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# Sensitivity of Mixed Populations of *Staphylococcus aureus* and *Escherichia coli* to Mercurials

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ABSTRACT

STUTZENBERGER, F. J. (University of Houston, Houston, Tex.), AND E. O. BENNETT. Sensitivity of mixed populations of *Staphylococcus aureus* and *Escherichia coli* to mercurials. Appl. Microbiol. **13**:570–574. 1965.—*Staphylococcus aureus* was found to have a higher resistance to merbromin and mercuric chloride in the presence of *Escherichia coli*. The protective effect of the gram-negative organism on *S. aureus* was due to the production of extracellular glutathione and hydrogen sulfide and to an unequal distribution of the inhibitor between the two species. *S. aureus* did not significantly influence the resistance of *E. coli* to mercurials.

Numerous reports have been published concerning inhibitors that can be used to control microbial growth under a variety of conditions. Most of these investigations measure the effectiveness of inhibitors in terms of their ability to kill or inhibit growth of pure cultures of microorganisms under highly artificial laboratory conditions. In actual practice, antimicrobial agents are generally employed against a mixed microbial flora rather than pure cultures.

Dean and Hinshelwood (1952) suggested that the sensitivity of an organism to an inhibitor can be markedly influenced by the presence of other bacteria in the environment. Bennett and Bauerle (1960) demonstrated that *Desulfovibrio desulfuricans* was more resistant to phenols in the presence of *Pseudomonas aeruginosa*, and that the pseudomonads were more resistant to mercurials in the presence of the sulfate reducers. Bachenheimer and Bennett (1961) showed that the sulfate reducers produced hydrogen sulfide which protected the pseudomonads from mercurials. Stutzenberger and Bennett (1963) reported that *Escherichia coli* could protect *Staphylococcus aureus* against numerous antibiotics and phenols in mixed cultures.

This investigation pertains to the protective effect of *E. coli* on the inhibitory activities of mercurials against. *S. aureus* and the mechanism of this protection.

## MATERIALS AND METHODS

*E. coli* and *S. aureus* were maintained on nutrient agar and Staphylococcus Medium No. 110 (Difco) slants, respectively, at a temperature of 4 C. A 1-ml amount of each culture was transferred to individual flasks containing 50.0 ml of sterile S medium (Table 1), and an aditional flask was inoculated with 0.5 ml of both cultures. These flasks were incubated for 10 hr at 37 C on a gyrotory shaker rotating at 180 rev/min and then standardized with sterile medium to an optical density of 1.4 with a Beckman model B spectrophotometer set at 625 mμ.

A 1-ml amount of the standardized suspension of *E. coli* was added to each of a series of tubes containing S medium and various concentrations of inhibitor. The same concentrations of *S. aureus* were inoculated into a second set of tubes, and the third set was inoculated with 1.0 ml of the mixed culture. The fourth set was inoculated with 0.5 ml each of the two pure cultures. All tubes were vigorously shaken and then incubated for 24 hr at 37 C.

*S. aureus* would not grow on Eosin Methylene Blue Agar (Difco) nor would *E. coli* grow in Staphylococcus Medium No. 110 (Difco). Thus, the bactericidal concentration for either species could be determined by plating 0.1 ml from each tube of the four sets onto these appropriate media. These cultures were incubated for 48 hr at 37 C and examined for the presence of viable cells. The results shown in the tables are given as the lowest concentration of inhibitor from which no viable cells could be recovered in the subcultures.

Stock solutions of each inhibitor were made in distilled water and sterilized by autoclaving for 10 min. These solutions were added to S medium to give total volumes of 9 ml, containing varying concentrations of the inhibitor in increments of 1 ppm (w/v).

Determinations of extracellular products released into the medium were performed on the cell-free culture medium after centrifugation at 1,400 × *g* at 4 C for 30 min. Free glutathione determinations were made by the method of Patterson and Lazarow (1955). That extracellular

ascorbic acid did not interfere with the glutathione determinations was established by the 2,6-dichlorophenol-indophenol method as described by Evelyn, Malloy, and Rosen (1938) and modified by Bessey (1938). Hydrogen sulfide determinations were performed according to the method of Delwiche (1951) and free extracellular cysteine by the method of Nakamura and Binkley (1948).

