Acta vet. scand. 1980, *21*, 209—223.

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THE EFFECT OF CATALASE ON THE TOXICITY OF CADMIUM IN CADMIUM-SENSITIVE AND CADMIUM-RESISTANT STAPHYLOCOCCUS AUREUS

By

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KORKEALA, H. and SATU SANKARI: *The effect of catalase on the toxicity of cadmium in cadmium-sensitive and cadmium-resistant Staphylococcus aureus.* Acta vet. scand. 1980, *21*, 209—223. — The effect of catalase on the toxicity of cadmium (Cd) in Cd-resistant Staphylococcus aureus 3719+ and its plasmid-negative Cd-sensitive variant 3719— was studied. Catalase on a solid medium increased the recovery of Cd-stressed S. aureus 3719— cells, and the addition of catalase into a liquid growth medium resulted in a shortened lag phase of growth especially in S. aureus 3719—. The catalase activity of S. aureus 3719+ cell suspensions was greater than the corresponding activity of S. aureus 3719— cell suspensions. Cd did not influence the activity of beef liver catalase or the catalase production of the bacterial cells.

Catalase reduced the toxicity of Cd especially for S. aureus 3719—. The greater catalase production of S. aureus 3719+ might be one factor in its resistance against the toxic effects of Cd. It is suggested that Cd together with hydrogen peroxide may induce oxidative damage to cells if there is not sufficient catalase available to decompose all the hydrogen peroxide formed.

cadmium resistance; catalase activity; lag phase; oxidative damage.

The enzyme catalase, produced by many organisms, converts the toxic compound hydrogen peroxide (H_2O_2) to water and oxygen. *Baird-Parker & Davenport* (1965) suggested that the catalase of Staphylococcus aureus may be destroyed or its activity reduced by heating or drying, and for that reason the recovery of heated or dried cells of S. aureus was best on such media, e.g. blood agar, as are able to destroy peroxide. *Flowers et al.* (1977) found that the addition of catalase onto several selective media

greatly increased the recovery of stressed (heated, reduced water activity, or freeze-dried) cells of S. aureus. The stressed cells have a reduced ability to repair themselves and form colonies in the absence of an exogenous decomposer of H_2O_2. Added catalase acts as such a decomposer.

The present study was undertaken to investigate the effect of catalase on the toxicity of cadmium (Cd) in a Cd-sensitive and a Cd-resistant S. aureus.

MATERIAL AND METHODS

The test organism

The microbial strain used in the studies was Staphylococcus aureus strain 3719+ and its plasmid-negative, penicillin- and Cd-sensitive variant 3719—. S. aureus 3719+ (phage type 52/80/81) carries a penicillin resistance plasmid and is resistant to Cd ions. Both variants were obtained from Dr. K. G. H. Dyke, the Department of Biochemistry, University of Oxford, England.

Chemicals and water

The HCl, H_2O_2, NaCl, $Na_2HPO_4 \times 2\ H_2O$, $NaH_2PO_4 \times 2\ H_2O$, and $CdCl_2 \times 2\frac{1}{2}\ H_2O$ used in the experiments were pro analysis grade. The HCl, H_2O_2, NaCl and $Na_2HPO_4 \times 2\ H_2O$ were obtained from E. Merck, Darmstadt, German Federal Republic, the $NaH_2PO_4 \times 2\ H_2O$ from BDH Chemicals, Poole, England, and the $CdCl_2 \times 2\frac{1}{2}\ H_2O$ from J. T. Baker, Phillipsburg, N.J., USA. Tris(hydroxymethyl)aminomethane and catalase (purified powder from bovine liver containing 2900 Sigma units/mg) were obtained from Sigma Chemical Co., St. Louis, Mo., USA. The titanium tetrachloride was a product of Riedel-de Haën Ag, Seelze, German Federal Republic. The water used throughout the experiments was double-distilled and deionized.

Effect of catalase on the recovery of Cd-stressed S. aureus 3719— cells

Washed cells of S. aureus 3719— were incubated for 4 h at 35°C in a shaker with or without Cd in an isotonic NaCl solution buffered to pH 8.0 with tris/HCl buffer. The buffer was prepared in an isotonic NaCl solution. In addition to the NaCl, 100 ml of the buffer contained 25.0 ml of 0.2 M tris(hydroxymethyl)aminomethane and 22.9 ml of 0.1 N-HCl. A pH 8.0 was chosen because most bacteria are generally more sensitive to Cd at high than at low pH (*Korkeala & Pekkanen* 1978). The Cd concentration used in the isotonic NaCl solution was 45 mg of Cd/l.

