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Aging of the Erythrocyte

V. Hydrolysis of Fluoresceine Diacetate in Red Cells

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Abstract. In contrast to situation found in other cell types, no linear dependence of product fluorescence vs time is observed when fluoresceine diacetate (FDA) is hydrolysed by erythrocytes and hemolysates. The rate of hydrolysis is increased by high concentrations of sucrose suggesting a positive effect of viscosity on the rate of the reaction. These peculiarities can be explained by assumption of a two-step hydrolysis of FDA. The FDA-hydrolytic activity decreases with increasing cell density (age).

Key words: Erythrocyte aging – FDA hydrolysis – Two-step hydrolysis – Effect of viscosity

1. Introduction

FDA has been proven to be a useful compound in cytological studies. This fluorogenic substance easily penetrates into cells where it is hydrolysed to yield a fluorescent product, fluoresceine (Rotman and Papermaster 1966). The rate of product formation, an index of cellular esterase activity, can be easily monitored in a fluorescence spectrophotometer (Augsten and Güttner 1975), under a fluorescence microscope (Sernetz 1973) or in a fluorescence-activated cell sorter (Szöllösi et al. in press). In the latter case, eventual separation of cells with abnormal esterase activity is possible.

Among other, the red blood cell is also capable of FDA hydrolysis and was studied from the standpoint of the effects of various physical and chemical factors on the rate of FDA hydrolysis and of the substrate permeation across the red cell membrane (Sontag 1977a, b). A complex kinetics of FDA hydrolysis was revealed and explained by a set of sophisticated assumptions. It seemed that a simpler explanation of this kinetics should be possible.

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On the other hand, it seemed noteworthy to examine the FDA-hydrolytic activity in erythrocytes of different age fractions. Activities of majority (but not all) of red cell enzymes decrease progressively as the red blood cell ages in the circulation and measurements of enzyme activities are often employed as an indirect index of efficiency of cell separation according to age using various experimental procedures (Chapman and Schaumburg 1967; Sass et al. 1964; Turner et al. 1974). The high sensitivity of the test of FDA hydrolysis, conditioned by the sensitivity of fluorimetric techniques, would make this test very convenient for this purpose especially in cases when small amounts of cells are available if FDA hydrolysis decreased in older cells.

Moreover, some aspects of the eventual changes could be interesting for understanding general mechanisms governing the progressive inactivation of erythrocyte enzymes and intracellular regulation of enzyme activities.

2. Materials and Methods

Bovine blood was obtained in a local abbatoir and citrated. Erythrocytes were separated according to density (and age) by the method of Murphy (Murphy 1973). The centrifugation was performed in a Beckman L3-50 ultracentrifuge (Central Research Laboratory, Medical University of Debrecen) using a 60 Ti (8 × 36) rotor. Stratified cell columns were divided into six equal-size fractions. Validity of this procedure with respect to fractionation of bovine erythrocytes will be discussed elsewhere (Bartosz et al. in preparation).

The cells were washed and suspended in phosphate-buffered saline (152 mM NaCl in10 mM sodium phosphate, pH 7.4) containing 1 g/l glucose (PBSG). Cells were counted in a Bürker chamber. Erythrocyte membranes were isolated according to Dodge et al. (1963). Hemolysates were prepared in 10 mM sodium phosphate, pH 7.4.

For estimation of the initial rate of FDA hydrolysis, a small aliquot of a stock FDA (Sigma) solution in acetone was injected into a microcuvette containing 400 µl of erythrocyte suspension in PBSG or hemolysate from an equivalent amount of cells. The cell concentration was usually $1-2 \times 10^6$/ml; in this range the rate of hydrolysis was proportional to the concentration of cells. Final acetone concentration was always 1% (v/v), higher acetone concentrations were found to be inhibitory. Fluorescence was monitored in a Hitachi MPF-4 fluorescence spectrophotometer at 516 nm using an excitation wavelength of 485 nm (Sontag 1977a) and resolution of 10/10 nm. Except for temperature dependence studies, the reaction was followed at 37.0 ± 0.1° C. Temperature was monitored with a thermocouple.