The protective effect of glutathione upon *S. aureus* cells treated with 5 ppm of mercuric chloride was determined by the addition of 10 ppm of glutathione to 10-ml cultures exposed to the inhibitor for various times. After 24 hr, all cultures were checked for viable cells by subculturing on Staphylococcus Medium No. 110.

Studies were conducted to determine the uptake of mercury-203 from the medium by pure cultures of *E. coli* and *S. aureus*. Pure cultures of each organism were washed twice with cold, sterile saline and calibrated to an optical density of 1.4. A 1-ml amount of the cell suspension was inoculated into 9 ml of S medium containing 5 ppm of mercuric chloride. Viable-cell counts were made on each culture after 24 hr.

Radioactive determinations with $Hg^{203}$ $Cl_2$ were performed on 0.1-ml samples of culture supernatant fluids and control after centrifugation, by use of a Nuclear Chicago D-47 gas-flow counter and a model 186 rate meter at 1,300 v.

TABLE 1. *Composition of S medium*

| Component* | Amt |
|---|---|
| $MgSO_4 \cdot H_2O$ | 1 mg |
| $MnSO_4 \cdot H_2O$ | 1 mg |
| $FeSO_4 \cdot 7H_2O$ | 1 mg |
| $KH_2PO_4$ | 500 mg |
| $K_2HPO_4$ | 500 mg |
| NaCl | 500 mg |
| Nicotinic acid | 1 mg |
| Thiamine | 1 mg |
| Glucose | 1,000 mg |
| Valine | 40 mg |
| Glycine | 40 mg |
| Arginine | 40 mg |
| Casein hydrolysate | 100 mg |
| Deionized water | 100 ml |

* To the components listed, 10 ml of the following solution were added: 1.3 mg/ml of lysine, 1.3 mg/ml of histidine, and 0.3 mg/ml of glutamic acid. The medium was adjusted to *p*H 6.8 and filter-sterilized through an ultrafine Morton fritted-glass filter.

## RESULTS

The sensitivities of the pure and mixed cultures to mercurials in nutrient broth are given in Table 2. It may be observed that *E. coli* had a significant effect on the sensitivity of *S. aureus* to merbromin and mercuric chloride.

Preliminary isotope studies with nutrient broth showed that a large amount of the mercury combined with constituents of the medium to form complexes which could be removed by centrifugation. For this reason, and also because of the difficulty encountered with colorimetric tests made in nutrient broth, S medium was selected for all subsequent work. The sensitivities of pure and mixed cultures to mercuric chloride in this medium are given in Table 3.

Mixed-culture studies frequently encounter a problem in that one organism exhibits a faster rate of growth than the other, thereby causing an undesirable repressive effect or the total elimination of one organism from the culture. Growth curves and viable-cell counts of pure and mixed cultures in S medium showed that both *E. coli* and *S. aureus* grew vigorously in the medium. In mixed culture, the growth of *S. aureus* was reduced 75% and *E. coli* 44% as compared with pure cultures.

Hydrogen sulfide production was determined for the pure and mixed cultures (Fig. 1). *S. aureus* produced a very small amount of hydrogen sulfide and *E. coli* produced a relatively greater quantity of this compound.

Extracellular glutathione production by pure

TABLE 2. *Sensitivities of pure and mixed cultures to mercurials in nutrient broth**

| Compound | Bactericidal concn (ppm) | | | | | |
|---|---|---|---|---|---|---|
| | *Escherichia coli* | | | *Staphylococcus aureus* | | |
| | Pure | Mixed | S + E† | Pure | Mixed | S + E† |
| *o*-Chloro-mercury-phenol | 35 | 30 | 30 | 20 | 15 | 20 |
| Phenylmercuric salicylate | 30 | 30 | 30 | 5 | 8 | 8 |
| Phenylmercuric chloride | 40 | 40 | 40 | 8 | 11 | 11 |
| Merbromin | 2,000 | 2,000 | 2,000 | 90 | 160 | 160 |
| Mercuric chloride | 300 | 300 | 300 | 45 | 90 | 95 |

* Results are the average of four determinations with a maximal experimental variation of 12%.
† Indicates inoculum grown as pure cultures and mixed at the time of exposure to the inhibitor.

