

Estimation of H₂O₂ gradients across biomembranes

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Abstract When cells are exposed to an external source of H₂O₂, the rapid enzymatic consumption of H₂O₂ inside the cell provides the driving force for the formation of the gradient across the plasma and other subcellular membranes. By using the concepts of enzyme latency, the following gradients – formed after a few seconds following the exposure to H₂O₂ – were estimated in Jurkat T-cells: [H₂O₂]_{cytosol}/[H₂O₂]_{peroxisomes} = 3; [H₂O₂]_{extracellular}/[H₂O₂]_{cytosol} = 7. The procedure presented in this work can easily be applied to other cell lines and provides a quantitative framework to interpret the data obtained when cells are exposed to an external source of H₂O₂. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Hydrogen peroxide; Glutathione peroxidase; Catalase; Steady state; Redox regulation; Jurkat T-cell

1. Introduction

Signal transduction, development, cell proliferation, apoptosis, and necrosis, among other processes, are believed to be regulated by the redox status of the cell [1–4]. H₂O₂ is often the oxidant of choice in studies of redox-regulated processes, because it is continuously produced in aerobic metabolism and diffuses easily across cellular compartments [5]. In spite of the high permeability of H₂O₂, gradients across membranes are indeed formed when a membrane separates the production and consumption sites of H₂O₂. When cells are exposed to external H₂O₂, the fast consumption of H₂O₂ inside the cells provides the driving force for setting up a gradient across the plasma membrane (Fig. 1). The intracellular concentration of H₂O₂ is lower than the extracellular one and, thus, to establish the actual concentration of H₂O₂ that is implicated in the intracellular signaling events, this gradient must be determined.

One possible strategy to estimate gradients across biomembranes is based on the well-known fact that enzymes entrapped in compartments show a lower activity than enzymes free in solution, an observation brought forward in the early 1950s by De Duve and co-workers in their seminal studies on subcellular fractionation [6]. In general, the reason for enzyme latency is the permeability barrier constituted by the compartment entrapping the enzyme, which limits the diffusion of the

substrate to the enzyme, and not some other factor, such as inhibition of the enzyme when trapped in the compartment.

Cellular H₂O₂ consumption is largely the domain of catalase and glutathione peroxidase; for the former enzyme, several studies firmly established that the permeability barrier is indeed the cause for the latency observed [6], while for the latter, as far as we know, no studies on its latency have been carried out. Under in vivo conditions, both catalase [5] and glutathione peroxidase [7] display first-order kinetics, not showing saturation. As such, the gradient between the concentration of H₂O₂ inside ([H₂O₂]_{in}) and outside ([H₂O₂]_{out}) the cell is independent of the concentration of H₂O₂ and is given by the equation:

$$\frac{[H_2O_2]_{in}}{[H_2O_2]_{out}} = R \quad (1)$$

where *R* is the ratio of activity of the first-order process between intact and disrupted cells [8]. To apply this equation, the consumption of H₂O₂ by intact cells and the sum of activities that consume H₂O₂ in disrupted cells must be determined.

In this work, we revisited the concepts of enzyme latency to estimate the H₂O₂ gradients produced in Jurkat T-cells – a cell line widely used in studies of redox regulation – upon exposure to an external source of H₂O₂. The procedure used can easily be applied to other cell types, thus constituting a general strategy to determine H₂O₂ gradients.

2. Materials and methods

2.1. Chemicals and biochemicals

Catalase (bovine liver), digitonin, and GSSG reductase (Baker's yeast) were from Fluka (Buchs, Switzerland). Glucose oxidase and NADPH were from Boehringer (Mannheim, Germany). DTPA, GSH, H₂O₂, NaN₃, and Triton X-100 were from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical grade.

