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Factors Affecting Catalase Level and Sensitivity to Hydrogen Peroxide in *Escherichia coli*

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Composition of the culture medium, growth phase, and temperature play important roles in the sensitivity of *Escherichia coli* to H_2O_2. The medium and growth phase affected the sensitivity of the cells to H_2O_2 by modifying the amount of catalase synthesized by them, whereas the effect of temperature was due to the thermolability of the enzyme. Since catalase is unstable in the presence of its substrate, the correlation between the catalase level in the cells and their sensitivity to H_2O_2 could be observed only when the H_2O_2 concentration was not excessive in proportion to the amount of catalase.

Hydrogen peroxide is a well known bactericidal agent that has been used extensively in medicine and sanitation. It is one of the components of antimicrobial and antifungal systems in leukocytes, saliva, and milk (16, 17, 19), and the virulence of *Mycoplasma pneumoniae* is partly attributed to the excretion of H_2O_2 into the tissues of the respiratory system (9). Since H_2O_2 accumulates in the cells during aerobic respiration, the cells dispose of it through peroxidases, mainly catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6). In microorganisms lacking catalase, such as pneumococci (2) and *M. pneumoniae* (18), H_2O_2 accumulates and arrests growth of the cultures.

Some authors (1) found correlation between the catalase content of the cells and their sensitivity or resistance to H_2O_2, whereas others did not (5, 11). The purpose of this work was to examine the catalase level in *Escherichia coli* K-12 as affected by growth conditions and its role in protecting the cells against H_2O_2.

MATERIALS AND METHODS

Bacterial strains. *E. coli* K-12 and *Micrococcus lysodeikticus* were obtained from the Institute of Microbiology, Hebrew University-Hadassah Medical School, Jerusalem.

Culture media. Nutrient broth, nutrient agar, and MacConkey agar were purchased from Difco Laboratories, Detroit, Mich. Mineral medium supplemented with glucose (M-9 medium) consisted of: (i) 0.7% (wt/vol) Na_2HPO_4, 0.3% KH_2PO_4, and 0.1% NH_4Cl (Merck, Darmstadt, Germany); (ii) 1 M $MgSO_4$ (Merck); (iii) 10^{-3} M $FeCl_3$ from BDH (The British Drug Houses Ltd., Poole, England), each of them autoclaved separately; and (iv) 4% glucose sterilized by filtration through a Seitz filter. Before inoculation, 9 parts of solution (i) was mixed aseptically with 1 part of the glucose solution, and solutions (ii) and (iii) were added to give a final concentration of 10^{-3} M $MgSO_4$ and 10^{-6} M $FeCl_3$.

Growth conditions. Erlenmeyer flasks (250 and 500 ml) with side arms, containing 50 and 100 ml of medium, respectively, were inoculated with cells grown on nutrient agar or M-9 medium adjusted to give a final density of 10^7 to 10^8 cells/ml. They were incubated in a water bath with shaking (100 strokes/min) at 37 or 20°C.

Kill experiments. Cells were harvested in the exponential or stationary phase by centrifugation at 2°C, washed once in 0.01 M phosphate buffer (pH 7), and suspended in M-9 medium. H_2O_2 was added to the desired concentration.

Turbidity. Turbidity was measured in a Klett-Summerson photoelectric colorimeter with filter no. 42 (420 nm).

Viable counts. Several dilutions of the samples were plated on nutrient agar and MacConkey agar, and colonies were counted after 24 h incubation at 35°C. Samples of cultures containing H_2O_2 were treated with 1 ml of a 1,000-fold dilution of bovine liver catalase (BDH; 150,000 U) before diluting and plating.

Determination of dry weight. Portions (100 ml) washed cell suspensions at a turbidity of 400 Klett units were evaporated and dried at 105°C to constant weight (1.07 mg). Enzyme activity was determined at a turbidity of 400 Klett units and calculated to 1 mg (dry weight).

Sonication. Sonication was performed in a Branson sonifier at 1.5 A for 3 min, with a layer of ice placed around the test tubes.