After 4 h of incubation with or without Cd the numbers of colony-forming units (CFU) were determined on Trypticase soy agar (TSA) and on TSA plus catalase. Five parallel experiments were made without Cd and 10 parallel experiments with Cd. The TSA contained

Trypticase (BBL, Cocceysville, Md., USA) 1.5 %, phytone peptone (BBL) 0.5 %, NaCl 0.5 % and agar 1.5 % (concentrations as % w/v in distilled water). Before seeding, catalase was added to the agar plates by spreading 0.1 ml of a 0.27 % filter-sterilized solution onto previously poured plates, resulting in approx. 780 Sigma units per plate. All bacterial enumerations were carried out using the spread plate technique.

Accommodation of S. aureus 3719— to Cd

Cells of S. aureus 3719— were acommodated to Cd by incubating them to the exponential phase of growth at 35°C in an autoclaved broth containing 0.08 mg of added Cd/l. The broth (denoted YG broth below) contained 10 g of yeast extract (Difco Laboratories, Detroit, Mich., USA) and 1 g of D-glucose (BDH Chemicals) per l distilled water. The pH of the broth was 7.0.

Effect of catalase on the growth of S. aureus 3719— and S. aureus 3719+ in Cd-containing broth

The autoclaved YG broth served as a basic medium. The filter-sterilized Cd and catalase solutions were added to the broth immediately before inoculation, keeping the concentrations of the nutrients constant. Cells from an overnight culture were used for inoculation (0.1 ml of staphylococcal suspension to 7 ml of broth). The size of the inoculum was determined by plate count agar (Difco). The tubes were incubated in a shaker at 35°C and growth was monitored with the Klett-Summerson photoelectric colorimeter (filter no. 42, Klett Manufacturing Co., N.Y., USA). The Cd and catalase concentrations used were 0.08 mg of Cd/l and 750, 1500 and 2250 Sigma units of catalase per tube in the case of S. aureus 3719—, 25 mg of Cd/l and 1500 Sigma units of catalase per tube in the case of S. aureus 3719+. Tubes without added catalase or Cd, tubes containing Cd without added catalase and in the experiments with S. aureus 3719— in addition tubes with added catalase without added Cd served as controls. For each catalase concentration with Cd, as also for the controls, five parallel tubes were incubated. Each such experiment was repeated at least three times.

In addition to the autoclaved broth, the effect of catalase (1500 Sigma units) on the growth of S. aureus 3719— was also studied in filter-sterilized YG broth containing 0.08 mg of Cd/l. Tubes without either added catalase or added Cd, and tubes containing added Cd without added catalase, served as controls. In these three series, as previously, five parallel tubes were incubated in each experiment and each experiment was repeated three times.

Catalase activity of S. aureus 3719—, S. aureus 3719+ and Cd-accommodated S. aureus 3719—

S. aureus 3719— and 3719+ were inoculated from an overnight culture on nutrient agar (Orion Diagnostica, Espoo, Finland). The

Cd-accommodated S. aureus was inoculated on nutrient agar containing 0.08 mg of Cd/l. After 10 h of incubation at 35°C, the cells were harvested from the plates by suspending them in 0.05 M phosphate buffer (pH 7.0). The densities of the suspensions thus obtained were measured with a spectrophotometer (Perkin-Elmer 550, Norwalk, Conn., USA) at 460 nm and the densities were adjusted to the same level with the buffer solution.

Catalase activity was measured spectrophotometrically by following the decomposition of H_2O_2 at 240 nm at 22°C, according to the method of *Beers & Sizer* (1952). 0.1 ml of 0.5 M-H_2O_2 was added to 3 ml of the respective cell suspension. Optical density readings were recorded every 5 s during the first minute of the reaction.

The catalase activity of both S. aureus 3719— and S. aureus 3719+ was determined by six parallel experiments and the catalase activity of Cd-accommodated S. aureus 3719— by four parallel experiments.

