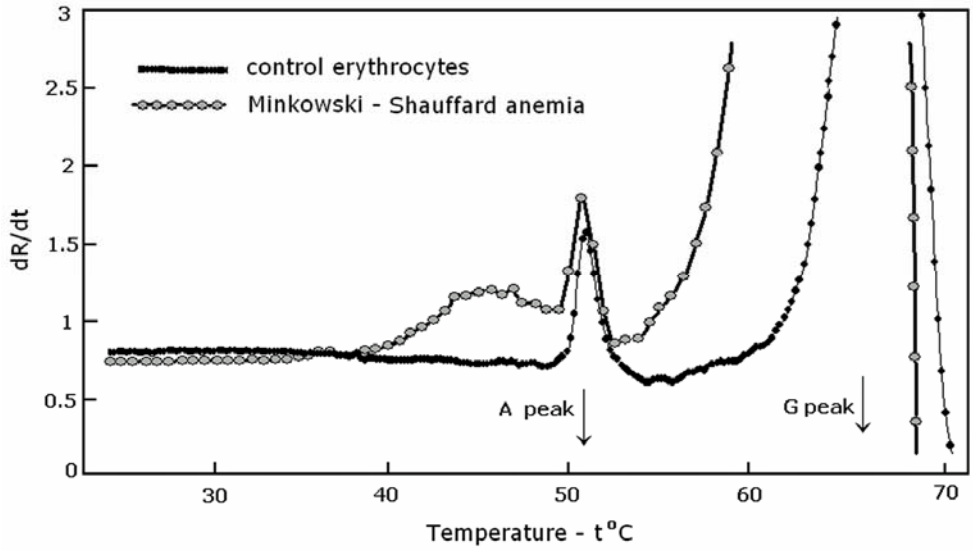


**Figure 4**.Temperature profile of the temperature derivative of suspension resistance, dR/dt. The suspension contained healthy erythrocytes (control) and erythrocytes of a patient with Minkowski-Chauffard anemia, suspended in isotonic 30 mM NaCl / sucrose solution. The hematocrit and frequency were 0.95 and 100 kHz, respectively.

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