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Evaluation of Hexose Monophosphate Shunt Activity in Isolated Murine Lens by Monitoring the Potential of the Ferricyanide–Ferrocyanide System

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Abstract—A method for evaluation of the activity of the hexose monophosphate shunt (HMS) in isolated lens is presented. The measurement of HMS activity is based on continuous monitoring of the potential of the ferricyanide–ferrocyanide system (where ferricyanide is an artificial electron acceptor) in the presence of a lens. The rate of reduction of ferricyanide increased after the addition of methylene blue (MB) or saponin. The ferricyanide reduction rate did not correlate with GSH content in the contralateral lenses of the same mouse in the absence of MB or saponin. Correlations between the ferricyanide reduction rate and GSH content in the lens were 0.67 ($\beta = 0.93$) in the presence of MB and 0.82 ($\beta = 0.95$) in the presence of saponin. We think that the measured curves of ferricyanide reduction are representative of: 1) normal level of HMS activity (in the absence of methylene blue and saponin); 2) maximal HMS activity (in the presence of methylene blue); 3) the intracellular concentration of reducing equivalents (in the presence of saponin).

Key words: hexose monophosphate shunt, lens, reducing equivalents, ferricyanide, ferrocyanide, methylene blue, saponin, reduced glutathione

The level of reduced glutathione (GSH) in lenses of vertebrates is very high [1], reaching in the metabolically most active lenticular epithelium of values of about 60 μmol GSH per g wet weight [2]. By comparison, other mammalian organs with high GSH content such as kidney or liver contain about 5 μmol GSH per g wet weight [3, 4]. Reduced GSH level decreases more than twofold in different types of cataract (diabetic, senile, ultraviolet) [1, 5-7] and even before the development of cataract [7, 8]. The redox system of the lens, including NADPH-dependent glutathione reductase, has a key role in maintaining high GSH content. Much of the NADPH in the lens is derived from the hexose monophosphate shunt (HMS), this being one of the main energetic systems for erythrocyte and lens metabolism [9]. Commonly used methods for estimation of HMS activity require destruction of the native structure of the lens. A colorimetric method for studying the HMS in living cells (erythrocytes) was proposed by Orringer and Roer [10]. This method is based on the oxidation of membrane impenetrable ferricyanide in the medium by cellular reducing agents that do penetrate into the medium. The decrease in ferricyanide concentration was determined by spectrophotometric measurement. Using potentiometric rather than optical registration of ferricyanide, the method was adapted for single-stage monitoring of ferricyanide reduction kinetics by intact cells [11, 12].

The purpose of this paper was to investigate the possibilities for evaluation of HMS activity in isolated lens using a platinum electrode for monitoring ferricyanide–ferrocyanide potential.

MATERIALS AND METHODS

Hybrid F1 mice (CBA×C57Bl/6) with mean initial body weight 25-30 g were used. Lenses were removed immediately after decapitation. Lenticular homogenates were prepared by homogenizing each lens in 1.1 ml 50 mM phosphate buffer (pH 7.4) at 20°C using a Potter–Elvehjem homogenizer.

As in previous works, we assumed that the GSH content in the lens equals content of non-protein reducing thiols [13]. The content of GSH was determined using the

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5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) reaction [14]. Protein was removed by precipitation with 7% (w/v) final concentration of trichloroacetic acid (0.5 ml of 21% trichloroacetic acid was added to 1.0 ml homogenate) followed by centrifugation at 8000 rpm for 10 min at 4°C. The reaction mixture for the assay consisted of 1.0 ml supernatant, 1.0 ml 0.8 M Tris-HCl (pH 8.6), and 50 μl 10 mM DTNB (ethanolic solution). Optical density was measured at 412 nm against a control sample. GSH concentration was determined from a standard curve constructed using GSH (Reanal, Hungary) as the standard. The content of GSH was expressed in terms of μmol GSH per g protein.

Protein concentration was determined by the microbiuret method [15] with the following changes. To 1 ml of protein solution, 2 ml of 6% NaOH and 0.1 ml of Benedict's reagent solution (17.3 g $C_6H_5O_7Na_3$, 10 g Na_2CO_3, and 1.73 g $CuSO_4$ dissolved in 100 ml of distilled water) were added. The samples were carefully mixed and left for 30 min at room temperature. Then the optical density was determined at 330 nm against a control sample. Protein concentration was determined from a standard curve constructed using BSA (Sigma, USA) as a standard.

Oxidation–reduction potential was measured using a platinum electrode referenced to a AgCl electrode (EVL-1M) (which was connected to the measuring cuvette by a salt bridge) with a linear pH-340 potentiometer and Gilson (France) recorder.