Effect of Cd and NaCl on the activity of catalase

Beef liver catalase (65 Sigma units/ml reaction mixture) was allowed to decompose H_2O_2 in 0.05 M phosphate buffer (pH 7.0) in reaction mixtures containing 0.8, 1.0, 10, 33 and 66 mg of Cd/l phosphate buffer and in phosphate buffer containing 7.5 % (w/v) NaCl. A reaction mixture without either added Cd or NaCl served as a control. Catalase activity was determined by the method of *Beers & Sizer.*

Effect of Cd and NaCl on the decomposition of H_2O_2

Solutions containing 0.08 and 25 mg of Cd/l or 7.5 % (w/v) and 10 % (w/v) of NaCl were prepared in 0.05 M phosphate buffer (pH 7.0). Three ml of each solution was mixed with 0.1 ml of 0.5 M-H_2O_2 and the change in absorbance was recorded during 30 min with a spectrophotometer at 240 nm.

Catalase activity of S. aureus 3719— incubated with or without Cd, as determined with a respirometer

One ml of an overnight culture of S. aureus 3719— was inoculated into 50 ml of YG broth containing 0 or 0.08 mg of Cd/l. The catalase activity of the cells was measured both immediately after inoculation and after 1 h of incubation at 35°C, using the Warburg constant volume respirometer (B. Braun Aparatebau, Melsungen, German Federal Republic). Two ml of the inoculated medium was diluted with 2 ml of 0.05 M phosphate buffer (pH 7.0) in the manometer flask. 0.5 ml of 0.9 M-H_2O_2 prepared in phosphate buffer was pipetted into the sidearm of the flask. After 5 min of incubation the substrate was added into the flask and the manometer readings were recorded at 1 min intervals during the first 10 min. Oxygen production was calculated from the manometer readings of several successive measurements giving equal results. Five parallel experiments were carried out.

H_2O_2 determination of the YG broth

The H_2O_2 concentration in the culture medium was determined by the acidic titanium method (*Wolfe* 1962). Two ml of different H_2O_2 concentrations in aqueous solution were mixed with 1 ml of titanium tetrachloride (4 mg Ti/ml 6 N-HCl). Absorbances were measured with a spectrophotometer at 415 nm. A calibration curve was drawn on the basis of the readings. For checking the concentration of H_2O_2 used its absorbance was determined at 230 nm (*Beers & Sizer, Maehly & Chance* 1961).

The H_2O_2 concentrations of autoclaved and of filter-sterilized YG broths were similarly measured before the addition of Cd and after 24 h of incubation at 35°C without Cd and with Cd, using 2 ml of broth instead of the H_2O_2 water solution. The Cd concentrations used were 0.08 mg/l and 25 mg/l. Five parallel experiments were performed on the autoclaved and on the filter-sterilized broths. The H_2O_2 concentrations of the broths were obtained from the calibration curve.

RESULTS

The effect of catalase on the recovery of Cd-stressed S. aureus 3719— cells is shown in Table 1.

The effect of catalase on the growth of S. aureus 3719— and 3719+ in autoclaved YG broth containing 0.08 mg of Cd/l and 25 mg of Cd/l is shown in Figs. 1 and 2, respectively. The size

Table 1. The effect of catalase on the recovery of cadmium (Cd)-stressed Staphylococcus aureus 3719—.

	Log number of bacteria per ml		
	before incubation	plated without catalase after 4 h of incubation	plated with catalase[1] after 4 h of incubation
No added Cd[2]	9.46±0.09	9.39±0.03	9.39±0.02
45 mg added Cd/l[3]	9.39±0.13	8.66±0.35	8.91±0.33

The cells were incubated 4 h in an isotonic NaCl solution buffered to pH 8.0 with tris/HCl buffer at 35°C in a shaker with or without Cd. The cell numbers were determined on Trypticase soy agar (TSA), using the spread plate technique. The plates were incubated at 35°C for 48 h. Catalase significantly increased the recovery of Cd-stressed staphylococcal cells ($P < 0.001$, paired-sample t-test).

[1] Catalase was added by spreading 0.1 ml of 0.27 % filter-sterilized solution onto previously poured TSA plates, resulting in approx. 780 units per plate.

[2] Mean of 5 parallel experiments.

[3] Mean of 10 parallel experiments.