3. Results

3.1. Relation of Fluorescence Increase to Enzyme Activity

I was unable to follow the spectrophotometric conditions reported by Sontag (1977a, b) since under the measurement conditions employed cell suspensions of

a concentration of 10^8/ml gave a strong inner filter effect even using 400-μl microcuvettes. Instead, I used $1-2 \times 10^6$/ml cell suspensions or equivalent hemolysates. In this case there was no significant inner filter effect but the rate of hydrolysis was respectively lower so that only the initial rate of FDA hydrolysis was measured within 10–15 min (less than 1% of the substrate hydrolysed).

In all cases studied, plots of fluorescence vs time were curvilinear and had a typical upward curvature (Fig. 1A), in agreement with previous data from erythrocytes (Sontag 1977a; initial part of appropriate curve in Fig. 2 from that paper). This made difficult quantitation of the initial rate of FDA hydrolysis. I considered possible reasons for this phenomenon:

(i) Fluorescence of the product may be quenched by hemoglobin and this quenching, more significant for lower product concentrations, might be responsible for the curvature. However, this was not the case as erythrocyte ghosts containing negligible amounts of hemoglobin yielded curves of the same shape.

(ii) The curvature might be due to an interplay between the transport of the substrate into the cell and its intracellular hydrolysis. But identical shape of curves was obtained for hemolysates.

(iii) It can be hypothesized that the hydrolysis of FDA proceeds in two steps:

$$FA_2 \rightarrow FA + A \tag{1}$$

$$FA \rightarrow F + A \tag{2}$$

where: FA_2 = fluoresceine diacetate,
FA = fluoresceine monoacetate,
F = fluoresceine,
A = acetate,

and the fluorescence reflects mainly (or exclusively) the concentration of the product (Rotman and Papermaster 1966), the contribution of the intermediate FA to the fluorescence intensity being negligible.

Under the assumption that the substrate (FA_2) concentration s and the intermediate (FA) concentration i are much lower then respective K_m values of the hydrolytic enzyme, the rate of substrate consumption is

$$-\frac{ds}{dt} = k_1 s \tag{3}$$

and the rate of intermediate formation is

$$\frac{di}{dt} = k_1 s - k_2 i\,, \tag{4}$$

where k_1 and k_2 are the rate constants of the two consecutive hydrolysis steps.

Table 1. Effect of sucrose on the FDA-hydrolytic activity of bovine erythrocyte lysates (mean values from triplicate experiments)

Medium	Relative activity
10 mM phosphate buffer, pH 7.4	1.00
0.5 m sucrose in phosphate buffer	1.41
1 m sucrose in phosphate buffer	1.70

fluorescence of the product of hydrolysis was reported to be a linear function of time (Guilbault and Kramer 1964; Cercek and Cercek 1973a, b). Apparently in the latter cases FDA was hydrolysed by enzyme(s) which did not release the intermediate. FDA is a substrate for a variety of enzymes including lipase, acylase and chymotrypsins (but not acetylcholinesterase of penicillinase) (Guilbault and Kramer 1964). The shape of the fluorescence vs time curve depends probably upon which enzyme (releasing or not releasing the intermediate) governs the FDA hydrolysis in a given system. Activity of enzymes which do not release the intermediate is expected to be decreased, not increased, by elevation of viscosity (Somogyi et al. 1978). Indeed, increased viscosity inhibited the FDA hydrolysis in such cell types as lymphoid (Szöllösi et al. in press; Sengbusch et al. 1976), yeast (Cercek and Cercek 1973a, b) and Chinese hamster ovary cells (Cercek et al. 1973) in which fluorescence of the reaction product increased linearly with time.

The FDA-hydrolytic activity of erythrocytes decreased with increasing ionic strength of the medium. The ratio of the FDA-hydrolytic activity of the hemolysates in 10 mM sodium phosphate buffer (pH 7.4) to that in 152 mM NaCl in the same buffer was 1.41 ± 0.01 ($n = 10$). This effect was not due to inhibition by Cl^- since the same dependence was observed when diluting the hemolysate with phosphate and chloride solutions of changeable ionic strength.