The experimental setup is shown in Fig. 1. A typical curve of potential monitoring of ferricyanide reduction is shown in Fig. 2.

The measured values (GSH in lenses) represented as mean values (M) from n ($n = 4$) results of measurements carried out on one technique and confidential interval (ε) calculated with probability $\beta = 0.95$ from the formula: $\varepsilon(\beta) = [t\,(p, f)\,s]/n^{0.5}$, where $t\,(p, f)$ is the tabulated value of Student's t-criterion, whose value depends on the degrees of freedom $f = n - 1$, and the significance p ($p = 1 - \beta$); s is standard deviation. The confidence probability of factors of linear correlation were determined using the z-transformation of Fisher, with subsequent analysis of the null hypothesis concerning the z-transformed factor of correlation based on tabulated values of critical points of Student's t-criterion [16].

Scheme of the experiment. In a typical experiment, 1 ml of the incubation solution was entered into the quartz measuring cuvette (1 × 1 cm), which was thermostatted at 36°C and stirred with a magnetic stirrer. The incubation solution consisted of 0.14 M NaCl, 5.6 mM glucose, and 10 mM Na-K-phosphate buffer at pH 7.4. When both electrodes (platinum electrode and AgCl electrode) were placed in the measuring cuvette, 5 μl of 10 mM ascorbic acid solution was added to the measuring cuvette and recording of the system potential was started. Ferricyanide (20 μl of 10 mM solution) was added to the measuring cuvette after 15 min. When the potential of the system stabilized at a level of constant drift (after 15 min), a cell with grid (where the lens is placed) was put on the platinum electrode. After 15 min of potential monitoring, 5 μl of 1% methylene blue solution was added to the

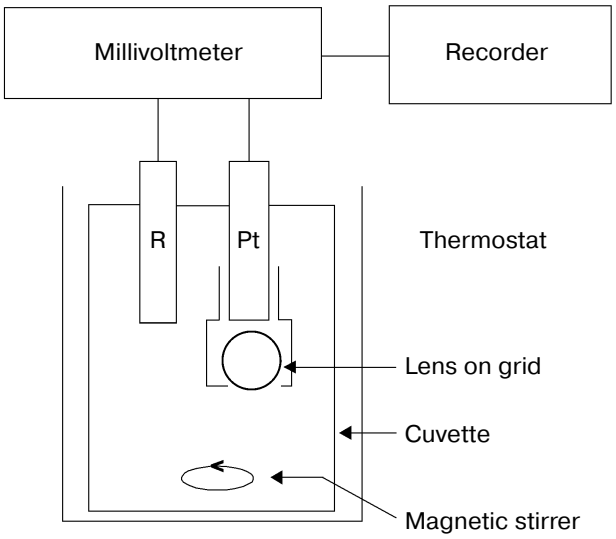

Fig. 1. Schematic of the experimental setup (t = 36°C). Designations: R, AgCl reference electrode; Pt, platinum electrode.

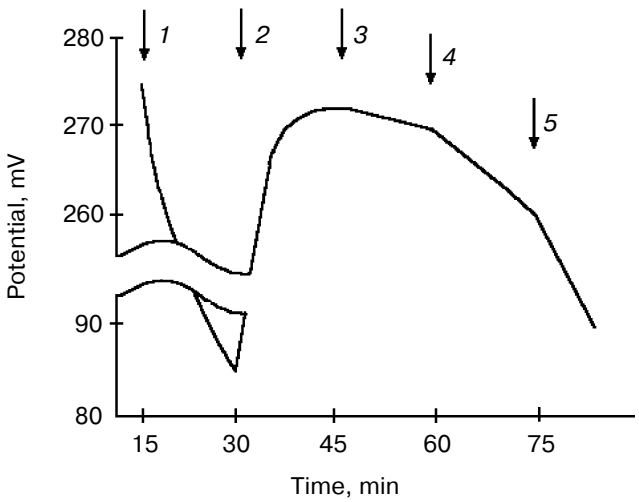

Fig. 2. Example of a typical experiment. Arrows in the figure designate: *1*) introduction of ascorbic acid into the system; *2*) introduction of ferricyanide; *3*) measurement of potential in the presence of a lens; *4*) introduction of methylene blue; *5*) introduction of saponin. A detailed description of the experiment is given in the text.

measuring cuvette, and then (after 15 min) 5 μl of 0.25% saponin solution was added. After 15 min, the potential monitoring experiment with the lens was terminated.

Calculation of the ferricyanide concentration (μM) at each time using formula (1) as been reported earlier [12]:

$$C = C_0 - C_0/(1 + 10^{(E - E_0)F/2.3RT}), \qquad (1)$$

where C_0 is initial concentration of ferricyanide; E_0 is the potential measured in a system with equal concentrations of ferricyanide and ferrocyanide; E is the potential which is measured; T is the absolute temperature; F is the Faraday constant; R is the gas constant.