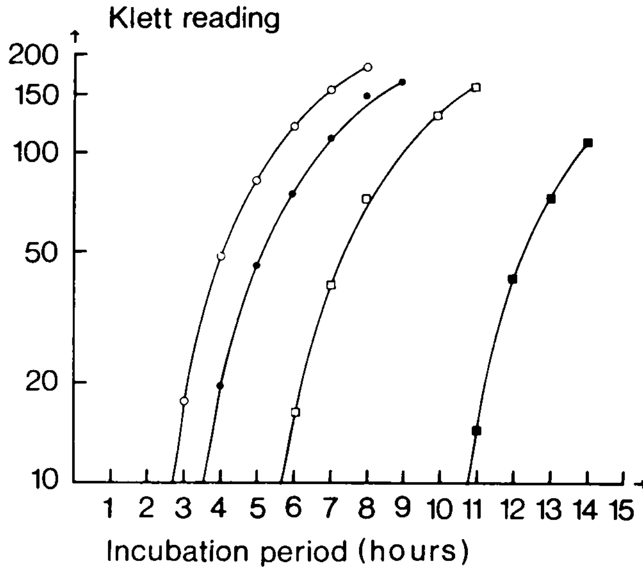

Figure 1. Growth of S. aureus 3719— in autoclaved YG broth with added filter-sterilized Cd solution to 0.08 mg of Cd/l and 1500 Sigma units of filter-sterilized catalase/test tube. Each test tube was inoculated in the same way from the same overnight culture. Turbidities were measured with the Klett-Summerson photoelectric colorimeter (filter no. 42). Each point represents the mean of five separate determinations. Symbols: (●) no added Cd or catalase, (○) 1500 Sigma units of added catalase, (■) 0.08 mg of added Cd/l, (□) 0.08 mg of added Cd/l + 1500 Sigma units of added catalase.

of the inoculum was 22×10^7 cells/ml on S. aureus 3719— and 28×10^7 cells/ml on S. aureus 3719+. The effect of different catalase concentrations on the growth of S. aureus 3719— in autoclaved YG broth containing 0.08 mg of Cd/l is shown in Fig. 3. The size of the inoculum was 74×10^7 cells/ml. Each figure gives the result of one experiment. The parallel experiments in each case gave mostly similar results.

The catalase activity of S. aureus 3719—, S. aureus 3719+ and Cd-accommodated S. aureus 3719— is presented in Fig. 4. The mean values of catalase activity per absorbance unit of the cell density at 460 nm of S. aureus 3719— and Cd-accommodated S. aureus 3719—, calculated as percentage of the corresponding mean catalase activity of S. aureus 3719+, are given in Fig. 5.

The effect of Cd and NaCl on the activity of beef liver catalase

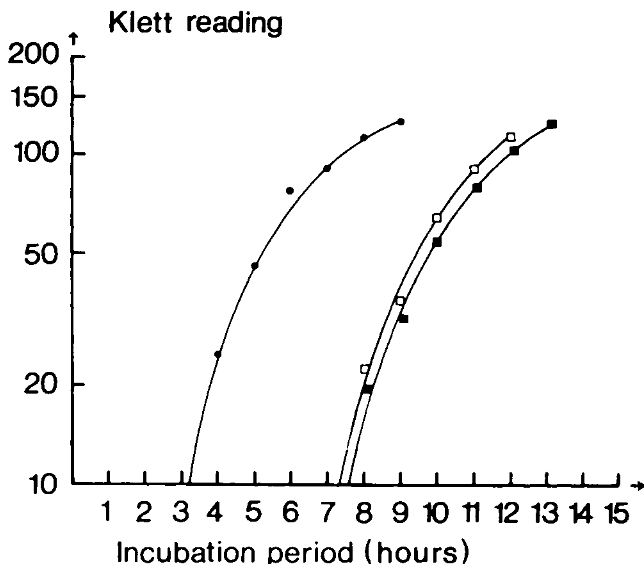

Figure 2. Growth of S. aureus 3719+ in autoclaved YG broth with added filter-sterilized Cd solution to 25 mg of Cd/l medium and 1500 Sigma units of filter-sterilized catalase/test tube. Each test tube was inoculated from the same overnight culture. Turbidities were measured with the Klett-Summerson photoelectric colorimeter (filter no. 42). Each point represents the mean of five separate determinations. Symbols: (●) no added Cd or catalase, (■) 25 mg of added Cd/l, (□) 25 mg of added Cd/l + 1500 Sigma units of added catalase.

is presented in Fig. 6; 0.8, 1.0, 10 and 33 mg of Cd/l did not influence the catalase activity of the reaction mixture; 66 mg of Cd/l or 7.5 % of NaCl in the reaction mixture reduced catalase activity.