TABLE 3. *Sensitivities of pure and mixed cultures to mercuric chloride in S medium**

| Culture | Bactericidal concn |
|---|---|
| | *ppm* |
| Pure *Escherichia coli* | 10 |
| Mixed *E. coli* | 7 |
| S + E *E. coli* | 9 |
| Pure *Staphylococcus aureus* | 3 |
| Mixed *S. aureus* | 6 |
| S + E *S. aureus* | 6 |

* Results are the averages of four determinations with a maximal experimental variation of 16%.

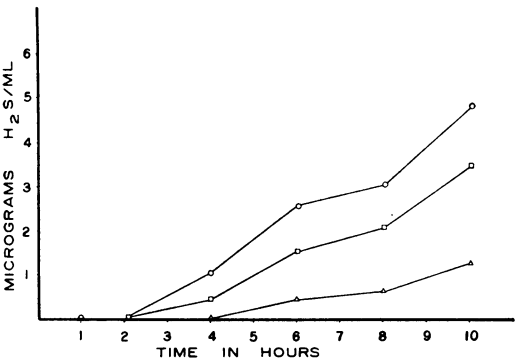


FIG. 1. *Hydrogen sulfide production by pure and mixed cultures. Symbols:* ○ = *Escherichia coli;* △ = *Staphylococcus aureus;* □ = *mixed culture.*

and mixed cultures is illustrated in Fig. 2. *E. coli* liberated approximately three times as much glutathione into the medium, and the mixture liberated 2.5 times as much, as did the pure *S. aureus* culture. It was also determined that 10 ppm of mercuric chloride complexed with 15 (±3) ppm of glutathione from the zone of mercury-glutathione equivalence to that of glutathione excess. Additional experiments established that *S. aureus*, inhibited by 5 ppm of mercuric chloride, could be revived by 10 ppm of glutathione after up to 5 hr of exposure to the inhibitor.

The pure *S. aureus* culture produced no free glutathione at any time during exposure to 5 ppm of mercuric chloride. The pure *E. coli* and mixed cultures showed free glutathione in the medium as early as 3 hr after initial exposure to the inhibitor. The cultures containing *E. coli* released from 1 to 2 μg of free glutathione per ml of medium during the first 5 hr of exposure to the mercurial.

Chromatographic analyses of extracellular products demonstrated that neither organism liberated cysteine into the medium. These findings were also confirmed by the colorimetric test of Nakamura and Binkley (1948).

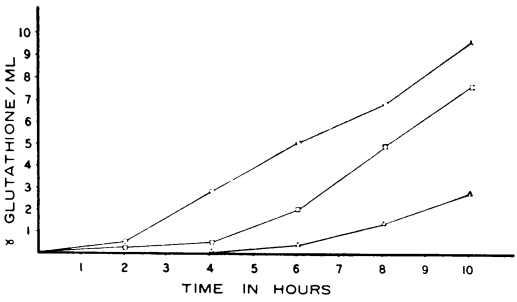


FIG. 2. *Glutathione production by pure and mixed cultures. Symbols:* ○ = *Escherichia coli;* △ = *Staphylococcus;* □ = *mixed culture.*

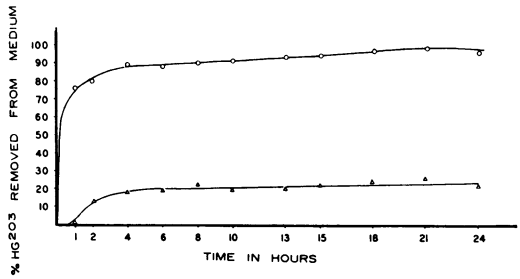


FIG. 3. *Mercury uptake by pure cultures of Escherichia coli* (○) *and Staphylococcus aureus* (△).

