Kinetic Studies on the Removal of Extracellular Hydrogen Peroxide by Cultured Fibroblasts*

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To investigate the function of antioxidant enzymes in intact cells, we examined the removal of extracellular H2O2 by cultured fibroblasts (IMR-90). H2O2 concentration dependence of the reaction rate was interpreted as that the process involves two kinetically different reactions (referred to as reactions 1 and 2). Reaction 1 was characterized by a relatively low Ka value (about 40 μM), and reaction 2 by linear dependence of the rate up to 500 μM H2O2. The magnitude of reaction 1 was reduced by treatment of the cells with diethyl maleate or 6-aminonicotinamide, while reaction 2 was inhibited by 3-amino-1,2,4-triazole treatment. It was concluded that reactions 1 and 2 are principally due to GSH peroxidase and catalase, respectively.

The values of kinetic parameters were estimated by curve-fitting, and it was inferred that 80 to 90% of H2O2 is decomposed by GSH peroxidase at H2O2 concentrations lower than 10 μM. The contribution of catalase increases with the increase in H2O2 concentration. The intact cells showed a low catalase activity (about 15%), as compared with the activity found in the solubilized cells. The low catalase activity was ascribed to the latency of the enzyme caused by localization in peroxisomes.

Fibroblasts also removed intracellular H2O2 generated by menadione. Treatment with diethyl maleate greatly impaired the H2O2-removing capability and caused H2O2 efflux into the medium.

In living cells, H2O2 is mainly generated from mitochondria, microsomes, and peroxisomes (1, 2). Peroxisomes contain H2O2-producing oxidases, while mitochondria and microsomes produce the superoxide anion as a by-product of the O2 reduction, and the anion quickly dismutates to H2O2 and O2 spontaneously or by the action of superoxide dismutase. H2O2 itself is not very reactive with cellular constituents, but in the presence of transition metal ions and appropriate reductants it is converted to the hydroxyl radical which is highly reactive with organic compounds and is hazardous to living cells. In this relevance H2O2 is regarded as a key substance in the oxygen toxicity, and its elimination is important for the cell.

GSH peroxidase and catalase are involved in the decomposition of H2O2, but they are differently localized in the cell. GSH peroxidase activity is found in cytoplasm and mitochondria (3), whereas catalase is localized in peroxisomes and lower density particles, and very little enzyme is free in the cytoplasm (4). The two enzymes are also different in kinetic behavior. Catalase shows a very high Ka value for H2O2, and its reaction apparently follows the first-order kinetics, but GSH peroxidase shows a relatively low Ka value, obeying the Michaelis-Menten kinetics. On the basis of their kinetics, it is believed that in intact cells GSH peroxidase is more effective at relatively low H2O2 concentrations, while catalase is more effective at high concentrations, as exemplified by the studies on erythrocytes (5-7) and hepatocytes (8).

The reduction of H2O2 by GSH peroxidase is accompanied by the oxidation of GSH, which is restored by GSSG reductase using NADPH as a reactant. The supply of NADPH is generally rate-limiting in the reaction sequence, and it is mainly produced by the pentose phosphate pathway and cytosolic isocitrate dehydrogenase. Kauffman et al. (9) have shown that the latter enzyme supplies the majority of NADPH in hepatocytes.

Although plenty of qualitative knowledge has been accumulated on the H2O2-eliminating enzymes, we still do not understand quantitatively their behavior in the living cells, principally because of the difficulty in determining the intracellular H2O2. In addition, hepatocytes have been used frequently in studies on the oxidative stress and the related enzyme systems, but the properties of other cells are not well understood.

In this study we evaluated the activity of cultured human fibroblasts (IMR-90) in removing extracellular H2O2 and analyzed the reaction in terms of the enzyme kinetics. To distinguish the actions of the H2O2-eliminating enzymes, the effects of several inhibitors (AT, DEM, and 6-AN) were examined. AT inactivates catalase irreversibly, only when the enzyme is functioning (10). DEM reacts with GSH irreversibly, depleting it from the cells. 6-AN, a nicotinamide derivative, is metabolized to the 6-amino derivative of NADP, which is a strong inhibitor of 6-phosphogluconate dehydrogenase and blocks the pentose phosphate pathway (11).

In this study we also used menadione to generate H2O2. Quinone derivatives including menadione are reduced by NADH- or NADPH-linked oxidoreductases and form H2O2 via superoxide anion (12-14). It can be used to estimate the capability of the cells to decompose intracellularly generated H2O2.