Toluenization. Cell suspensions were shaken with 1% toluene at 37°C for 5 min, and the enzymatic activity was determined. Ten minutes of shaking with 2% toluene at 37°C gave the same results.

Storage and determination of H_2O_2. Perhydrol (30%, wt/vol) (pro analysis, Merck) was diluted to 3% in 0.01 M phosphate buffer (pH 7), and further

dilutions were prepared from this stock solution. All dilutions were kept at 10°C.

The amount of H_2O_2 in the cultures was determined according to Chance and Maehly (6) by pipetting 5-ml portions into 50 ml of 2% H_2SO_4 (Merck) and titrating with 0.01 $KMnO_4$ (BDH). One milliliter of 0.01 N $KMnO_4$ is equivalent to 5 μmol of H_2O_2.

Measurement of catalase activity. In preliminary experiments, it was found that a single concentration of H_2O_2 was unsatisfactory for measurement of catalase activity because the enzyme was inactivated by excess subtrate and no saturation curve could be obtained. Catalase activity was therefore measured at several substrate concentrations and extrapolated to the $1/V_{max}$ by the Lineweaver-Burk plot, and the V_{max} was calculated. This value was designated as the number of units.

For each determination, six parallel analyses were set up, each with 1 ml of toluenized cells and 1 ml of H_2O_2 to final concentrations of 0.1 (3×10^{-2} M), 0.15, 0.2, 0.25, 0.4, and 0.5%. After 1 min of incubation at 25°C, the reaction was stopped by adding rapidly 50 ml of 2% H_2SO_4, and the residual amount of H_2O_2 was determined by titration with 0.01 N $KMnO_4$ for the 0.1 to 0.25% and 0.02 N for the 0.4 and 0.5% dilutions of H_2O_2. The amount of the decomposed H_2O_2 was calculated by subtraction from blanks of the same H_2O_2 concentrations determined daily.

One catalase unit was defined as the amount of enzyme that would decompose 1 μmol of H_2O_2/min at 25°C (V_{max}). Specific activity was calculated as units of enzyme per milligram (dry weight).

RESULTS

Catalase levels in cells growing on nutrient broth and mineral medium supplemented with glucose. When cells with a high catalase level (grown on nutrient agar) were inoculated into nutrient broth, there was a lag in catalase synthesis during the first generation (Fig. 1). Since the mass of cells had doubled, the catalase level in the cells dropped to half the original amount; then synthesis began and the level rose gradually until it reached its maximum towards the end of the exponential phase. When the catalase level of the inoculum was low (grown on M-9 medium), the drop was rapid during the first 15 min, and then synthesis started and continued until the level for nutrient broth was reached (50 U). In cells with a high catalase level (grown on nutrient agar) inoculated into M-9 medium (Fig. 2), the level remained high during the first generation and then started to drop gradually since synthesis was repressed. When cells grown in M-9 medium to the stationary phase were transferred to fresh M-9 medium, the catalase level remained low throughout the growth cycle.

Selection of medium for kill experiments.

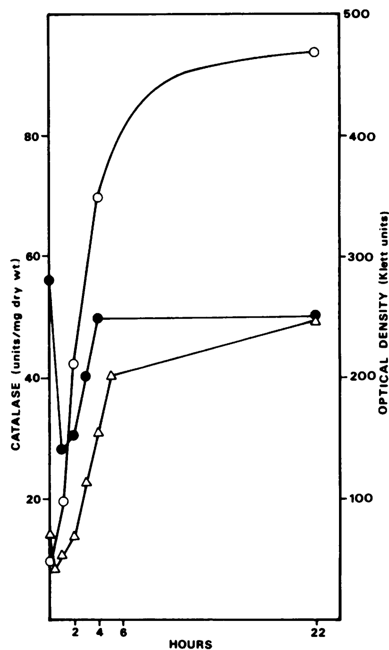

FIG. 1. *Catalase levels in cells of E. coli grown in nutrient broth at 37°C. Symbols:* (●) *Inoculated with cells grown on nutrient agar;* (△) *inoculated with cells grown on M-9 medium;* (○) *growth with both types of inoculum.*