RESULTS AND DISCUSSION

As noted above, measurement of HMS activity is based on continuous monitoring of the potential of the ferricyanide–ferrocyanide system (where ferricyanide is the artificial electron acceptor) in the presence of living cells (see the scheme of the experimental setup on Fig. 1). In the first stage of the experiment, ascorbate was added to the measuring cuvette to final concentration 50 μM. In response to ascorbate addition, the system potential decreased to 80 mV (Fig. 2). The ascorbate–dehydroascorbate system was used as a shuttle transferring reducing equivalents through the lens epithelium membranes. The concentration of ascorbate was selected so as to convert part of the ferricyanide, which was added to system later, to ferrocyanide.

Ferricyanide was added to the ascorbate-containing sample to final concentration 200 μM. This concentration was empirically chosen to achieve the best signal-to-noise ratio of the device. On addition of ferricyanide into the cuvette, the measured potential increased considerably to a mean of about 280 mV.

For the measurements, the cell with a lens was put on the platinum electrode (the lens was placed on the grid at the bottom of the cell) when the potential settled at a level of constant drift, and then the kinetics of reduction of ferricyanide was recorded. The kinetics of ferricyanide reduction in the presence of a lens was found to be linear, as revealed earlier for erythrocytes [11]. The measured potential fell linearly due to reduction of ferricyanide by the lens. The slope of the curve was used to determine the rate of ferricyanide reduction. Then this rate was evaluated in μmole/min by formula (1).

The overall rate of ferricyanide reduction by two lenses simultaneously was found to equal the sum of the rates of ferricyanide reduction for each lens separately. After 15 min recording of the basal ability of a lens for ferricyanide reduction, methylene blue was added to the system to final concentration 0.005%.

Methylene blue has a nonspecific effect on the HMS due to its participation in shuttle transfer of reducing equivalents, the specific effect of removing the limitation of the work of the shunt by oxidation NADPH to $NADP^+$ [17]. Methylene blue increased the rate of ferricyanide reduction by the lenses of mice, as was observed earlier for erythrocytes [11].

Significantly, this method allows estimation of the impairment of membrane barrier function that occurs both during the development of cataract and during the action of agents that can damage the membrane [18]. In the given work, the membrane-damaging substance used was saponin. It is known that saponin forms complexes with cholesterol, nonspecifically increases membrane permeability [19], and thereby leads to the issue of intracellular reducing equivalents into the medium. On addition of saponin to final concentration 0.001% to isolated lens, the rate of ferricyanide reduction increased considerably (Fig. 2). This fact can be explained by the damage to the lens epithelial membranes and by the issue of intracellular contents.

The reducing power of a lens is attributable not only to the transport of reducing equivalents through membrane, but also to the issue of their intracellular content into the medium as well.

To test this suggestion, the kinetics of GSH-release from mouse lenses was measured. GSH was selected as a marker of the release of reducing equivalents from lentic-

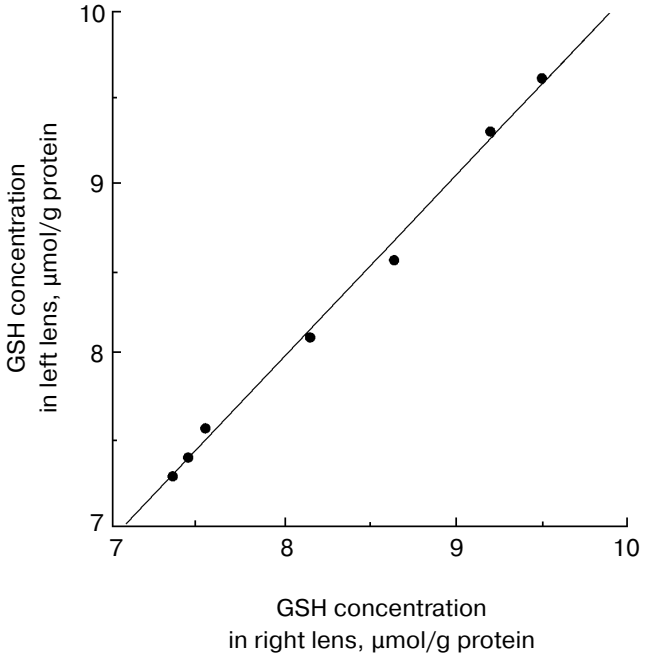

Fig. 3. Dependence of GSH concentration (μmol/g protein) in the left lens on GSH concentration in the right (contralateral) lens of the same mouse.