EXPERIMENTAL PROCEDURES

Reagents and Chemicals—Catalase (C-30), GSSG reductase, NADPH, GSH, 6-aminonicotinamide (6-AN), and menadione (menadione sodium bisulfite or 2-methyl-1,4-naphthoquinone sodium bisulfite) were purchased from Sigma. Bis(2,4,6-trichlorophenyl)oxalate, perylene, and 3-amino-1,2,4-triazole (AT) were purchased from Tokyo Kasei (Tokyo). Diethyl maleate (DEM) was obtained from Wako (Osaka). DEM was dissolved in 40% ethanol, and other reagents were dissolved in PBS without dialyzer cations. Menadione sodium bisulfite was dissolved in PBS immediately before use.

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1 The abbreviations used are: AT, 3-amino-1,2,4-triazole; DEM, diethyl maleate; 6-AN, 6-aminonicotinamide; PBS, phosphate-buffered saline.
Cell Culture—Human fibroblasts, strain IMR-90, were grown in Eagle's basal medium (Life Technologies Inc.) supplemented with 10% fetal bovine serum (Whittaker). They were subcultivated every 3 or 4 days with the split ratio of 1:2. For the experiments, the cells between the 30th and 50th passages were used. They were plated in dishes with 60-mm diameter (2-4 x 10^6 cells per dish) and were incubated for 2 days before the experiments.

When the effects of inhibitors were examined, the cells were treated with 10 mM AT (for 6 h), 1 mM DEM (for 3 h), or 0.5 mM AN (for 5 h) before the experiments. The cell viability was tested by exclusion of nigrosin (0.05% in PBS). No change in the viability was observed after the treatment with inhibitors.

Measurement of H2O2 Removal by Fibroblasts—The cells grown in a 60-mm dish were rinsed three times with warmed PBS (140 mM NaCl, 2.7 mM KCl, 0.9 mM CaCl2, 0.5 mM MgCl2, and 10 mM glucose at pH 7.4) plus 5.6 mM glucose. The dish was placed in a small plastic box, of which temperature was maintained at 37°C in a water bath. For the measurement of removal rate, 5 ml of PBS + glucose containing an appropriate concentration of H2O2 (2-500 µM, warmed at 37°C) was added to the dish, and the dish was shaken at 100 cycles/min. At appropriate time intervals (1 to 3 min), 50-µl portions of the medium were withdrawn and were subjected to the H2O2 determination, as described below. No change in the cell viability was observed after incubation with 500 µM H2O2 for 10 min, as tested by the nigrosin exclusion.

Determination of H2O2—The method of Williams et al. (15) using the peroxoxyate luminescence was applied with some modifications. A vial (17 mm in diameter) contained 60 µl of water, 90 µl of PBS, and 200 µl of 0.005% (w/v) triethylamine solution in methanol, to which 50 µl of a test solution was added. The vial was placed in a chemiluminescence detector (Aloka BLR-201), and the reaction was started by addition of 200 µl of ethyl acetate containing 0.06% bis(2,4,6-trichlorophenyl) oxalate and 0.005% perylene. The intensity of luminescence was integrated between 10 and 20 s after the reaction was started. H2O2 was quantified by comparison with the results for standard H2O2 solutions. The method gave linear response between 0.1 nmol and 0.1 µmol of H2O2. The concentration of standard H2O2 solution was determined from the absorbance at 230 nm and the extinction coefficient of 61 cm^-1. 

Calculation of H2O2 Removal Rate—Time courses of H2O2 removal by fibroblasts were measured as described above, and natural logarithms of H2O2 concentrations were plotted against the reaction time. The pseudo first-order rate constant k' (min^-1) was obtained from the slope of the linear portion of the plots by the least-square method and was converted to the reaction rate u (nmol/min/mg of protein) as:

\[ u = k' (H_2O_2)_o \] (Eq. 1)

where (H2O2)o is the H2O2 concentration at the midpoint of the measurement. In the absence of inhibitors, the plot gave a straight line after 1 min of reaction (cf. Fig. 2), and k' was calculated from the data obtained between 1 and 10 min. When using the cells treated with the inhibitors, one had to wait for 3-4 min before the reaction reached a steady-state. Usually, 6-10 data points obtained in the initial part of the reaction (less than 30% of the total) were used for the calculation.