Cd and NaCl had no effect on the decomposition of H_2O_2. The Cd concentrations studied were 0.08 and 25 mg of Cd/l and the NaCl concentrations were 7.5 % (w/v) and 10 % (w/v).

In the manometric experiment, the mean oxygen production of S. aureus 3719— incubated 1 h at 35°C in YG broth without Cd was 231±73 ml/min per absorbance unit of the cell density at 460 nm. When S. aureus 3719— was similarly incubated with Cd, the corresponding mean was found to be 235±72.

The mean H_2O_2 concentration in autoclaved YG broths before and after 24 h of incubation at 35°C was 1.9±0.3 μg/ml and 2.2±0.2 μg/ml, respectively, and after 24 h of incubation at 35°C

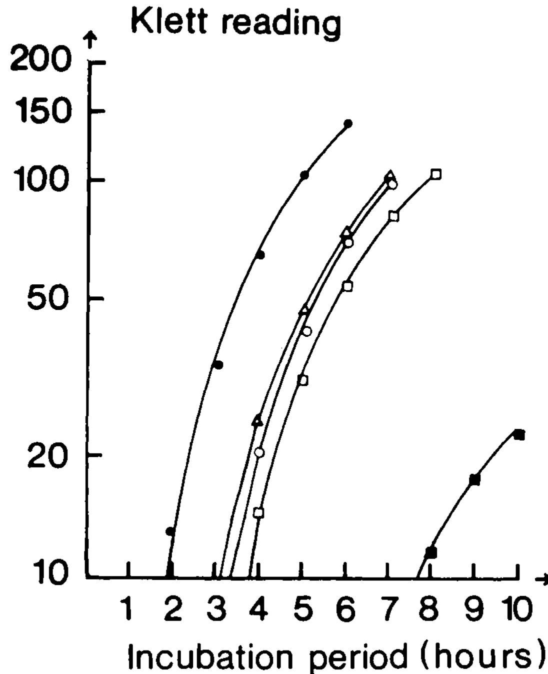

Figure 3. Growth of S. aureus 3719— in autoclaved YG broth with added filter-sterilized Cd solution to 0.08 mg of Cd/l and 750, 1500 or 2250 Sigma units of filter-sterilized catalase/test tube. Each test tube was inoculated similarly from the same overnight culture. Turbidities were measured with the Klett-Summerson photoelectric colorimeter (filter no. 42). Each point represents the mean of five separate determinations. Symbols: (●) no added Cd or catalase, (■) 0.08 mg of added Cd/l, (□) 0.08 mg of added Cd/l + 750 Sigma units of added catalase, (○) 0.08 mg of added Cd/l + 1500 Sigma units of added catalase, (△) 0.08 mg of added Cd/l + 2250 Sigma units of added catalase.

with 0.08 and 25 mg of Cd/l 2.2±0.2 μg/ml and 1.9±0.2 μg/ml, respectively. The H_2O_2 concentration in filter-sterilized YG broths was in all cases below the lowest limit of detection (1.5 μg/ml).

The effect of catalase on the growth of S. aureus 3719— in filter-sterilized YG broth containing 0.08 mg of Cd/l is presented in Fig. 7. The size of the inoculum was 90×10^7 cells/ml. The figure gives the result of one experiment. Parallel experiments gave mostly similar results.

