Alteration of Cl^- Transport in Erythrocytes from Patients with Huntington's Disease

W. A. Białas

Institute of Physics, Technical University of Wrocław, Wybrzeże Wyspiańskiego 27, 50-370 Wrocław, Poland

Abstract. Rate constants for Cl^- transport across erythrocyte membranes were measured in experiments performed simultaneously on normal erythrocytes (control) and erythrocytes from patients with Huntington's disease (HD). The rate constants were observed to decrease in HD cells during aging in vitro from 12 % higher to lower than normal values. Young HD cells, separated from blood, quickly decreased their permeability for anions by about 30 %, while old cells seemed to increase it. Rate constants in control erythrocytes remained steady and equal in both fractions. A hypothesis has been proposed that there might be two populations of erythrocytes in HD blood, one abnormal, characterized by an initially high anion permeability, which decreases significantly during the cell life. A theoretical analysis of the results has suggested that the abnormal fraction of erythrocytes in HD blood may be associated with an increased population of stomatocytes observed by electron microscopy.

Key words: Erythrocytes — Anion transport — Huntington's disease

Introduction

Huntington's disease (HD) is a hereditary neurological disorder transmitted by an autosomal dominant gene. It is characterized by a late onset of clinical symptoms involving choreiform movements and dementia, which progress with the duration of the disease (Heathfield 1973). Autopsies performed on deceased HD patients reveal pathological changes involving the central nervous system, and characterized by a loss of and deformation of cells. The changes are, however, inequally pronounced in the studied parts of the system, and may depend on patient's age (Heathfield 1973; Bruyn et al. 1979). The pathogenesis of HD is unknown.

The possibility that HD may also involve tissues other than nervous (Appel 1979) raised interest in such investigations. Biochemical, morphological, and biophysical studies of red blood cells revealed certain erythrocyte membrane alterations (Butterfield and Markesbery 1979). These include altered ESR spectra of membrane proteins, but not lipids, a significantly increased population of

stomatocytes in HD blood (23 % vs. 8 % in normal blood), observed by electron microscopy, and a decreased deformability of HD erythrocytes as expressed by a longer time of filtration of an erythrocyte suspension through a 3 μm filter. The mean dimensions of the cells are normal. Studies on self-exchange transport of Cl^- across erythrocyte membrane in HD (Białas et al. 1980) revealed a slightly increased rate constant in fresh blood from HD patients as compared to normal control. This suggested the possibility of a molecular defect involving the anion transport system of the erythrocyte membrane. The anion transport in erythrocytes, specifically HCO_3^-/Cl^- exchange, is the rate limiting step for gas transport by blood (Wieth et al. 1982). Genetic malfunction of erythrocytes in HD may throw new light on the pathogenesis of this disease and also help to understand the mechanism of anion transport across the erythrocyte membrane. Results of further studies on this problem are presented in this paper.

Materials and Methods

Blood

Blood samples from drug-free HD patients (6 females, aged 25—46, and 6 males, aged 29—59) and matched healthy controls were drawn into heparinized syringes, stored on ice, and immediately delivered to the laboratory where the experiments were performed.

Separation of erythrocytes by age

The youngest and oldest fractions of erythrocytes were obtained by high speed centrifugation in plasma, according to the method of Murphy (1973). The cell fractions were collected in 1 ml syringes, resuspended in 5 ml of physiological buffer solution (PBS), containing 150 mmol/l NaCl in 5 mmol/l sodium phosphate buffer, pH 8 at 20°C, washed 3 times, and prepared for isotopic experiments. Small amounts of the suspensions of both fractions of cells, with the hematocrits adjusted to the same value, were lysed in pure water, and the concentration of released hemoglobin was measured photometrically at a 540 nm wavelength. The concentration in old cells was increased by 14.5%, which corresponded well to the decrease in volume of old cells (Murphy 1973).