2.2. Cell culture

Jurkat cells obtained from ATCC (clone E6-1) were cultured in complete medium (RPMI 1640 medium supplemented with 10% fetal calf serum, L-glutamine, and antibiotics from Life Technologies (Rockville, MD, USA)). Cells were incubated at 37°C in humidified air with 5% CO₂, and kept in logarithmic phase by routine passage every 2 days. Before use, cells were spun down, resuspended in fresh medium at 1 × 10⁶ cells/ml, and incubated for at least 1 h. Cell viability was determined by propidium iodide uptake.

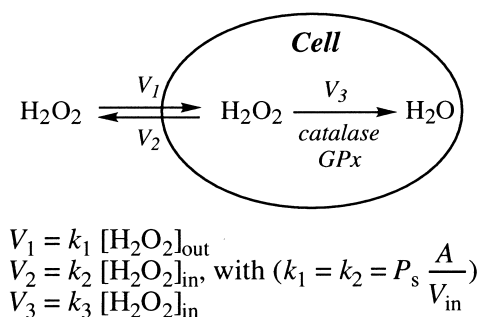
2.3. Biochemical measurements

H₂O₂ was measured with an oxygen electrode following the addition of catalase, which caused a rapid conversion of H₂O₂ to H₂O and O₂, the release of the latter being monitored by the oxygen electrode. With a clean and stable electrode, H₂O₂ concentrations as low as

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Abbreviations: GPx, glutathione peroxidase; GSH, glutathione; GSSG, glutathione disulfide; *P*_s, permeability coefficient



At steady-state conditions

$$V_1 = V_2 + V_3$$

$$[\text{H}_2\text{O}_2]_{\text{in}} = \frac{k_2 [\text{H}_2\text{O}_2]_{\text{out}}}{k_2 + k_3}$$

Fig. 1. Why a H_2O_2 gradient? At steady-state conditions, if the intracellular consumption of H_2O_2 (k_3) is non-negligible, the concentration of H_2O_2 inside the cell ($[\text{H}_2\text{O}_2]_{\text{in}}$) will be lower than the extracellular one ($[\text{H}_2\text{O}_2]_{\text{out}}$). The magnitude of the gradient forced depends on the relative value of the rate of intracellular consumption (k_3) compared to the rate of diffusion across the plasma membrane (k_2), and is given by $k_2/(k_2+k_3)$. P_s , permeability constant for H_2O_2 ; A/V_{in} , ratio between the area and the volume of the cell.

5 μM could be measured. A calibration curve was made with H_2O_2 standards before each experiment. Catalase activity was measured as previously described [9] in 0.5 M potassium phosphate buffer, pH 7.0, containing 0.01% digitonin in the presence of 1×10^6 cells. H_2O_2 (10 mM initial concentration) consumption was followed at 240 nm at room temperature for 2 min ($\epsilon_{240} = 43.4 \text{ M}^{-1} \text{ cm}^{-1}$). Alternatively, catalase activity was measured with a cell lysate. H_2O_2 concentrations were plotted on a semi-logarithmic graph against time and the first-order rate constant (catalase activity) was calculated. Titration of catalase activity with digitonin was carried out in a similar manner, but digitonin was added using dimethyl sulfoxide (DMSO) as a vehicle (corrections accounting for the slight inhibition of catalase activity by DMSO at the level added (1% v/v) were considered). Glutathione peroxidase (GPx) activity was measured by studying the kinetics of the enzyme in the whole cell applying a well-established method [10]. The assay mixture contained (final concentrations): 10×10^6 cells/ml, 0.05 M potassium phosphate buffer, pH 7.0, 1 mM DTPA, 50 μM NaN_3 , 1.1 U/ml glutathione reductase, 0.1 mM NADPH, 35 μM H_2O_2 , and 1% (v/v) Triton X-100; the concentration of GSH varied between 0.335 and 3.35 mM. Alternatively, glutathione peroxidase activity was measured on a cell lysate without the addition of Triton X-100 to the assay mixture. All reactants, with the exception of H_2O_2 , were pre-incubated at 37°C for 10 min. NADPH consumption was followed at 340 nm ($\epsilon = 6.2 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) at 37°C until all the hydroperoxide was used, recording the absorbance every 0.1 s. For the kinetic analysis, the part of the curve corresponding to 1.6–16 μM H_2O_2 was used as suggested [10]. Glucose oxidase activity was measured by following O_2 consumption with an oxygen electrode.