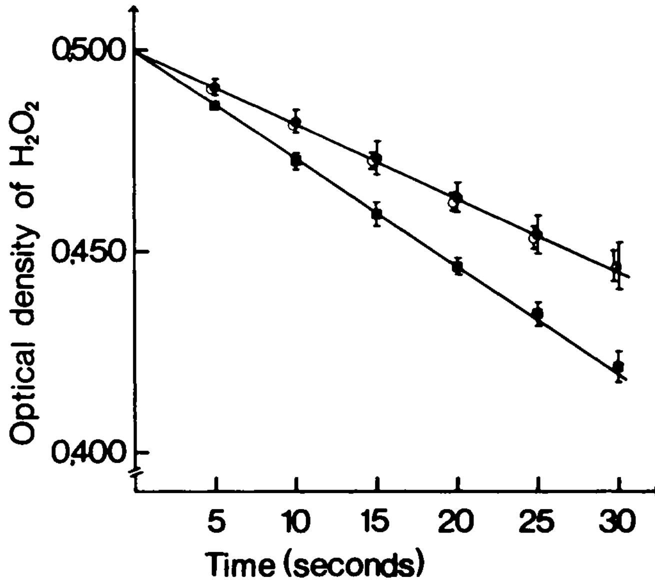

Figure 4. Catalase activity of S. aureus 3719—, S. aureus 3719+ and Cd-accommodated S. aureus 3719— cell suspensions measured by following the decomposition of H_2O_2 spectrophotometrically at 240 nm, as a function of time. Cells were suspended to phosphate buffer from nutrient agar after 10 h of incubation at 35°C. The densities of the cell suspensions were measured spectrophotometrically at 460 nm and adjusted to the same level prior to the catalase determinations. Each point represents the mean of six parallel experiments on S. aureus 3719— and 3719+ and the mean of four parallel experiments on Cd-accommodated S. aureus. Symbols: (○) S. aureus 3719—, (■) S. aureus 3719+, (●) Cd-accommodated S. aureus 3719—.

DISCUSSION

The results presented in Table 1 show that catalase on a solid medium significantly increased the recovery of Cd-exposed cells of S. aureus 3719— ($P < 0.001$, paired-sample t-test). Although 45 mg of Cd/l in isotonic buffered saline obviously is toxic to S. aureus 3719— cells, the damage seems to be partially reversible. This could mean, in terms of catalase and H_2O_2, that Cd exposure either reduces the catalase activity or production of the cells, increases the concentration of the catalase substrate (H_2O_2), or increases cell susceptibility to H_2O_2; any or all of these effects may be valid.

The lag phase of growth of S. aureus 3719— and S. aureus

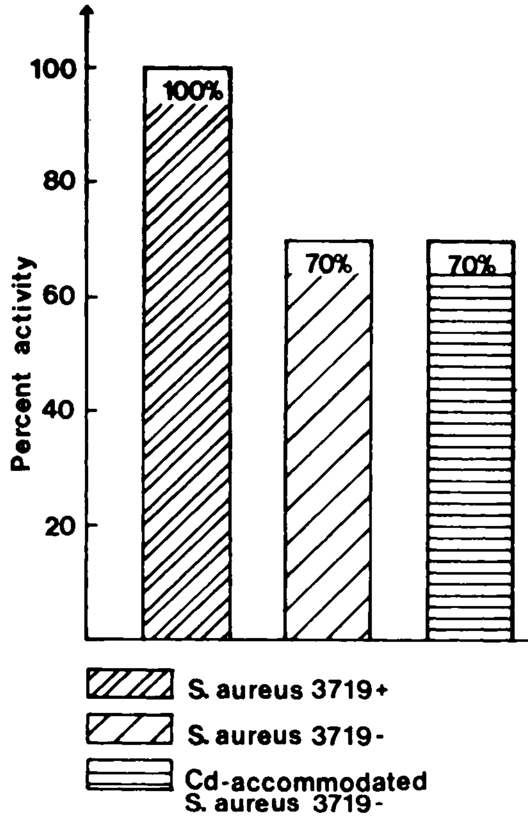

Figure 5. Mean values of catalase activity per absorbance unit of S. aureus 3719— and Cd-accommodated S. aureus 3719— cell suspensions, given as percentage of mean catalase activity per absorbance unit of S. aureus 3719+ cell suspensions. For initial cell densities and determination of catalase activity see legend to Fig. 4.