3.3. Properties of the FDA-Hydrolysing Enzyme

The presence of the FDA-hydrolytic activity was demonstrated both in hemolysates and in erythrocyte membrane preparations. Comparison of this activity in whole hemolysates (cells hemolysed 1 : 100 (v/v) with 10 mM sodium phosphate buffer, pH 7.4) and in hemolysates centrifuged (20,000 *g*, 30 min) to sediment the membranes revealed that under these conditions cell membranes contained about 5% of the total cellular activity, in a full agreement with the data of Sontag (1973a). This value did not differ among cell fractions of different mean age.

Apparent activation energy for FDA hydrolysis measured in the range of 293.9–312.7 K was 90.7 ± 1.6 kJ/mol^{-1} for unfractionated erythrocyte populations with no significant differences between cell fractions of various mean age.

The dependence of hemolysate FDA-hydrolytic activity on the substrate concentration fitted the Michaelis-Menten model (Fig. 2). Apparent K_m value

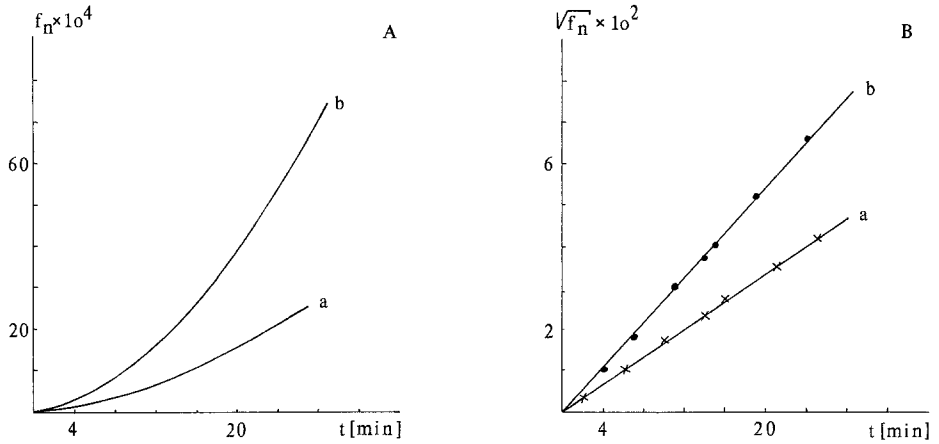

Fig. 1 A and B. Time dependence of fluorescence (**A**) and square root of fluorescence (**B**) during the initial stage of FDA hydrolysis by erythrocyte suspensions in PBSG. Medium: (*a*) PBS, (*b*) 1 m sucrose in PBS

(f_∞ – fluorescence corresponding to complete hydrolysis of the substrate) and plotting the square root of the (normalized) fluorescence vs time should yield a straight line. This was indeed the case (Fig. 1B).

The fluorescence curves were analysed therefore by replotting them in $(t, f_n^{1/2})$ coordinates and determination of the mean FDA-hydrolysing capacity of the cells (hemolysates) from these plots.

3.2. Effect of Sucrose and Ionic Strength on FDA Hydrolysis

The two-step mechanism of FDA hydrolysis by erythrocytes and hemolysates proposed here, proceeding with a release of the intermediate from the enzyme should involve a considerable effect of viscosity on the reaction rate. The more viscous the medium is, the lower the diffusion rate of the intermediate and the higher is the probability of the intermediate to react for the second time with the parent enzyme molecule (Somogyi et al. 1978). The observed effect of high concentrations of sucrose on FDA hydrolysis is compatible with this assumption. The rate of hydrolysis was significantly increased in the presence of high concentrations of sucrose as in hemolysates (Table 1) as in erythrocytes (Fig. 1). Though the effect of sucrose on the FDA-hydrolytic activity of hemolysates may be due to some specific properties of the solute not related to viscosity, sucrose does not penetrate the red cell membrane but microviscosity of cell interior is increased when erythrocytes are in a hypertonic sucrose solution (Bartosz and Leyko 1980) and in this case the viscosity seems to be the main factor mediating the effect of sucrose on FDA hydrolysis.