Preparation of erythrocytes for isotopic experiments

The erythrocytes were separated from plasma by centrifugation at $2000 \times g$ for 10 min in 15 ml tubes, and then washed 3 times in PBS. Subsequently, the cells were resuspended in PBS to a hematocrit of about 50 % and a final volume of 3 ml. A small amount of PBS labelled with radioactive Cl^{36} (Amersham) was added, to a final specific radioactivity of 0.5 μCi/ml. The suspension was stirred and incubated at room temperature for 10 min. It was then distributed to 1 ml plastic tubes (5×50 mm), centrifuged at $2000 \times g$ for 5 min, and the radioactive supernatant removed along with the topmost layer of cells. The tubes, with packed erythrocytes loaded with radioisotope, were stored at 4°C until used in experiments. All the preparatory procedures were completed within 3 h of the drawing of the blood samples.

Table 1. Rate constants for Cl^- self-exchange transport in erythrocytes from HD patients and matched controls as measured during aging in vitro. The values shown are means and their standard errors. The rate constants corresponding to 3h aging (Białas et al. 1980) were evaluated from triplicate runs made on blood samples from 15 patients and controls, the other rate constants came from triplicate runs made on blood from 5 patients and controls.

Time of aging	k_c (s^{-1})	k_{HD} (s^{-1})
3 h	0.116 ± 0.003	0.130 ± 0.004
6 h	0.112 ± 0.005	0.110 ± 0.005
24 h	0.110 ± 0.004	0.083 ± 0.003

Isotopic experiments

The small 1 ml plastic tube with 0.4 ml of packed erythrocytes loaded with Cl^{36} was connected to a 5 ml disposable syringe containing 3 ml of ice-cold PBS. The bottom of the tube was cut off, and, at time $t = 0$, the erythrocytes were rapidly flushed down into a 100 ml plastic beaker with 80 ml of vigorously stirred PBS, kept at 1.5 ± 0.5°C. Six samples of the extracellular medium were filtered (by the method of Dalmark and Wieth 1972) in time intervals of 4 s. The equilibrium sample was obtained after 180 s. An aliquot of 0.1 ml of each filtrate was added to 4 ml of "Biofluor" (New England Nuclear) and placed in a liquid scintillation counter (Packard Tri-Carb 3255) for radioactivity counting.

The rate constant and its standard error were computed by linear regression analysis of the relation between ln $(1 - a_t/a_\infty)$ and time. The rate constant for Cl^- efflux is, under the conditions chosen (hematocrit 0.5 %), equal to the negative slope of this relation (see Appendix). Results showing correlation coefficients below 0.99 were not considered as significant.

Results

In fresh HD blood samples Cl^- transport was found to be about 13 % faster than in normal erythrocytes (Table 1). HD cells suspended in plasma or PBS were gradually losing this increased anion permeability. Normal, control, erythrocytes maintained the rate constant practically unchanged. Results of experiments carried out on young and old erythrocytes separated from the same blood sample revealed that the normal cells apparently maintained the rate of chloride transport at a normal level throughout their life in circulation, and were essentially insensitive to aging in vitro for several hours (Fig. 1.). The young and old HD erythrocytes differed, however, with respect to their anion permeabilities (Fig. 2.). The rate of Cl^- exchange in the young cells was initially by about 22 % higher than normal, but during aging in vitro it dropped and became by about 13 % lower than normal after 10 h. In old HD cells, the permeability seemed to increase from a slightly lower than normal, to the normal level (Fig. 3.). This change was, however, small and difficult to demonstrate.