Assay for GSH Peroxidase Activity and Total Glutathione—Fibroblasts cultured in 60-mm dishes were solubilized with 0.5% Triton X-100 and 4 mM EDTA in 0.1 mM phosphate buffer (pH 7.4). Samples from two dishes were combined and were subjected to assays for GSH peroxidase activity and total glutathione content. The GSH peroxidase activity was measured by the method of Awasthi et al. (16) with some modifications. A 2-ml portion of the solubilized cell, containing 0.2 mg of protein, was added to 2.7 ml of 4 mM EDTA, and 4 mM sodium azide in 0.1 M phosphate buffer at pH 7.4, plus 0.2 mg of protein, the reaction was started by addition of 2.4 mM H2O2 (50 µl), and the absorbance change of NADPH at 340 nm was followed at 37°C. Under the assay conditions, the rate of the non-enzymatic reaction was considered to be negligible (less than 30% of the total) and was used for the calculation. The activity was expressed as nanomoles of GSH peroxidase activity and total glutathione concentration.

Superoxide Dismutase Activity—Fibroblasts were treated with 0.5 µg of menadione and were incubated for 6-10 h. The cells were then solubilized with 0.5% Triton X-100 and 4 mM EDTA in 0.1 M phosphate buffer (pH 7.4) plus 5.6 mM glucose. The dish was placed in a small plastic box, of which temperature was maintained at 37°C in a water bath. For the measurement of removal rate, 5 ml of PBS + glucose containing an appropriate concentration of H2O2 (2-500 µM, warmed at 37°C) was added to the dish, and the dish was shaken at 100 cycles/min. At appropriate time intervals (1 to 3 min), 50-µl portions of the medium were withdrawn and were subjected to the SOD determination, as described below. No change in the cell viability was observed after incubation with 500 µM H2O2 for 10 min, as tested by the nigrosin exclusion.

Determination of SOD—The method of Tietze (17) using the peroxoxyate luminescence was applied with some modifications. A 50-pl portion of 0.005% (v/v) triethylamine solution in methanol, to which 50 µl of a test solution was added, was placed in a chemiluminescence detector (Aloka BLR-201), and the reaction was started by addition of 200 µl of ethyl acetate containing 0.06% bis(2,4,6-trichlorophenyl) oxalate and 0.005% perylene. The intensity of luminescence was integrated between 10 and 20 s after the reaction was started. Superoxide dismutase was quantified by comparison with the results for standard SOD solutions. The method gave linear response between 0.1 and 0.1 µmol of SOD. The concentration of standard SOD solution was determined from the absorbance at 230 nm and the extinction coefficient of 61 cm^-1.

Calculation of SOD Activity—Time courses of SOD removal by fibroblasts were measured as described above, and natural logarithms of SOD concentrations were plotted against the reaction time. The pseudo first-order rate constant k (min^-1) was obtained from the slope of the linear portion of the plots by the least-square method and was converted to the reaction rate v (nmol/min/mg of protein) as:

\[ v = k (SOD)_o \] (Eq. 2)

where (SOD)o is the SOD concentration at the midpoint of the measurement. In the absence of inhibitors, the plot gave a straight line after 1 min of reaction (cf. Fig. 2), and k was calculated from the data obtained between 1 and 10 min. When using the cells treated with the inhibitors, one had to wait for 3-4 min before the reaction reached a steady-state. Usually, 6-10 data points obtained in the initial part of the reaction (less than 30% of the total) were used for the calculation.

Results

Permeability and Diffusibility of H2O2 in the Cells—In this work, the H2O2 removal activity of fibroblasts was measured by determination of extracellular H2O2, and it is important whether H2O2 diffuses into the cell rapidly enough. The fibroblasts attach to the surface of a culture plate and spread out forming a thin monolayer, of which thickness is estimated as small as 1 µm or less. Unfortunately, the diffusion coefficient of H2O2 in the cell membrane is unknown, but its diffusion behavior is thought to be similar to that of O2 in cellular environments. The diffusion coefficients of O2 in the cell membrane and matrix have been reported to be 4-15 x 10^-6 cm^2 s^-1 (20, 21). The mean diffusion distance (d) in time t for particles with a diffusion coefficient of D is given as d = 2√Dt, and the mean time required for O2 (or H2O2) to diffuse for 1 µm is calculated to be less than 0.4 ms. Thus, it is thought that H2O2 diffuses into the cell quickly enough in the time span of the experiments.