The present and previous (Sontag 1977a, b) results on the kinetics of FDA hydrolysis in erythrocytes and the effect of sucrose on this reaction are in disagreement with data from other cells and enzyme preparations in which

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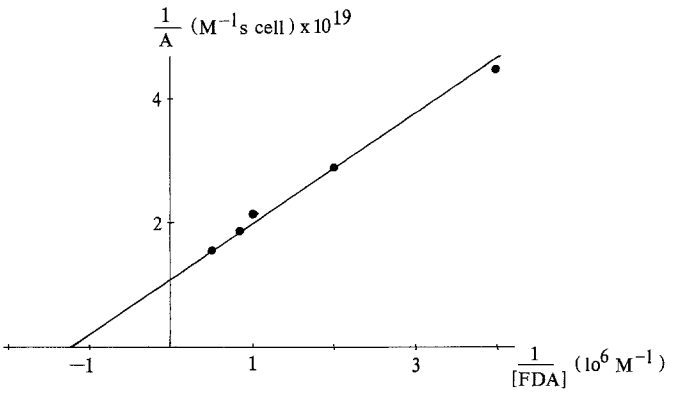

Fig. 2. Lineweaver-Burk plot for FDA hydrolysis by hemolysates of bovine erythrocytes. Medium: 10 mM phosphate buffer, pH 7.4

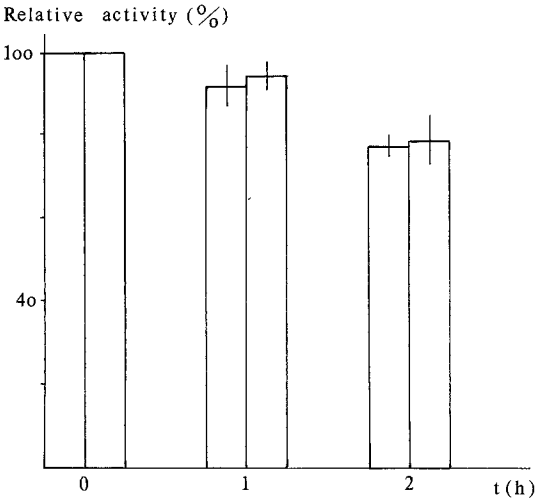

Fig. 3. Heat stability of the FDA-hydrolytic activity in hemolysates of youngest and oldest erythrocytes. Medium: 10 mM phosphate buffer, pH 7.4; $n = 6$

determined from the Lineweaver-Burk plot was 0.78 ± 0.11 μM and V_{max} amounted to $8.2 \pm 1.4 \times 10^{-20}$ $M/s^{-1}/cell^{-1}$. These values coincided for the fractions of youngest and oldest cells.

No differences were revealed in heat stability of the FDA-hydrolysing enzyme between the youngest and oldest cell fractions when erythrocyte lysates were incubated at 318 K for varying time intervals (Fig. 3).

3.4. Effect of Cell Age on the FDA-Hydrolytic Activity

Comparison of the rate of FDA hydrolysis by hemolysates of various density (and age) fractions of erythrocytes showed a progressive loss of the FDA-hydrolytic activity during erythrocyte aging in vivo (Table 2).

Table 2. FDA-hydrolytic activity of hemolysates of red cell fractions of different mean age. 10 mM sodium phosphate, pH 7.4; $n = 6$; mean ± SD

Fraction No.	FDA-hydrolytic activity
1 (youngest)	$52.5 \pm 8.6 \times 10^{-21}$ M/s^{-1}/cell^{-1} = 100%
2	87.9 ± 8.5%
3	81.3 ± 10.0%
4	72.1 ± 9.4%
5	68.1 ± 7.7%
6 (oldest)	66.5 ± 7.8%

3.5. Possible Effect of Microviscosity of Cell Interior on FDA Hydrolysis

The in vivo aging of the red blood cell involves an increase in the microviscosity of cell interior (Bartosz and Leyko 1980). One may expect therefore some viscosity-dependent elevation of the activity of the FDA-hydrolysing enzyme in senescent erythrocytes, superimposed on the more pronounced cell age-related inactivation of the enzyme. Such an effect could be revealed by comparison of ratios of the FDA-hydrolytic activity in native red cells of various ages and in hemolysates obtained from these cells. Respective activity ratios $A_{\text{hemolysate}}/A_{\text{intact cell}}$ were: 1.47 ± 0.02 and 1.41 ± 0.02 for the youngest and oldest cell fractions ($n = 5$), a result in favour of this hypothesis (in these measurements FDA hydrolysis was monitored in whole cells suspended in PBSG and in hemolysates made with 10 mM sodium phosphate). Older cells, of higher internal microviscosity, had a higher relative FDA-hydrolytic activity (referred to the activity observed in a low-viscosity medium).