$Hg^{203}Cl_2$ with a specific activity of approximately 1,000 disintegrations per min (dpm) per μg was employed to determine the relative amounts of mercury taken up by *E. coli* and *S. aureus*. Figure 3 shows that *E. coli* suspensions removed approximately five times as much mercury from the medium as did *S. aureus* cultures of the same turbidity. At the termination of the 24-hr exposure period, viable-cell counts revealed $8 \times 10^6$ *E. coli* cells per milliliter, whereas there were no viable *S. aureus* cells present. It was calculated that *E. coli* bound $2.7 \times 10^7$ mercury ions per cell and *S. aureus* bound $1.8 \times 10^7$ ions per cell. *E. coli* cells bound mercury very quickly, removing 75% of a 5 ppm concentration during the first hour of exposure, whereas *S. aureus*, although inhibited at a much lower concentration of mercuric chloride, did not take up appreciable mercury until after 1 hr of exposure, and did not remove more than 20% of the inhibitor even after 24 hr of exposure.

## DISCUSSION

In recent years, increasing attention has been given to studies on the interrelationships of mi-

croorganisms in mixed cultures. These investigations have shown that the presence of one microorganism can greatly influence the activity of another under various conditions. Rakhman (1958) found that *S. aureus* decreased the toxicity of diptheria bacilli when grown together in mixed cultures, and Arndt and Ritts (1961) demonstrated a synergistic relationship between *S. aureus* and *Proteus vulgaris*, in that the virulence of the latter organism was greatly enhanced in mice by staphylococci or their extracts. Okuda and Kobayashi (1961) demonstrated that mixed cultures of *Rhodopseudomonas capsulatus* and *Azotobacter vinelandii* produced profuse quantities of slime substances not found in either pure culture, accompanied by a 12-fold increase in nitrogen fixation.

The data in Table 2 show that the presence of *E. coli* markedly reduced the susceptibility of *S. aureus* to mercurials. Merbromin and mercuric chloride showed the greatest difference, and the other inhibitors showed the same trend to a lesser degree.

In comparing the resistance of washed and unwashed cells of both species to mercuric chloride, it was quite evident that most of the protective effect afforded *S. aureus* by *E. coli* could be attributed to the extracellular culture fluid, rather than to the cells themselves. This information indicated that the inhibitor was inactivated by some substance released into the medium before it made contact with the cells.

Numerous reports have appeared in the literature pertaining to the antagonistic effects of various chemicals upon mercurials. It has been shown that hydrogen sulfide (McCalla, 1940), cysteine (Cavallito et al., 1945), and glutathione (Cook and Steel, 1959) are detrimental to these inhibitors.

It is an established fact that *E. coli* can produce hydrogen sulfide under a variety of conditions. Chemical analyses showed that the culture used in this investigation was an active hydrogen sulfide producer and that this material was responsible for a portion of the protective effect on *S. aureus*.

The production of glutathione by *E. coli* and its release into the medium could have, on the basis of combining ratios, accounted for a portion of the protective effect. Furthermore, the presence of free glutathione in the medium as nearly as 3 hr after initial contact with 5 ppm of mercuric chloride indicated that *E. coli* was instrumental in reviving inactivated *S. aureus* cells, as well as offering initial protection at the time of contact with the inhibitor.

Mercury-203 absorption studies revealed an unequal distribution of the inhibitor between the two species. This observation accounted for that portion of the protective effect which could not be attributed to mercurial inactivation by extracellular compounds. It is apparent that the environmental concentration can be reduced to a subinhibitory concentration for *S. aureus* by *E. coli*. A similar phenomenon was reported by Russell (1955) when he noted that *Penicillium roqueforti* absorbed large amounts of phenyl mercuric acetate and therefore allowed growth of *Sterium sanguinolentum* in the same environment.

In interpreting the data obtained in the mercury-203 absorption studies, it is necessary that a linear relationship exists between the number of cells present and the amount of mercury taken up by the cells. Steel (1960) found that such a relationship was correct for *E. coli* cells when plotting the logarithm of the numbers of organisms in the inoculum against the logarithm of the percentage of mercuric chloride utilized. The *E. coli* cells in mixed cultures removed about one-half as much inhibitor as was removed in a pure culture, or 1.9 ppm (38% of a 5 ppm concentration) in 1 hr, leaving a residual concentration of 3.1 ppm in solution. *E. coli* produced sufficient hydrogen sulfide and glutathione to inactivate approximately 2 ppm of the remaining uncombined inhibitor. Thus, a subinhibitory concentration of 1.1 ppm of free mercuric chloride was left in the environment, allowing *S. aureus* to survive at this concentration.

ACKNOWLEDGMENT

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