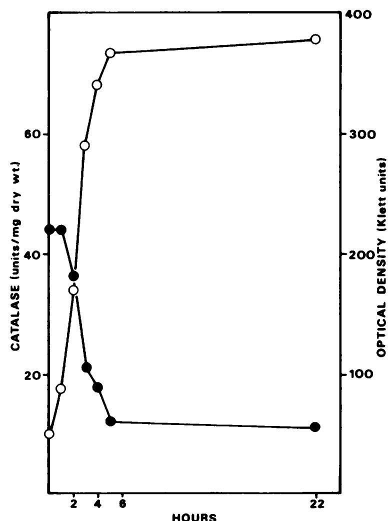

FIG. 2. *Catalase levels in cells of E. coli grown in glucose-mineral medium (M-9), inoculated with cells grown on nutrient agar. Symbols:* (●) *Catalase levels;* (○) *growth.*

The effect of mineral medium, with and without glucose or $FeCl_3$, on the stability of H_2O_2 and death rate of *E. coli* was examined. None of

the components affected the H_2O_2 stability. Although the death rate of the cells was slightly enhanced by the presence of glucose, it was desirable to have the carbon source in the medium to allow the cells that were partially damaged, in order to resume their growth.

Effect of growth medium on cell sensitivity to H_2O_2. Cells grown in nutrient broth decomposed most of the H_2O_2 that had been introduced within the first 30 min (Fig. 3). In such a case, the reduction in cell number was small and remained constant for 2 h, whereupon the growth resumed. After 22 h the count showed about 5×10^9 cells/ml. On the other hand, cells grown in M-9 medium decomposed only a small fraction of the H_2O_2, and the residual amount was high and constant even after 22 h. The number of viable cells decreased by five orders of magnitude in 4 h, and no viable organisms were detected after 22 h.

Effect of growth phase on cell sensitivity to H_2O_2. Cells at the exponential phase were more sensitive to the detrimental action of H_2O_2 than those of the stationary phase (Fig. 4) even though they exhibited more catalase activity than the latter and reduced the H_2O_2 to much lower levels.

Effect of temperature. The effect of temperature on catalase activity during 1 h of incubation is shown in Fig. 5. At 37 and 21°C, the activity was high during the first few minutes; however, it declined rapidly and no activity was noted at 37°C after 20 min, whereas it continued at a slow rate at 21°C. At 10 and 0°C, though slow at the beginning, the enzyme activity continued for the entire hour so that at the end of this period more H_2O_2 had been decomposed than at the higher temperatures.

The correlation between the catalase stability at various temperatures and the death rate of the organisms in the presence of H_2O_2 is shown in Fig. 6, at the high cell concentration (about 3×10^8/ml), where the amount of catalase was sufficient to decompose the H_2O_2 introduced. Ninety percent of the H_2O_2 was decomposed in 3 h; the number of viable organisms decreased by four orders of magnitude in 4 h, and then growth resumed, though at a slow

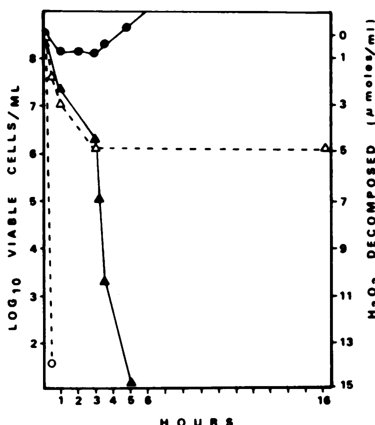

FIG. 3. *Effect of growth medium of E. coli cells on their sensitivity to 0.05% H_2O_2 (15 μmol/ml). Symbols: (●○) Cells grown in nutrient broth; (▲△) cells grown in glucose-mineral medium; (——) viable count; (- - -) catalase activity.*