The ratio of the FDA-hydrolytic activity : hypotonic buffer/intact cell can be therefore a measure of changes in intracellular microviscosity. However, it cannot be employed as an absolute estimate of the microviscosity of cell interior and compared with the previously reported ratio for hemolysate diluted with low-ionic strength and high-ionic strength media. Microenvironment of the enzyme inside the cell and in the hemolysate differs markedly not only with respect to microviscosity but also with respect to many other parameters (e.g., local dielectric constant) which may also affect the enzyme action. The different rates of product accumulation inside the cell and in the hemolysate can influence the reaction rate, too.

4. Discussion

FDA can be hydrolysed by various hydrolases (Guilbault and Kramer 1964) and, depending on the actual enzyme set of a cell, this hydrolysis may be governed by different enzymes in various cells.

In the majority of cells studied hitherto (Cercek and Cercek 1973a, b; Cercek et al. 1973; Rotman and Papermaster 1966; Sengbusch et al. 1976; Szöllösi et al. in press) fluorescence of the product of FDA hydrolysis was a linear function of time during the initial period of reaction. The same was found for preparations of such enzymes as lipase, acylase and chymotrypsin (Guilbault and Kramer 1964). However, in erythrocytes kinetics of FDA hydrolysis was proven to be more complex (Sontag 1977a). In order to explain the mechanism of this kinetics a complicated model was elaborated and the presence of two conformers of the substrate, hydrolysed at different rates, was postulated (Sontag 1977a).

In the present study this kinetics was explained in a simpler way basing on the assumption of a two-step hydrolysis in which the intermediate is released from the enzyme molecule and must encounter the enzyme for the second time. In agreement with this assumption, fluorescence of the product was found to be proportional to the second power of time during the initial period of FDA hydrolysis by erythrocytes or hemolysates.

It seems that in contrast to those in other cells, the enzyme responsible for FDA hydrolysis in the erythrocyte is an intermediate-releasing enzyme. Its activity is increased, not decreased by high concentrations of sucrose and its apparent Michaelis constant (determined from resultant activity values) of about 0.8 μM is lower than those reported for FDA-hydrolysing enzymes from L cells (10 μM) (Sengbusch et al. 1976), macrophages (μM) (Augsten and Güttner 1975) and Chinese hamster ovary cells (4–8 μM) (Cercek et al. 1973).

In red cells the FDA-hydrolysing enzyme is located mainly in the cytosol, about 5% of its activity being bound to cell membranes under conditions of hypotonic hemolysis. Its activity is decreased by increasing ionic strength. Attempts are made to identify and isolate this enzyme from erythrocytes.

Activity of the FDA-hydrolysing enzyme decreases during the in vivo aging of the red blood cell. Because of the simplicity and sensitivity of the method of its fluorimetric estimation, it can be used as an indicatory enzyme of red cell age, especially when small amounts of erythrocytes are available. The FDA-induced fluorochromasia (Rotman and Papermaster 1966) can be also postulated to enable age separation of erythrocytes in a fluorescence-activated cell sorter (Dolbeare and Smith 1979) taking advantage of differences in the rate of FDA hydrolysis in cells of different age.

No conclusion can be drawn on the mechanism of inactivation of the FDA-hydrolysing enzyme during red cell aging. Neither the substrate affinity nor the sensitivity to heat inactivation are altered in senescent erythrocytes, a situation found also for some other red cell enzymes (Bartosz et al. submitted).

On the other hand, the age-related elevation of the microviscosity of red blood cell interior increases the specific activity of the enzyme. This phenomenon may represent one of the mechanisms of intracellular modulation of activities of biologically active proteins.

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