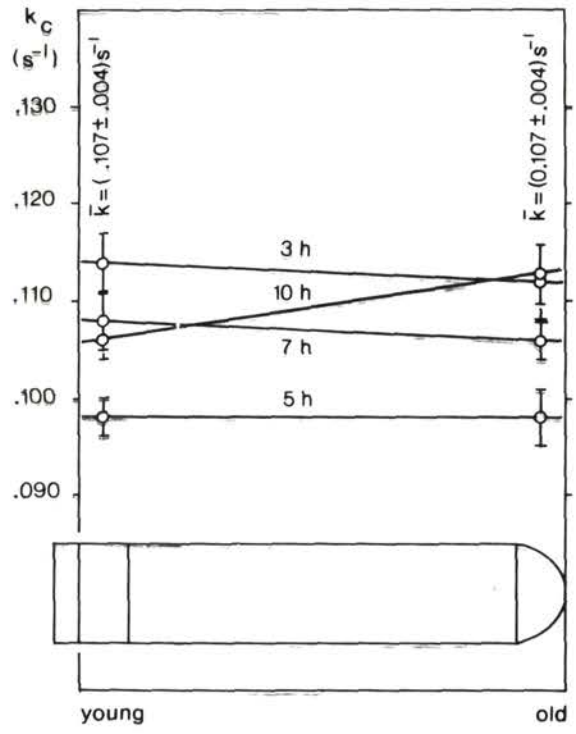

Fig. 1. Rate constants for Cl^- transport in young and old erythrocytes separated from control blood, as measured after different times of aging in vitro. The rate constants were measured at 1.5°C, in cells collected from top (the young cells) and bottom (the old cells) of the tube, after the separation of erythrocytes by age by high speed centrifugation.

Discussion

The qualitative difference observed in the behavior of HD erythrocytes during aging in vivo and in vitro was not associated with any abnormal change in their osmotic fragility during aging in vitro for 24 h (Białas 1981). Therefore, the alteration of anion transport cannot be associated with any structural alteration of the membrane components responsible for its mechanical properties. The results of standard hematological tests performed on blood from 6 patients (I. Sniecinski, unpublished) suggested that the glycoproteins and glycolipids of the erythrocyte membrane, on which the receptors for antibodies are located were normal. Thus, the membrane alteration associated with the decreasing anion permeability seemed to be localized in a specific protein, which was consistent with the altered ESR spectra of HD erythrocyte membrane proteins (Butterfield and Markesbery 1979).

The following hypothesis provides an explanation for the observations: in

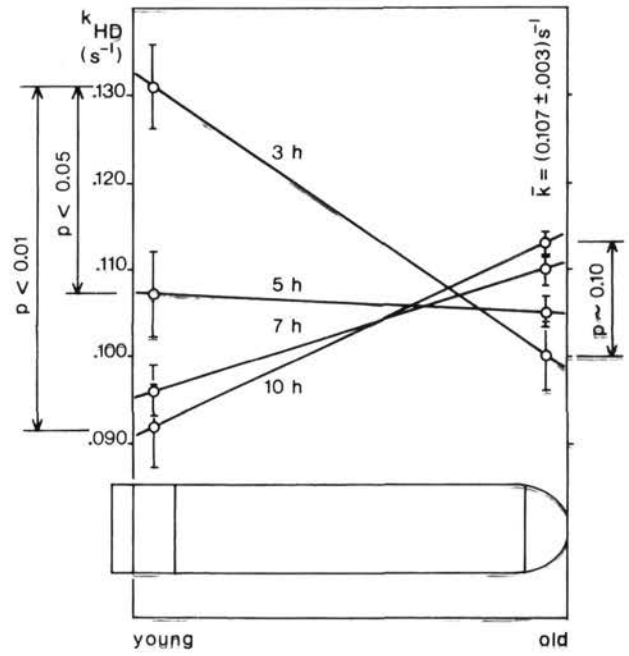

Fig. 2. Rate constants for Cl^- transport in young and old erythrocytes separated from blood of 5 HD patients, as measured during aging in vitro. The experiments were run simultaneously with those on control blood, and under the same conditions. Statistical p values were computed by paired t test.

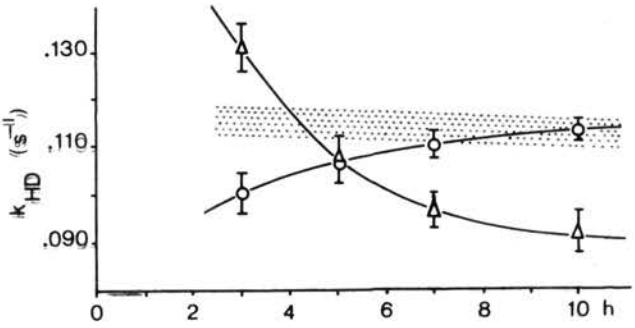

Fig. 3. Another representation of the results from Fig. 2. The shaded region corresponds to the normal values of Cl^- transport rates in "full" blood, shown in Table 1. (Δ) young HD erythrocytes; (o) old HD erythrocytes