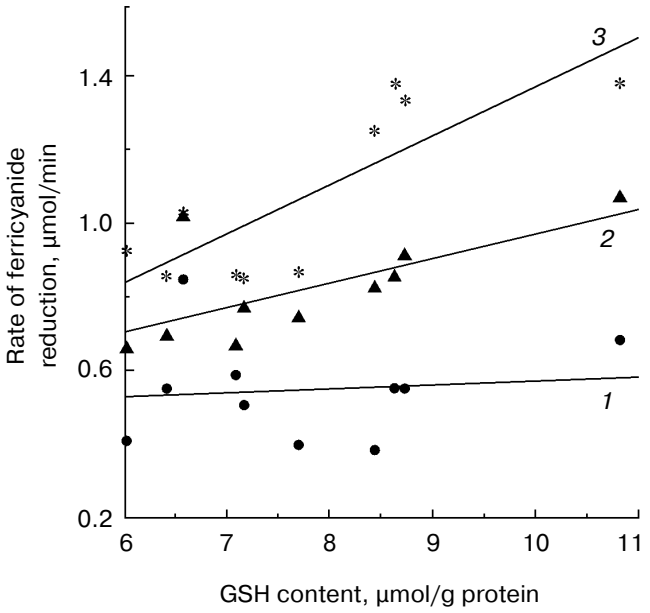

Fig. 4. Rate of ferricyanide reduction by murine lenses (μmol/min) as a function of GSH content in the contralateral lenses of the same mouse: *1*) in the absence of methylene blue and saponin; *2*) in the presence of methylene blue; *3*) in the presence of methylene blue and saponin.

ular cells. Ten murine lenses were incubated for 1 h in 5 ml of medium without ferricyanide, and the GSH concentration was measured at quarter-hourly intervals. The content of GSH in the medium of samples (0.5 ml) remained practically constant for the time of incubation and was not more than 2.5 ± 0.2% of the total GSH content of the lenses taken for experiments. On the basis of these results, it can be concluded that the observed curve for ferricyanide reduction is determined by the ascorbate-depended transport of reducing equivalents through the membrane, but not due to the release of the intracellular content of the lenses into the medium (including GSH).

As noted earlier, much of the NADPH (and thus GSH) is derived from the HMS in the lens [9]. The current interest is evaluating the influence of the content of GSH in lenses on the level of the activity of the HMS.

Using a single lens, it is impossible to determine the GSH level and the activity of the HMS simultaneously, because the experiment requires damage to the intactness of the lens membranes by saponin. So, we evaluated the possibility of assessing the correlation between GSH content and HMS activity in contralateral lenses from a single mouse. An investigation of the correlation between the GSH content in contralateral lenses of the same mouse is shown in Fig. 3. The high correlation shows that the GSH concentrations in contralateral lenses are equal. Also, it was shown that contralateral lenses from the same mouse give practically equal contribution to the rate of ferricyanide reduction.

Based on this fact, the relationship between HMS activity and GSH content was studied by measuring the appropriate parameters in contralateral lenses from the same mouse.

The reducing power of the lenses (in the absence or presence of methylene blue and saponin) is shown in Fig. 4 as a function of the GSH level.

The results of the study of 10 animals indicate that there was no correlation ($R = +0.11$) between the individual GSH content and the lens reducing power in the absence of methylene blue and saponin. On the contrary, there is the linear correlation between the rate of ferricyanide reduction by lenses in the presence of methylene blue and the GSH level ($R = 0.67$, $\beta = 0.93$) (see Fig. 4). The high linear correlation ($R = 0.82$, $\beta = 0.95$) between the rate of ferricyanide reduction by lenses in the presence of methylene blue and saponin and the GSH level was shown (Fig. 4).

The absence of a correlation between the GSH content and the lens reducing power in the absence of methylene blue and saponin can be explained on the assumption that the registered HMS activity under physiological conditions is less than maximal. New centers for the transport of reducing equivalents through membrane by means of a shuttle mechanism are probably formed by the addition of methylene blue. Besides, the NADPH oxidation caused by methylene blue is an activating factor for HMS [11, 12]. Thus, the presence of methylene blue stimulates the highest possible HMS activity.

It thus appears that the measured reduction curves are representative of different parameters of the lens. Namely, the rate of ferricyanide reduction in the absence of methylene blue and saponin is representative of the normal level of HMS activity. The rate of ferricyanide reduction in the presence of methylene blue is representative of the maximum of HMS activity. The rate of ferricyanide reduction in the presence of saponin is representative of the intracellular concentration of reducing equivalents.

We think the method proposed in this paper is especially suited for the study of the effects of different chemical compounds and the influence of physical factors (UV light, temperature, etc.) and anti-cataract drugs on the HMS activity of the lens.

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