3719+ in YG broth lengthened as the concentration of Cd in the broth increased (*Korkeala* 1979). Figs. 1 and 2 show that the addition of catalase into the growth medium containing Cd resulted in a definitely shorter lag phase of growth in S. aureus 3719— but had hardly any effect on the lag phase of S. aureus 3719+. The addition of catalase also shortened the lag phase of S. aureus 3719— in the broth without Cd but the effect on the Cd-exposed cells was more pronounced. The results given in Fig. 3, however, indicate that the addition of catalase apparently cannot totally eliminate the toxic effects of Cd on the test microbe, as measured by the length of the lag phase of growth. The

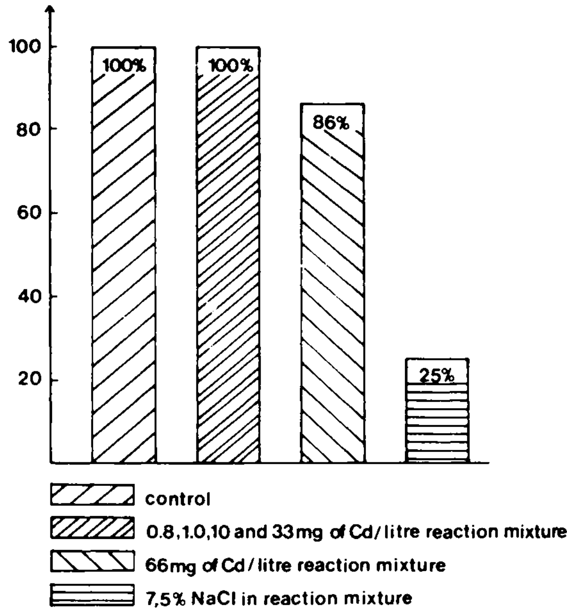

Figure 6. The effect of Cd or NaCl on the activity of beef liver catalase, expressed as percentage of the catalase activity of the control.

mechanism of Cd toxicity for bacteria thus seems to include other factors than those explainable by catalase alone, especially in the case of the Cd-resistant S. aureus.

The present results (Figs. 4 and 5) further show that the catalase activity of S. aureus 3719+ cell suspensions is greater than the corresponding activity of S. aureus 3719— cell suspensions. Previous work on various S. aureus strains has shown that low catalase activity and sensitivity to H_2O_2 are linked together (*Amin & Olson* 1968). The tolerance of S. aureus 3719+ cells to H_2O_2 may similarly be greater than the H_2O_2 tolerance of S. aureus 3719— cells. The increased catalase activity of S. aureus 3719+ may well be bound to the functions of the same plasmid as Cd resistance. For the Cd-resistant S. aureus, the greater catalase production may be one of the factors of resistance against Cd. Nevertheless the present study and those reported earlier (reviewed by *Babich & Stotzky* 1978) show that the toxicity of Cd for microbes includes various mechanisms.

Martin et al. (1976) found that either heat or NaCl, or these

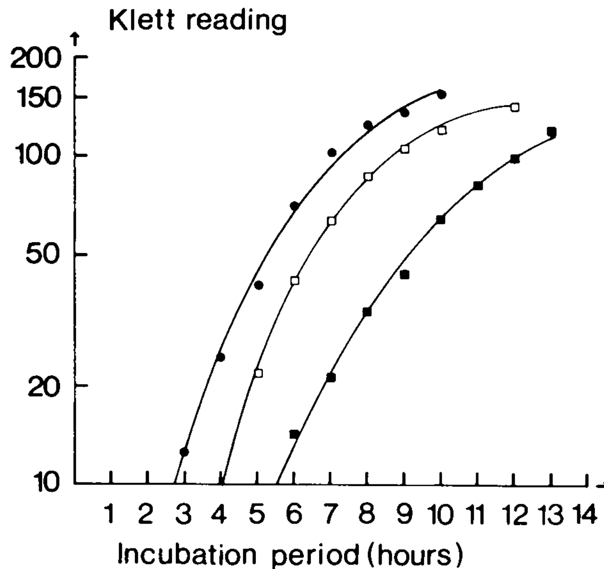

Figure 7. Growth of S. aureus 3719— in the filter-sterilized YG broth with added filter-sterilized Cd solution to 0.08 mg of Cd/l and 1500 units of filter-sterilized catalase/test tube. Each test tube was inoculated from the same overnight culture. Turbidities were measured with the Klett-Summerson photoelectric colorimeter (filter no. 42). Each point represents the mean of six separate determinations. Symbols: (●) no added Cd or catalase, (■) 0.08 mg of added Cd/l, (□) 0.08 mg of added Cd/l + 1500 Sigma units of added catalase.