3. Results and discussion

3.1. Consumption of H_2O_2 by intact Jurkat T-cells

The consumption of H_2O_2 by intact Jurkat T-cells was examined by two different experimental approaches: (a) exposure of cells to a bolus addition of H_2O_2 and (b) exposure of cells to a continuous flow of H_2O_2 . Neither approach altered cell viability during the experiment(s).

In the former instances, the decay of H_2O_2 concentration after supplementing Jurkat T-cells with 100 μM H_2O_2 fol-

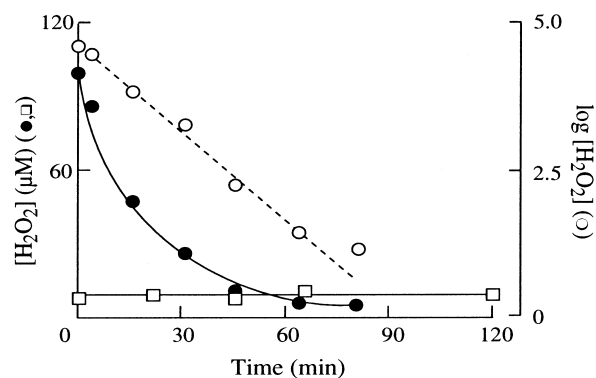


Fig. 2. Intact Jurkat T-cells consume H_2O_2 with a time scale of minutes. The consumption of H_2O_2 was determined by exposing cells to a bolus addition (\bullet) or to a steady state of H_2O_2 (\square). The steady state was initiated by simultaneous addition of H_2O_2 (9 μM) and glucose oxidase (producing 9 nM s^{-1} of H_2O_2). The time course of H_2O_2 concentration obtained after the bolus addition was linearized in a semi-logarithmic plot (\circ). Cells were resuspended to $10^6/\text{ml}$ for 2 h in fresh medium before exposure to H_2O_2 . Other assay conditions as described in Section 2.

lowed first-order kinetics (Fig. 2) with a k_{cell} value of $1.0 \pm 0.1 \times 10^{-3} \text{ s}^{-1}$ per 10^6 cells ($n = 16$). The growth medium (used within a few hours after resuspending cells) did not exhibit significant H_2O_2 consumption (not shown).

In the latter instances, cells were incubated with a low steady state of H_2O_2 (9 μM), by simultaneously exposing them to H_2O_2 and glucose oxidase, an enzyme that reduces O_2 to H_2O_2 during glucose oxidation. Cells were able to maintain the steady state of H_2O_2 up to 2 h (Fig. 2); hence, their capacity to consume H_2O_2 did not decrease with time. The k_{cell} obtained with this steady-state incubation approach was similar, within experimental error, to that obtained with a bolus addition of H_2O_2 and independent of cell density.

It may be surmised that a k_{cell} value of $1 \times 10^{-3} \text{ s}^{-1}$ per 10^6 cells is a reliable measure of the capacity of Jurkat T-cells to consume H_2O_2 , for low to moderate concentrations of H_2O_2 . Furthermore, the similar k_{cell} values obtained with both experimental approaches, suggest that no substantial changes in GSH levels –sufficient to compromise H_2O_2 removal by glutathione peroxidase – occurred following a bolus addition of H_2O_2 to cells.

3.2. Consumption of H_2O_2 by disrupted Jurkat T-cells

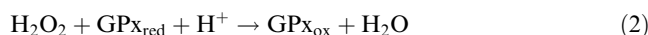
To determine the consumption of H_2O_2 in disrupted Jurkat T-cells, the enzymatic activities that mainly remove H_2O_2 , catalase and glutathione peroxidase were examined.