Removal of Extracellular H2O2 by Fibroblasts—Fibroblasts decomposed extracellular H2O2 considerably rapidly (Fig. 1). Fig. 2 shows semilogarithmic plots of the reaction at different H2O2 concentrations. The plots gave straight lines between 1 and 30 min after the start of the reaction. The half-time of the

\[ H_2O_2 \text{ Removal by Fibroblasts} \]
reaction was 20–30 min at 10–100 μM H₂O₂ under the experimental conditions (with 5 ml of medium and 0.11–0.17 mg of protein), and it became longer with an increase in the H₂O₂ concentration. At a low H₂O₂ concentration (3 μM), the plot deviated from the straight line, indicating a deviation from the first-order kinetics.

As described under "Experimental Procedures," the removal rate was calculated from the initial part of the reaction (less than 30% of the total) and was plotted against the H₂O₂ concentration (Fig. 3). The rate of H₂O₂ removal was proportional to the cell density, showing that the activity is apparently independent of the stage of proliferation.

Fig. 4 shows the concentration dependence of the H₂O₂ removal reaction. The plot was biphasic with respect to H₂O₂ concentration; the slope at lower concentrations was definitely higher than that observed at higher concentrations.

The Effects of Inhibitors—Fig. 5 shows the effect of pretreatment of the cells with a catalase inhibitor, AT. The slope of the plot at higher H₂O₂ concentrations was considerably decreased, whereas the reaction at lower concentrations was much less influenced.

Fig. 6 shows the effect of DEM treatment. In contrast to the results for the AT-treated cells (Fig. 5), the slope at lower H₂O₂ concentration was definitely reduced, whereas the reaction at higher concentrations was not appreciably affected.

Table I and II show the results of enzyme and glutathione assays of solubilized cells. As shown in Table I, AT reduced the catalase activity to 1/8 of the original value, but DEM showed no appreciable effects. As shown in Table II, DEM treatment decreased the glutathione content to 5% of the control value, but AT showed no effect. GSH peroxidase activity was not affected by either treatment.

Fig. 7 shows the effect of 6-AN on the H₂O₂ removal reaction. It reduced the reaction rate at lower H₂O₂ concentrations, but little affected the slope of the plot at higher concentrations. The effect of 6-AN was similar to that of DEM, although the extent was significantly less.

Curve-fitting Study—From the results shown in Figs. 4–7, it is presumed that two different types of kinetics are involved in the H₂O₂ removal reaction. One is of the Michaelis-Menten type with a relatively low Kₘ value, and its rate is close to the saturation at 100 μM H₂O₂. The other is characterized by a large Kₘ value, and the rate increases linearly up to 500 μM.

**Fig. 2.** Semilogarithmic plots of the extracellular H₂O₂ removal reaction at a different initial H₂O₂ concentration. Cell protein: 0.12 to 0.13 mg.

**Fig. 3.** The rate of extracellular H₂O₂ removal by fibroblasts as a function of cell density. IMR-90 was plated at different densities, and the H₂O₂ removal activity was measured after 2 days. Initial [H₂O₂] = 100 μM (○) and 10 μM (○).

**Fig. 4.** The rate of extracellular H₂O₂ removal by fibroblasts as a function of H₂O₂ concentration. The data (○) were obtained at cell densities ranging from 0.15 to 0.35 mg as protein. The solid lines show the fitted curve on the basis of Equations 3–5. The fitted parameter values are given in Table III. The broken lines show the rate of reaction 2, which is proportional to the H₂O₂ concentration. In panels A and B, the same data are shown in different scales.

On the basis of this idea, the reaction rate (v) can be expressed as a sum of two different reactions (1 and 2) as follows:

\[ v = v_1 + v_2 \]  \hspace{1cm} \text{(Eq. 3)}

where

\[ v_1 = \frac{V_{\text{max}}}{K_m + c} \]  \hspace{1cm} \text{(Eq. 4)}

\[ v_2 = k \cdot c \]  \hspace{1cm} \text{(Eq. 5)}

v₁ and v₂ represent the rates of reactions 1 and 2, which follow the Michaelis-Menten and first-order kinetics, respectively. c is the H₂O₂ concentration. Vₘₜ₉ and Kₘ are the maximum velocity and the Michaelis constant for reaction 1, respectively, and k is the first-order rate constant for reaction 2.

Equation 3 (together with Equations 4 and 5) was fitted to the H₂O₂ removal data by the nonlinear least-square method. As seen in Fig. 4, the fitting was satisfactory. Table III shows the estimated parameter values, together with the 70% confidence limits, which roughly correspond to the range for ±1 S.D. in the normal distribution.