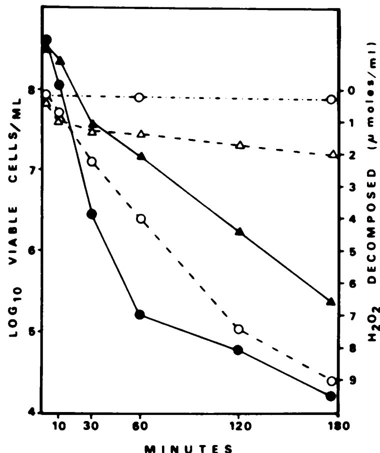

FIG. 4. *Effect of growth phase of E. coli cells on their sensitivity to 0.034% H_2O_2 (10 μmol/ml). Symbols: (●○) Cells grown in M-9 medium harvested in the exponential phase and (▲△) stationary phase; (——) viable count; (----) catalase activity; (-·-·-) H_2O_2 level in control of mineral glucose medium without cells.*

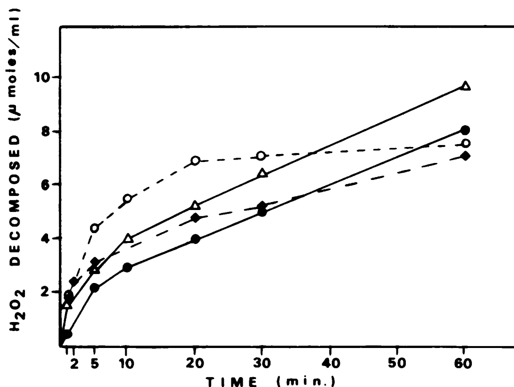

FIG. 5. *Effect of temperature on catalase activity in E. coli. Cells were grown in nutrient broth, washed, and resuspended in phosphate buffer (10 mM, pH 7) to a turbidity of 40 Klett units (ca. 4×10^8 cells/ml). H_2O_2 (10 μmol/ml) was added. Symbols: (●) 0°C; (△) 10°C; (◆) 21°C; (○) 37°C.*

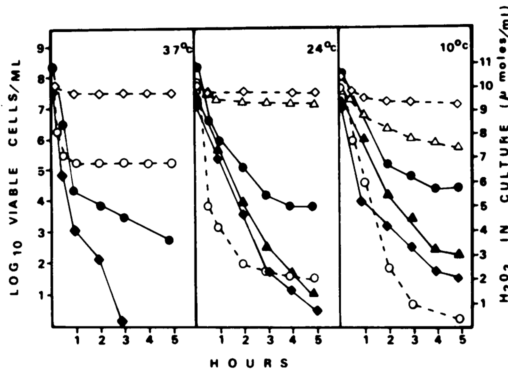

FIG. 6. *Effect of temperature on catalase activity and sensitivity of E. coli to 0.034% H_2O_2, at three bacterial concentrations. The cells were grown in M-9 medium, harvested in the exponential phase, washed, and resuspended in fresh M-9 medium. Initial cell concentration per milliliter* (◆◇) 3×10^7; (▲△) 6×10^7; (●○) *about* 3×10^8. (——) *Number of viable organisms;* (- - -) H_2O_2 *level.*

rate due to the low temperature (10°C). At 24°C, 80% of the H_2O_2 was decomposed in 4 h and the remaining amount was evidently bacteriostatic, for after a reduction of 4.5 orders of magnitude the number of viable cells remained almost constant for 22 h. At 37°C, 32% of the H_2O_2 had been decomposed by the end of the first hour; then the level remained constant for 22 h and was sufficient to kill all organisms.

At the lower cell densities the concentration of H_2O_2 was excessive in proportion to the amount of catalase present; therefore only a small fraction of it was decomposed at all temperatures examined. Yet the time required for completing the kill varied from 3 h at 37°C to more than 6 h at the lower temperatures.

Comparison of catalase activity in intact, toluenized and sonicated cells of *E. coli* and *M. lysodeikticus*. Cells grown in nutrient broth to the stationary phase were washed and resuspended in 0.01 M phosphate buffer (pH 7). A part of the suspensions was sonicated, another part was toluenized, and the catalase activity of each of them was compared with that of intact cells from the same lots. In *E. coli* all analyses showed catalase activity of 50 U/mg (dry weight). Toluenization of *M. lysodeikticus* doubled the catalase activity from 2,000 U/mg (dry weight) in intact cells to 4,000 U, and lysis with lysozyme released additional 16,000 U/mg (dry weight).