3.2.1. Catalase activity. In most tissues, but not all, this enzyme is present in peroxisomes. The subcellular location of catalase in Jurkat T-cells was examined by titration with digitonin, a drug that binds to cholesterol present in membranes forming pores; with this approach, membranes with higher levels of cholesterol are disrupted preferentially, thus allowing the study of enzyme compartmentation and latency [11]. Because only one latency threshold to digitonin was observed (Fig. 3), it may be surmised that in Jurkat T-cells all catalase activity is entrapped within one type of membrane, most likely the peroxisomal membrane.

Catalase activity in fully disrupted cells was $1.65 \pm 0.13 \times 10^{-3} \text{ s}^{-1}$ per 10^6 cells ($n = 5$). As with the plasma membrane, the peroxisomal membrane constitutes a barrier to

H₂O₂ diffusion and, thus, it is responsible for the latency of catalase activity. In intact cells, or in cells with only the plasma membrane disrupted, catalase activity represented 35% of the total catalase activity measured in fully disrupted cells (Fig. 3). Accordingly, the overall contribution of catalase to the removal of external H₂O₂ in intact cells was 35% of the value found in disrupted cells: $0.58 \pm 0.05 \times 10^{-3} \text{ s}^{-1}$ per 10^6 cells.

3.2.2. Glutathione peroxidase activity. Evaluation of the contribution of glutathione peroxidase to cellular H₂O₂ removal is more complex, because its reaction mechanism involves an oxidation–reduction cycle of the Se-cysteine moiety at the active center using GSH as the reducing agent [7]:



Glutathione peroxidase activity is usually measured by following the oxidation of GSH under relatively high concentrations of H₂O₂; under these conditions, catalysis is mainly limited by the reduction step (Eqs. 3 and 4, with their respective constants, k_3 and k_4). Conversely, under conditions entailing relatively low H₂O₂ concentrations (e.g. most in vivo conditions or addition of H₂O₂ to intact cells), catalysis is mainly limited by the oxidation step (Eq. 2, with a rate constant k_2) [7]. The integrated approach in Eq. 5 [10] considers both the reductive and oxidative steps, though the latter (comprising Eq. 2 and applying to low [H₂O₂]) is of interest for this study.

$$\frac{[\text{GPx}]_{\text{total}} \times t}{[\text{H}_2\text{O}_2]_0 - [\text{H}_2\text{O}_2]_t} = \phi_2 / [\text{GSH}] + \phi_1 \times \frac{\ln([\text{H}_2\text{O}_2]_0 / [\text{H}_2\text{O}_2]_t)}{[\text{H}_2\text{O}_2]_0 - [\text{H}_2\text{O}_2]_t} \quad (5)$$

where t is time, $[\text{GPx}]_{\text{total}}$ refers to total concentration of glutathione peroxidase, $\phi_1 = 1/k_2$ and $\phi_2 = 1/k_3 + 1/k_4$. The kinetics of glutathione peroxidase in the whole cell homogenate fitted

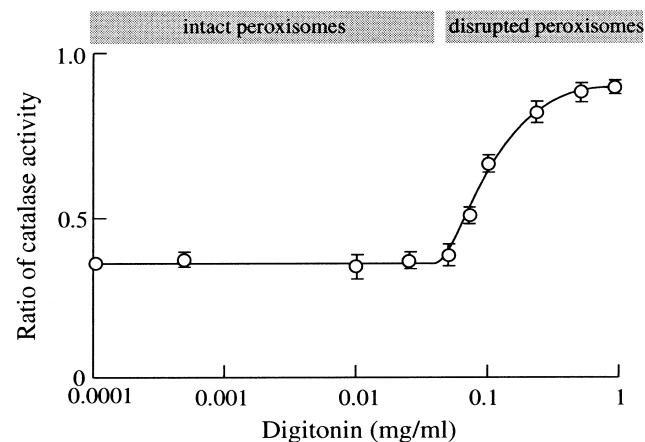


Fig. 3. Titration of catalase activity with digitonin in Jurkat T-cells. The ratio between catalase activity in the presence of digitonin and in fully disrupted cells (0.1% Triton X-100 added) is shown. 10^6 cells were used in each assay. Assay conditions described in Section 2. Median and standard deviations of at least three measurements are shown.