From the kinetic properties and the effects of inhibitors, it is
presumed that reactions 1 and 2 are attributable to GSH peroxidase and catalase, respectively. On this assumption, we tried to calculate the H\textsubscript{2}O\textsubscript{2} removal rate by the AT- and DEM-treated cells from Equations 3–5 as a function of H\textsubscript{2}O\textsubscript{2} concentration. The values of kinetic parameters were estimated from the glutathione contents and enzyme activities of the AT-treated cells (Table I) and the values of the kinetic parameters for the untreated cells (Table III), as described under "Results."
peroxidase roughly equals 0.8–0.9 but decreases with increases in the H$_2$O$_2$ concentration. Rates of the two reactions are comparable between 0.1 and 1 mM H$_2$O$_2$.

Removal of H$_2$O$_2$ Generated by Menadione—Fig. 9A shows the H$_2$O$_2$ generation observed on addition of menadione to the cells. In the control cells, the H$_2$O$_2$ concentration reached a plateau in 20 to 30 min. Presumably, a steady-state was reached between the H$_2$O$_2$ generation and decomposition. The extracellular H$_2$O$_2$ concentration was less than 1 µM under the experimental conditions. As shown in Fig. 9A, pretreatment of the cells with AT or 6-AN exerted no appreciable effects. This is interpreted as that catalase does not contribute much to the H$_2$O$_2$ removal at the low H$_2$O$_2$ concentration and that 6-AN does not completely inhibit the NADPH supply. In the DEM-treated cells, on the other hand, a considerable increase in the H$_2$O$_2$ generation was observed (Fig. 9B), indicating that DEM greatly impaired the H$_2$O$_2$-removing capability of the cell. The rate of H$_2$O$_2$ generation increased with time and, after 20 min, reached 1.5 and 3.5 nmol/min/mg of protein at, respectively, 0.1 and 0.2 mM menadione. The results confirm that the GSH-dependent system is more important in removing H$_2$O$_2$ at a low generation rate.

**DISCUSSION**

The Kinetics of H$_2$O$_2$ Removal by Fibroblasts—In the present study we analyzed quantitatively the H$_2$O$_2$-removing activity of fibroblasts by determining the rate of H$_2$O$_2$ removal in the extracellular medium. The kinetic measurement was made possible by the chemiluminescence method, which allowed quick and sensitive determination of H$_2$O$_2$. In principle, the present technique is applicable to other any other adhesive cells, but in the case of thicker cells the rate of H$_2$O$_2$ diffusion may have to be considered in kinetic analyses.

Contributions of the Enzymes to H$_2$O$_2$ Elimination—The present results indicate that the removal of extracellular H$_2$O$_2$ by cultured fibroblasts involves two reactions showing different H$_2$O$_2$ concentration dependences. From the effect of DEM (Fig. 6 and Table III), it is thought that reaction 1 is mostly due to GSH peroxidase. The nonenzymic reaction between GSH and H$_2$O$_2$ is negligibly slow at physiological pH (5). Reaction 2 was effectively inhibited by AT (Fig. 5 and Table III), and it is mostly attributable to catalase. Nonenzymic catalysts such as transition metal ions may participate in the reaction, but their contribution is estimated to be small, because their concentrations are low, and because the specific activity of catalase is extremely high.

It is generally believed that at low H$_2$O$_2$ concentration GSH peroxidase is more effective in removing H$_2$O$_2$, but the contribution of catalase increases as the H$_2$O$_2$ concentration increases. The idea was confirmed quantitatively by the present study, as shown in Fig. 8. It was calculated that, at H$_2$O$_2$ concentrations lower than 10 µM, 80–90% of H$_2$O$_2$ is decomposed by GSH peroxidase (or reaction 1), and its contribution is lower at higher H$_2$O$_2$ concentrations. Under normal conditions, the intracellular H$_2$O$_2$ concentration is low, and it is thought that GSH peroxidase is the primary enzyme in removing H$_2$O$_2$.

Latency of Catalase Activity—The k value of reaction 2 for the untreated cells was about 0.2 nmol/min/mg of protein/µM concentration (Table III), where the unit (nmol/min/mg of protein/µM concentration) is equivalent to ml/min/mg of protein. On the other hand, the catalytic activity of the solubilized cell was 0.485/min/mg at 10 mM H$_2$O$_2$ (Table I). Because the volume of the test solution was 3 ml, the activity is equivalent to the k value of 1.38 ml/min/mg. On the assumption that the rate of the reaction is proportional to H$_2$O$_2$ concentrations up to 10 mM, it is calculated that only 15% (8–20% for 70% confidence range) of the total catalytic activity was effective in removing the extracellular H$_2$O$_2$. In intact cells, catalase is confined in peroxisomes and smaller structures (4), and isolated peroxisomes show only 10–15% of the total activities (23, 24). It is thought that the low enzyme activity is due to the localization of the enzyme.