Huntington's disease, a certain fraction of erythrocytes released into circulation may have an abnormal anion transport system, characterized by an initially very high permeability. The fresh fraction of young erythrocytes, which is a mixture of normal and abnormal cells, would, therefore, have an apparent rate constant (characteristic for the mixture of different cells) of a higher than normal value. During aging in vitro, the normal cells maintain a constant rate of anion transport, while it decreases in the defective fraction of erythrocytes. Since no new cells are supplied in vitro, the apparent rate decreases below normal level. The fresh sample of old HD erythrocytes is also a mixture of normal and defective cells. The latter are characterized by a very low already, and still decreasing, permeability. The apparent rate constant, which is initially lower than normal, would approach the normal level in the course of time, if the transport rate in the abnormal cells drops below a certain critical value, that depends upon the conditions of measurements (for an explanation, see Appendix). For the same reason, the rate constant for "full" blood is initially slightly higher than normal (13 %) and quickly decreases during aging. This decrease accounts for the spread of data previously published (Białas et al. 1980).

The molecular mechanism leading to such alterations of the transport cannot be postulated since the mechanism of the anion transport itself remains unresolved. The involvement of some membrane enzymes, which has been suggested as a primary factor, should be rejected because the young HD erythrocytes, just released from the bone marrow, have already displayed an abnormal rate of anion transport. Its decrease during the life of the cell, related to a process involving perhaps the anion transport protein, may, however, be caused by some enzymes associated with the membrane (Tarone et al. 1979). Also, the question whether the malfunction of the anion transport system reflects the primary result of the genetic defect, or whether the defect is to be looked for somewhere in the bone marrow, cannot be answered yet. However, the irregularities found in some HD blood cells other than erythrocytes (for review, see Appel 1979) may suggest that the latter may be true.

It is also intriguing how big is the population of abnormal erythrocytes in HD blood. A theoretical reconstruction of the results (see Appendix) yielded values of apparent rate constants, for mixtures of normal and abnormal cells, similar to those obtained experimentally when the population of the defective cells was assumed to be about 23 %, as that of the fraction of stomatocytes in HD blood (Butterfield and Markesbery 1979). Attempts are to be made to separate the abnormal cells for detailed studies on the mechanism of the alteration of anion transport and the defect's nature.

The possible relation of the existence of an abnormal erythrocyte population in HD blood to the pathology of Huntington's disease exceeds the scope of this paper, and has been discussed elsewhere (Białas 1981).

Appendix

Under steady state conditions, the Cl^- transport follows first order kinetics, and the relation between the specific radioactivity of the medium and time can be written as:

$$a_t = a_\infty(1 - e^{-bt}) + a_0 e^{-bt} \tag{1}$$

where a_0, a_t and a_∞ are, respectively, specific radioactivities of the medium at time $t = 0$, t, and at equilibrium, when the concentrations of the tracer anions inside and outside the cells are the same. Equation (1) can be rewritten as:

$$\ln\left(1 - \frac{a_t}{a_\infty}\right) = -bt + \ln\left(1 - \frac{a_0}{a_\infty}\right) \tag{2}$$

This relation is linear and, at low hematocrit (less than 1 %), the value of b equals the value of the rate constant k (Brahm 1977).

In the experiments, samples of extracellular medium were taken within 21 s of the initiation of the process. The equilibrium sample was filtered at $t = 180$ s. It is interesting to analyze the behavior of the apparent rate constant k, measured under such conditions, for a mixture of two populations of erythrocytes: one normal, characterized by a steady rate constant $k_n = 0.116\ s^{-1}$, and another abnormal, with the rate constant k_x decreasing from a high value (e.g. twice that of normal cells) to zero. The fraction of the abnormal cells in the total cell population will be denoted by x. Let the initial radioactivity of the medium, a_0, equal zero. The increase in time of the specific radioactivity of the medium, a_t, is due to the presence of two different populations of erythrocytes, the contribution of each described by Eq. (1). Therefore, for normal cells:

$$a'_t = a'_\infty (1 - e^{-k_n t})$$

and for the abnormal ones:

$$a''_t = a''_\infty (1 - e^{-k_x t})$$

since

$$a'_\infty = (1 - x)a_\infty \quad \text{and} \quad a''_\infty = x\, a_\infty$$

and also

$$a_t = a'_t + a''_t \quad \text{and} \quad a_\infty = a'_\infty + a''_\infty$$

then for a mixture:

$$a_t = a_\infty [x(1 - e^{-k_x t}) + (1 - x)(1 - e^{-k_n t})] \tag{3}$$

The "equilibrium" radioactivity as measured at $t = 180$ s equals:

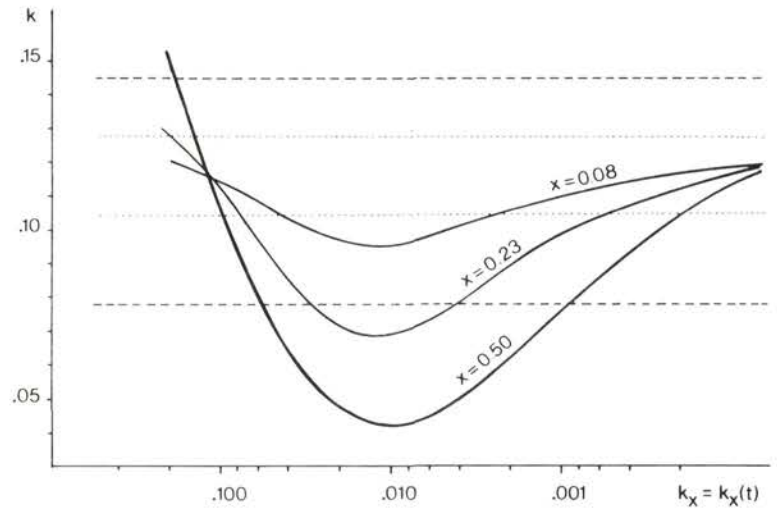

Fig. 4. Theoretical behaviour of the apparent rate constant k in a mixture of normal erythrocytes, with a steady rate constant $k_n = 0.116\ s^{-1}$, and a certain fraction (x) of abnormal cells, with the rate constant k_x decreasing in time. The curves were computed for the experimental conditions specified in Methods. The regions within which lay the experimental values of k_{HD} and k_c are limited by dashed and dotted lines respectively.

$$a_{180} = a_\infty \left[x(1 - e^{-180k_x}) + (1 - x)(1 - e^{-180k_n})\right] \tag{4}$$

From Eq. (2), (3), and (4) the relation

$$f(t) = -\ln\left[1 - \frac{x(1 - e^{-k_x t}) + (1 - x)(1 - e^{-k_n t})}{x(1 - e^{-180k_x}) + (1 - x)(1 - e^{-180k_n})}\right] \tag{5}$$

can be derived, from which the theoretical values of $f(t)$ for the times of sample taking in experiments (1; 5; 9; 13; 17; and 21 s) can be computed. The linear regression analysis of this set of data yields an apparent rate constant for the mixture of cells. It should be noticed that the function $f(t)$ is not linear. However, the correlation coefficient of the theoretical points thus obtained to the best fitting straight line does not drop below 0.99, which satisfies the condition of a linear approximation.

Since the radioactivity of the medium, as measured at $t = 180$ s, depends on k_x, the apparent rate constant for the mixture, k, will change differently for high and low values of k_x (Fig. 4.). For k_x greater than $0.01\ s^{-1}$ the abnormal fraction of cells can reach equilibrium during 180 s. The apparent rate constant will decrease as k_x decreases, but when k_x drops below $0.01\ s^{-1}$ the abnormal cells do not equilibriate

during 180 s, the measured "equilibrium" radioactivity of the medium, a_{180}, is then smaller than the actual a_∞, and the apparent rate constant will rise to normal values, when the abnormal cells become impermeable for anions. Indeed, the old HD erythrocytes seemed to behave in this way (Fig. 3.). The values of k (Fig. 4.) were evaluated for three fractions of abnormal cells in the total cell population. The spread of k at $x = 0.50$ far exceeds the experimentally measured rate constants in HD, but at $x = 0.23$ the values of k correspond well to those obtained experimentally. At $x = 0.08$ the values of k lie within the range of rate constants of normal, control blood.

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