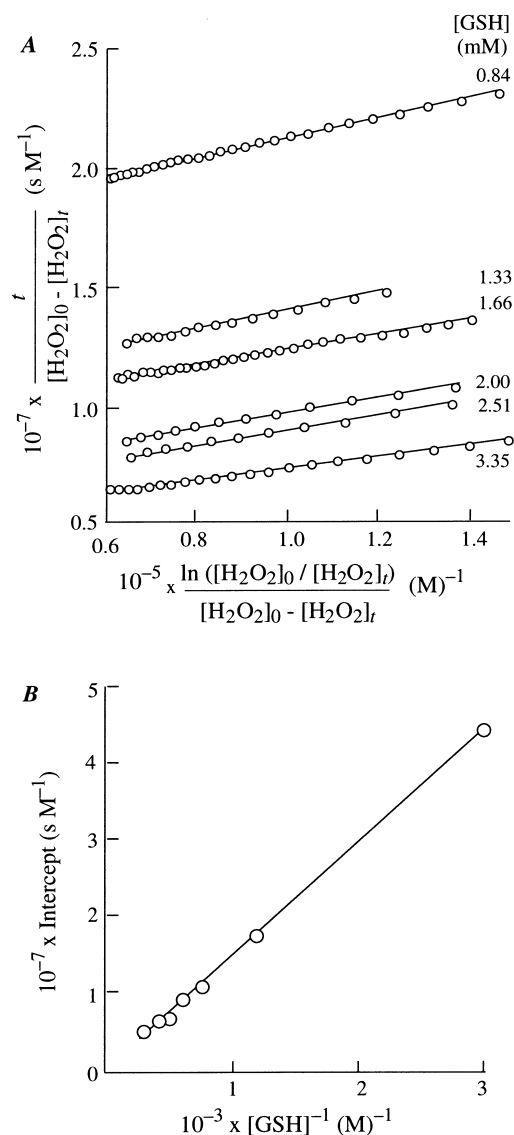


Fig. 4. Kinetic study of glutathione peroxidase activity in Jurkat T-cell homogenates. A: Kinetic analysis was carried out as described in Section 2 and the results were fitted to the integrated rate law of glutathione peroxidase ($n = 6$). B: The intercepts obtained in A were plotted as a function of $[\text{GSH}]^{-1}$ ($n = 7$).

Eq. 5 well (Fig. 4A,B); the following parameters were obtained from Fig. 4A:

$$\begin{aligned} [\text{GPx}]_{\text{total}} / \phi_1 &= k_2 \times [\text{GPx}]_{\text{total}} \\ &= 6.0 \pm 0.1 \times 10^{-3} \text{ s}^{-1} \text{ per } 10^6 \text{ cells} \end{aligned}$$

and from Fig. 4B:

$$[\text{GPx}]_{\text{total}} / \phi_2 = 1.27 \pm 0.02 \times 10^{-5} \text{ s}^{-1} \text{ per } 10^6 \text{ cells.}$$

Because the concentration of glutathione peroxidase in Jurkat T-cells is not known, the kinetic parameters ϕ_1 and ϕ_2 could not be determined¹. However, the ratio ϕ_2/ϕ_1 (reflecting the redox properties of glutathione peroxidase) obtained in

¹ Glutathione peroxidase is a non-saturable enzyme and, therefore, its concentration cannot be calculated from its 'specific activity'.