The Michaelis Constant of GSH Peroxidase—According to Flohé et al. (22), the apparent $K_m$ for H$_2$O$_2$ of the enzyme is expressed as a function of GSH concentration, as follows:

$$K_m(H_2O_2) = (4.4 \times 10^{-2}) \times [GSH] \text{ (pH 7.0)} \quad (\text{Eq. 6})$$

$$K_m(H_2O_2) = (1.13 \times 10^{-2}) \times [GSH] \text{ (pH 7.7)} \quad (\text{Eq. 7})$$

The above coefficient values have been obtained for bovine GSH peroxidase, but they would give a good approximation for human enzyme. The total glutathione content of fibroblasts
was about 39 nmol/mg of protein (as GSH), as shown in Table II. Since the protein content of fibroblasts is about 0.2 mg/µl H₂O₂, the GSH concentration is calculated to be 7.8 µM. Assuming that glutathione is mostly reduced in the cell, one obtains the Kₐ values of 34 and 88 µM from Equations 6 and 7, respectively. At physiological pH (about 7.4), the value should lie in between. If the amount of GSSG is not negligible, the Kₐ values would be smaller. Therefore, the Kₐ (H₂O₂) value estimated in the present study (38.5 µM, shown in Table III) is in good agreement with the calculated values.

The Rate of NADPH Supply—It is generally thought that the NADPH supply is rate-limiting in the H₂O₂-removing system using GSH. This seems to be true also in fibroblasts, because the estimated Vₘₐₓ value (Table III) was smaller than the GSH peroxidase activity in the solubilized cells (Table II).

The NADPH supply rate in hepatocytes has been estimated to be 5–8 nmol/min/10⁶ cells (3–5 nmol/min/mg of protein) from the rate of NADPH-consuming reactions (25, 26) or from the rate of GSSG reduction (27). On the other hand, Sies and Summer (28) have reported a much higher activity (3–4 nmol/min/mg of liver, approximately equivalent to 20–25 nmol/min/mg of protein) from the removal rate of an organic hydroperoxide in hepatocytes. The Vₘₐₓ value of reaction 1 determined in the present study (about 39 nmol/min/mg) was relatively close to that obtained by Sies and Summer (28) but considerably greater than the other values. The discrepancy may arise from the difference in the experimental methods. It has been reported that glucose-6-phosphate dehydrogenase is inhibited by NADPH, and the inhibition is counteracted by GSSG (29). As a result, the pentose phosphate pathway is stimulated under the oxidative stress (30). It is thus probable that, in the experiments using high concentrations of organic hydroperoxide or H₂O₂, a higher NADPH supply rate is observed as a result of the NADPH and GSH oxidation. A reported value for the activity of GSSG reductase in fibroblasts is high enough (36 nmol/min/mg of protein) (31) to support the H₂O₂ removal rate observed here.

The 6-AN treatment caused a decrease in GSH peroxidase activity to 30% (Fig. 7). It has been reported for fibroblasts that 6-AN decreases the activity of the pentose phosphate pathway to 14% of the original value (11), but the H₂O₂ elimination was inhibited to a significantly less extent. The results suggest that the pentose phosphate pathway supplies the majority of NADPH in fibroblasts and also suggest the presence of another NADPH source, such as isocitrate dehydrogenase (9, 32).

Removal of Intracellularly Generated H₂O₂—In this study we also examined the H₂O₂ removal activity of fibroblasts by using menadione. The method is rather qualitative but allows us to estimate the action of the H₂O₂-removing enzymes on intracellularly generated H₂O₂. Menadione is reduced by intracellular enzymes to the semiquinone radical or hydroquinone (12–14). Since the semiquinone reacts very rapidly with O₂ (33), it would form the superoxide anion (and H₂O₂) mostly before leaking out of the cell, although the possibility remains that a part of H₂O₂ is generated in the cell membrane, as recently reported for yeast (34). The present results conform to those obtained from the experiments with extracellularly added H₂O₂, showing that H₂O₂ is eliminated principally by GSH peroxidase at a low H₂O₂ generation rate. It is suggested that the intracellular H₂O₂ production and its metabolism can be studied by the present method.

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