DISCUSSION

The data indicate that growth medium and the growth phase modify the amount of catalase synthesized by the cells. While in stationary phase, in M-9 medium-grown cells the catalase level was low, and in nutrient broth-grown cells it was maximal. Consequently, the amount of H_2O_2 that killed the first culture had only a slight and temporary effect on the latter (Fig. 3). However, the catalase level in the cells was not the only factor determining their sensitivity to H_2O_2. In spite of the fact that exponential-phase cells grown on M-9 medium decomposed more H_2O_2 than stationary-phase cells (Fig. 4), they were more sensitive to H_2O_2. According to Dimmick (10), it is well established that "young" actively growing cells are more sensitive to almost any trauma than are "mature" cells. He found exponential-phase cells of *Serratia marcescens* to be more sensitive to elevated temperatures than mature ones.

Reduced levels of catalase activity in organisms grown in the presence of glucose were noted by Engel and Adler (11) in *E. coli*, by Sulebele and Rege (22) in *Saccharomyces cerevisiae*, by Avi-Dor and Yaniv (3) in *Pasteurella tularensis*, and by Jones et al. (15) in two strains of *Streptococcus faecalis*. Clayton (8) found that growth conditions affected the amount of catalase in *Rhodopseudomonas spheroides*. Stapleton and Engel (21) modified the sensitivity of *E. coli* to inactivation by X rays (where H_2O_2 is a byproduct) by alterations in the growth medium of the cells before irradiation. O'Barr and Eagle (20) state that the presence of glucose, as well as that of several other sugars, increases the sensitivity of isoniazide-resistant strains of *Mycobacterium tuberculosis* to H_2O_2, probably due to the reduction in catalase levels.

We have demonstrated a correlation between the amount of catalase in the cells and their sensitivity to H_2O_2, but it is manifested only when the H_2O_2 concentration is not excessive in proportion to the amount of catalase and at low temperatures. Since catalase is an enzyme that is inactivated by excess substrates (4, 7, 23), their amount will determine wheter H_2O_2 will be decomposed or the catalase will be inactivated. Catalase is a thermolabile enzyme (4; Fig. 5). It was inactivated rapidly at 37°C, and the residual amount of H_2O_2 was sufficient to kill the entire culture. At 10°C the catalase was stable for several hours and was able to decompose most of the H_2O_2, and the cells that survived resumed growth.

The higher death rate observed at 37°C in cell densities of 3×10^7/ml (Fig. 6) can be attributed either to enhanced peroxide effect on biological systems or to greater vulnerability of the cells growing at an optimal temperature.

These results provide an explanation for earlier data (1), which show a correlation between the amount of catalase in staphylococci and their resistance to H_2O_2 at 0.025 to 0.05%; when cells with low catalase levels (grown on peptone with glucose) and 0.6% H_2O_2 (11) or 3% H_2O_2 (5) were used, no correlation was found.

Sonication or toluenization of *E. coli* cells did not increase catalase activity. Similar results were reported by Amin and Olson (1) in certain strains of staphylococci. Yet rupture of the cells increases catalase activity up to 10-fold in *M. lysodeikticus* (13, 14; our findings), *Proteus mirabilis* (24), and *Leptospira* (12). Clayton (7), who studied permeability barriers and determined catalase activity in intact cells, found that in cultures of *R. spheroides* low in catalase production, toluenization had no effect on catalase activity and sonication resulted in a slight inactivation, whereas in cultures with high catalase levels, sonication or toluenization increased catalase activity. He explained this difference by the fact that in the catalytic reaction, the rate-limiting entry cannot be circumvented by raising the concentration of substrate since the enzyme is inactivated by the substrate excess. Comparison of the catalase levels in *E. coli* and *M. lysodeikticus* showed that it was about 400 times higher in the latter. It appears that the concentration of H_2O_2 required for measuring catalase activity in intact cells of *E. coli* is not sufficiently high to suppress it in 1-min duration of the reaction. The H_2O_2 concentration required for measuring catalase activity in *M. lysodeikticus* would have to be much higher and therefore toxic for the cell and the enzyme.

ACKNOWLEDGMENT

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