Jurkat T-cells (4.7×10^2) compares well with those obtained for the rat liver (5.3×10^2) [10] and the bovine erythrocyte enzyme (2.1×10^2) [7]. At low H_2O_2 concentrations, the rate of H_2O_2 consumption by glutathione peroxidase is given by:

$$-d[H_2O_2]/dt = k_2 \times [GPx]_{red} \times [H_2O_2] \quad (6)$$

and, hence, the term $k_2 \times [GPx]_{red}$ gives the pseudo first-order rate constant that characterizes the consumption of H_2O_2 by glutathione peroxidase. Under in vivo conditions, most of the enzyme is in the reduced form [7] (99.8% according to a mathematical model [12]) and, therefore, the term $k_2 \times [GPx]_{total}$ represents a good estimation of the desired parameter ($k_2 \times [GPx]_{red} = 6.0 \pm 0.1 \times 10^{-3} \text{ s}^{-1}$ per 10^6 cells).

Glutathione peroxidase is mainly present in cytosol and mitochondria [13], but also in the nucleus [14]. As mentioned above, glutathione peroxidase requires GSH to complete the catalytic cycle. GSH is not transported into cells [15] and, hence, titration of glutathione peroxidase activity with digitonin is not as useful as for catalase, because the latency of glutathione peroxidase will be determined by both H_2O_2 and GSH. Because most of the glutathione peroxidase activity is present in cytosol, an approximation of the cytosolic activity was inferred by considering the value found in the cell lysate: $6.0 \times 10^{-3} \text{ s}^{-1}$ per 10^6 cells. H_2O_2 consumption values in intact and disrupted Jurkat T-cells are listed in Table 1.

3.3. H_2O_2 catabolism

The information gathered on the activities of catalase and glutathione peroxidase above serves two purposes: on the one hand, it is essential for the calculation of an H_2O_2 gradient and, on the other hand, it makes it possible to characterize the catabolism of H_2O_2 .

The turnover time of H_2O_2 may be calculated from the sum of the pseudo first-order rate constants for H_2O_2 consumption. Assuming that glutathione peroxidase and catalase represent the main H_2O_2 sinks, the capacity of Jurkat T-cells to consume H_2O_2 is $6.6 \times 10^{-3} \text{ s}^{-1}$ per 10^6 cells, which, after conversion to units referred to the cell volume², corresponds to 4.5 s^{-1} , thus yielding a turnover time of 0.2 s (1/4.5) (Table 1). This turnover time is slower than that estimated for hepatocytes ($3 \times 10^{-3} \text{ s}$) [12], an observation that may be explained by the very high activities of glutathione peroxidase and catalase present in the liver. Nevertheless, a turnover time of 0.2 s indicates that when Jurkat T-cells are exposed to extracellular H_2O_2 or to an agent that increases intracellular H_2O_2 production, an intracellular steady state will be reached in a few seconds.

The relative importance of catalase and glutathione peroxidase for the elimination of H_2O_2 can be calculated from the pseudo first-order rate constants obtained for these enzymes. Glutathione peroxidase activity ($6 \times 10^{-3} \text{ s}^{-1}$ per 10^6 cells = 4.1 s^{-1}) is responsible for most of the H_2O_2 consumption in cytosol (91%), whereas catalase ($0.58 \times 10^{-3} \text{ s}^{-1}$ per 10^6 cells = 0.4 s^{-1}) appears to play a minor role (9%), as

Table 1

Parameters of H_2O_2 catabolism and gradients across biomembranes in Jurkat T-cells