factors combined, reduce the catalase activity of S. aureus. The effect of Cd and NaCl on beef liver catalase activity is shown in Fig. 6. NaCl (7.5 % w/v) decreased the activity of beef liver catalase, while the Cd concentrations used in the lag phase experiments had no measurable effect on catalase activity. Since it has been suggested that heavy metals may decompose H_2O_2 (*Aebi* 1974), and could thus influence the measuring system used, the effect of Cd on the decomposition of H_2O_2 was studied. The concentrations of Cd were 0.08 and 25 mg/l. No measurable effect was observed. Similarly it was confirmed that NaCl did not decompose H_2O_2. This indicates that the effect of NaCl on the activity of beef liver catalase is a direct one. The Cd toxicity studied in the present case thus cannot be due to a decrease in catalase activity, as was the case in the experiments of *Martin et al.* who heated the cells or treated them with NaCl.

The results obtained with the respirometer showed that the Cd concentration used (0.08 mg/l) apparently did not influence the synthesis of catalase by S. aureus 3719— cells. No difference was likewise observed when the catalase activity of Cd-accommodated S. aureus 3719— cells was compared to the corresponding activity of non-accommodated S. aureus 3719— cells (Figs. 4 and 5). This further indicates that the adaptation of the cells is not due to changes in catalase activity.

According to *Albert* (1973), H_2O_2 can initiate a self-propagating chain reaction in the presence of an oxidizable substrate and a trace of a heavy-metal cation. It is possible that Cd together with H_2O_2 formed in the cells could initiate a similar auto-oxidative chain reaction in S. aureus 3719—. If there is not enough catalase to decompose all the H_2O_2 formed, the Cd with the H_2O_2 might thus induce oxidative damage to cells.

The formation of H_2O_2 through auto-oxidation in bacteriological culture media has been known for some time (*Proom et al.* 1950, *Barry et al.* 1956). When the formation of H_2O_2 was studied in autoclaved YG broth at 35°C without Cd and after the addition of 0.08 and 25 mg of Cd/l by the acidic titanium method (*Wolfe* 1962), it was found that Cd had no effect on the concentration of H_2O_2 in the YG broth. *Carlsson et al.* (1978) found that media containing phosphate and glucose had a tendency to accumulate H_2O_2 if the media were autoclaved under anaerobic conditions and were then exposed to atmospheric oxygen. The work of *Flowers et al.* (1977) showed that unstressed cells of S. aureus treated with H_2O_2 at concentrations ranging from 33 to 3333 μg/ml showed no loss of viability or increased sensitivity to NaCl. The mean H_2O_2 concentration of the autoclaved YG broths used in the present experiments was found to be 1.9±0.3 μg/ml. The corresponding value of the filter-sterilized YG broths was below the lowest limit of detection (1.5 μg/ml). Nevertheless a lag phase of growth, which was shortened by catalase, was observed when S. aureus 3719— was grown in filter-sterilized YG broth containing Cd (Fig. 7). The stress from the H_2O_2 formed during the autoclaving of the broth is obviously not responsible for the toxic effect of Cd on the bacteria. The toxicity of Cd influenced by catalase is thus associated with effects on the bacterial cell rather than with phenomena outside the cell.

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SAMMANFATTNING

Effekten av katalas på kadmiums toxicitet på kadmiumkänslig och kadmiumresistent Staphylococcus aureus.

Effekten av katalas på kadmiums (Cd) toxicitet på en Cd-resistent Staphylococcus aureus stam 3719+, och dess plasmid-negativa, Cd-känsliga variant 3719— undersöktes. Enzymet katalas på ett fast substrat ökade cellantalet av den Cd-belastade S. aureus 3719— stammen. Tillsatsen av katalas till det flytande näringssubstratet förkortade lagfasen särskilt i S. aureus 3719—. Katalasaktiviteten i S. aureus 3719+ cellsuspensionerna var större än den motsvarande aktiviteten i S. aureus 3719— cellsuspensionerna. Cd påverkade inte aktiviteten av katalas från nötlever eller katalasproduktionen av bakteriecellerna.

Katalas minskade Cd:s toxicitet på speciellt S. aureus 3719—. Den större katalasproduktionen av S. aureus 3719+ kunde vara en av dess resistensfaktorer mot Cd:s toxiska effekt. Det är möjligt, att Cd tillsammans med väteperoxid kunde förorsaka en oxidativ skada på celler om det inte finns tillräckligt med katalas at nedbryta den producerade väteperoxiden.

(Received December 17, 1979).

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