Parameters measured	
<i>H₂O₂ consumption by intact cells</i>	
k_{cell} (bolus addition)	$1.0 \times 10^{-3} \text{ s}^{-1}$ per 10^6 cells
k_{cell} (continuous flow)	$1.0 \times 10^{-3} \text{ s}^{-1}$ per 10^6 cells
<i>H₂O₂ consumption by disrupted cells</i>	
Catalase pathway	
disrupted peroxisomal membranes	$1.65 \times 10^{-3} \text{ s}^{-1}$ per 10^6 cells
intact peroxisomal membranes	$0.58 \times 10^{-3} \text{ s}^{-1}$ per 10^6 cells
Glutathione peroxidase pathway	
	$6.0 \times 10^{-3} \text{ s}^{-1}$ per 10^6 cells
Parameters estimated	
<i>H₂O₂ catabolism</i>	
Turnover time	0.2 s
Glutathione peroxidase pathway	4.1 s^{-1} (91%)
Catalase pathway	0.4 s^{-1} (9%)
<i>H₂O₂ profile and gradients</i>	
P_s	$2 \times 10^{-4} \text{ cm s}^{-1}$
Equilibrium $[H_2O_2]_{in}/[H_2O_2]_{out}$	0.9 s
$[H_2O_2]_{cytosol}/[H_2O_2]_{peroxisomes}$	3
$[H_2O_2]_{out}/[H_2O_2]_{in}$	7

also observed in other cell types, such as hepatocytes and endothelial cells [17,18]. Therefore, most of the H_2O_2 added to Jurkat T-cells is probably removed via glutathione peroxidase with the concomitant change of the thiol/disulfide status inside the cell within a few seconds.

3.4. Profile of H_2O_2 in Jurkat T-cells

The ratio between the concentration of H_2O_2 inside (cytosol) and outside the cells (R ; Eq. 1) may be inferred from the consumption of H_2O_2 by intact cells over the sum of activities that consume H_2O_2 in disrupted cells:

$$R = [H_2O_2]_{in}/[H_2O_2]_{out}$$

$$R = k_{cell}/(k_{GPx} + k_{catalase}) =$$

$$1.0 \times 10^{-3}/(6.0 \times 10^{-3} + 0.58 \times 10^{-3})$$

$$R = k_{cell}/(k_{GPx} + k_{catalase}) = 0.15$$

This estimated gradient is a lower limit, because only the glutathione peroxidase and catalase pathways were considered for H_2O_2 removal; if other enzymes consume H_2O_2 , the gradient will be steeper.

To determine how fast the gradient is established, the permeability coefficient (P_s) of the plasma membrane of Jurkat T-cells for H_2O_2 must be obtained. A P_s value of $2 \times 10^{-4} \text{ cm s}^{-1}$ may be estimated from Eq. 7 [8]:

$$P_s = \frac{k_3 R}{\frac{A}{V_{in}}(1-R)} \quad (7)$$

(where A is the area of the plasma membrane, V_{in} is the intracellular volume, k_3 is the pseudo first-order rate constant for H_2O_2 consumption) and by considering that (a) Jurkat T-cells are spheres with an area of $627 \pm 21 \mu\text{m}^2$ [16], (b) a gradient R

² Assuming that the average volume of a Jurkat T-cell (clone E6.1) is $627 \pm 21 \mu\text{m}^2$ (estimated by electrophysiological studies [16]), the ratio between the volume of cells in the reaction mixture and the total volume in the reaction mixture (1 ml in these experiments) is estimated to be 1.5×10^{-3} .

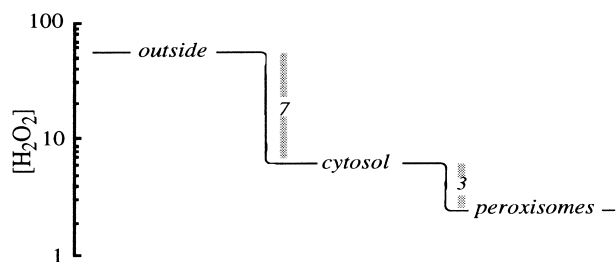


Fig. 5. Profile of H_2O_2 concentration in Jurkat T-cells upon incubation with an external source of H_2O_2 . $[\text{H}_2\text{O}_2]$ log scale axis has arbitrary units.

of 0.15, and (c) a k_3 of 4.5 s^{-1} . This P_s is smaller than that obtained for erythrocytes [8] ($6 \times 10^{-4} \text{ cm s}^{-1}$), which is expected in view of the higher water permeability of erythrocytes compared with other cell types [19]; in particular, erythrocytes show a P_s for water that is more than one order of magnitude higher than those of lymphocytes [20]. For a spherule of the size of a Jurkat T-cell, a P_s of $2 \times 10^{-4} \text{ cm s}^{-1}$ indicates that the time scale for the equilibration with H_2O_2 outside the cell is 0.9 s (Table 1). Therefore, when Jurkat T-cells are exposed to an external source of H_2O_2 , the intracellular components sense the presence of H_2O_2 within 1 s. This fast diffusion of H_2O_2 is important for the postulated signaling roles that have been attributed to this molecule.

In addition to the gradient across the plasma membrane, a gradient is expected across the membrane of cellular organelles that consume significant amounts of H_2O_2 , like peroxisomes [5] (which contain catalase), nucleus [14], mitochondria [13], and endoplasmic reticulum [13] (all of which contain glutathione peroxidase). For peroxisomes, results shown in Fig. 3 allow the estimation of the gradient across the peroxisomal membrane: in fact, the ratio measured between catalase activities in intact and in lysed peroxisomes was 0.35, which, according to Eq. 1, is the gradient between cytosolic and peroxisomal H_2O_2 concentrations upon incubation with an external source of H_2O_2 . H_2O_2 gradients were not estimated for organelles containing glutathione peroxidase, but upon exposure to an external source of H_2O_2 , the concentration of H_2O_2 in these organelles may be anticipated to be lower than in the cytosol. In organelles like lysosomes, that do not contain H_2O_2 -consuming enzymes, a concentration similar to that in cytosol may be expected. Fig. 5 shows the H_2O_2 profile that is expected to form in Jurkat T-cells upon incubation with an external source of H_2O_2 . Because H_2O_2 permeation and catabolism are fast, this profile will form, within a few seconds, whether the source is a steady state or a bolus addition.

4. Concluding remarks

When cells are exposed to external sources of H_2O_2 , gradients across cell membranes are established, with a magnitude that depends on the intensity of the intracellular consumption of H_2O_2 and on the permeability characteristics of the membrane to H_2O_2 . Once the profile of H_2O_2 concentration is known, the actual intracellular concentration of H_2O_2 that triggers the process under study can be easily estimated, which is useful for several reasons. First, the physiological relevance of the observations obtained can be discussed on

a basis of comparing the concentration that was found to trigger the process in vitro with the concentration of H_2O_2 in vivo. For example, in Jurkat T-cells, a bolus addition of $50 \mu\text{M}$ of H_2O_2 is sufficient to induce apoptosis [21]. According to the results presented in this work, the maximal intracellular concentration of H_2O_2 reached inside the cells was around $7 \mu\text{M}$, and in organelles like mitochondria it was probably around $2 \mu\text{M}$. These values are much more likely to be reached in vivo than the $50 \mu\text{M}$ added, thereby supporting the physiological relevance of the findings. Second, different cell types, with distinct properties in terms of H_2O_2 -consuming enzymes and of permeability to H_2O_2 , form different gradients and consequently, the knowledge of the actual intracellular concentrations of H_2O_2 that elicit the same process in the various cell types helps to compare results between cell lines. It may be concluded that the procedure presented in this work provides a quantitative framework to interpret the data obtained upon incubation of cells with an external source of H_2O_2 .

The data presented here provide further understanding on the bactericidal and bacteriostatic activities of H_2O_2 in body fluids [22–25]: on the one hand, bacteria – being small organisms – have a large ratio between cellular area and volume and, hence, the gradient established between extra- and intracellular concentrations of H_2O_2 is small or negligible [24]; on the other hand, the larger host cells would be subjected to a gradient as described in this work. As a corollary, for the same extracellular concentration of H_2O_2 , the intracellular medium of bacteria will be subjected to a higher concentration of H_2O_2 than that of host cells, thus contributing to a selective toxicity of the peroxide towards pathogens.

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