Substituted Diazenes: Effect on the Growth of Enterobacteria and Possible Use as Selective Agents for Isolation of Pseudomonads

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Incorporation of various diazenes into Trypticase soy media appeared selectively to permit the growth of pseudomonads while inhibiting the growth of a variety of enterobacteria. One of these diazenes, diamide (diazenedicarboxylic acid bisdimethylamide), was shown to be bactericidal for pure cultures of Escherichia coli, Proteus sp., and Salmonella enteritidis and to cause a 1- to 2-hr delay in the growth of Pseudomonas aeruginosa. When mixtures of these four organisms were inoculated into Trypticase soy broth or Trypticase soy agar (TSA) containing diamide, P. aeruginosa grew in overnight cultures. TSA containing diamide was also used successfully to isolate pseudomonads from soil, clinical urine specimens, fish, ground beef, ground pork, and ground veal.

Selenite and tetrathionate, often incorporated into media as selective agents for the growth of salmonellae, have been found, respectively, by Painter (6) and Parker and Allison (7) to act as thiol-oxidizing agents. Diamide, a diazene originally synthesized by Crawford and Raap (1), similarly is capable of oxidizing glutathione within mature red blood cells without affecting cellular function (5) and within Escherichia coli cells causing a bacteriostatic effect (7). In the course of a study designed to examine the effect of diamide, as well as a series of diazenes synthesized recently by Kosower and his associates (3), on the growth of various enterobacteria and their possible use as selective agents for the growth of salmonellae, it was observed that concentrations of diamide which inhibited the growth of salmonellae permitted the growth of Pseudomonas aeruginosa. This observation seemed of practical significance because pseudomonads are frequent contaminants of food products, and their detection is a problem for both food and clinical microbiologists. In this report, we describe the selective action of diazenes on the growth of pseudomonads and other enteric microorganisms.

MATERIALS AND METHODS

Cultures. Many of the Pseudomonas cultures employed in this study were generously supplied by William C. Haynes, Fermentation Laboratory, U.S. Depart-

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2 Retired.
### Table 1. Samples of diazenes from the American Cyanamid Co.

| Sample | Reference | Compound |
|--------|-----------|----------|
| 22     | R-6680-22 | 1,1′-Azobis-(N-2-methoxyethyl)formamide |
| 26     | R-7405-26 | 1,1′-Azobis-(N-n-butylformamide) |
| 146    | R-7260-146| N-n-Butyl-N′-cyclohexyl-1,1′-azobisformamide |
| 155    | R-7270-155| N-n-Butyl-N′-isopropyl-1,1′-azobisformamide |
| 171    | R-6680-171| 1,1′-Azobis-(N-sec-butylformamide) |
| 174    | R-6680-174| N-Ethyl-N′-phenyl-1,1′-azobisformamide |

Additional diazenes were also obtained from the American Cyanamid Co. but were found to be insoluble in water, dioxane, methanol, ethanol, butanol, acetone, 1 N HCl, CCl₄, and petroleum ether and, consequently, were not tested (Table 2).

Compound R-6850-177 (American Cyanamid), 1,1′-azobis-(N-cyclohexyl-formamide),

\[
\text{CH}_2\text{CH}_2\text{CHNHNCN} = \text{NCHNHCH}_2\text{CH}_2\text{CH}_3
\]

was dissolved in methanol in a final concentration of 0.67%. The compound was not inhibitory for *E. coli*, *S. enteritidis*, *Proteus* sp., and *P. aeruginosa* up to and including concentrations of 0.03% (final methanol concentration 5.0%) and was not tested further.

Diamide (Calbiochem, Los Angeles, Cal.) is the

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Diazines. We are grateful to the American Cyanamid Co. for samples of the diazenes listed in Table 1. Of these compounds, only compound 22 was directly water-soluble; it was prepared as a 3.3% solution (w/v) in distilled water. Compounds 146, 155, and 174 were prepared as 3.3% solutions in dioxane, compound 26 was prepared as a 0.67% solution in dioxane, and compound 171 was prepared as a 3.3% solution in methanol. All solutions were stored at 6°C and used within 1 week of their preparation by mixing with TSB in the desired concentration (final pH 7.2). When this resulted in precipitation of the compound in the water-solvent mixture (146 and 171), the media were used as prepared without further attempts to dissolve the chemicals.

model B spectrophotometer at 540 nm or by the standard plate count method. All plating was done in triplicate.
trivial name for diazenedicarboxylic acid bisdimethylamid e \((\text{CH}_2\text{NH})_2\text{NCON}=\text{NCON}\) \((\text{CH}_2\text{NH})_2\). The yellow crystalline material was stored at 6°C and dissolved in distilled water before use in a final concentration of 10.0% (w/v), pH 9.05. Kosower and Kosower (4) have reported that the hydrolytic stability (approximate half-life for hydrolysis in aqueous buffer, pH 7.4) is 3,000 hr. All solutions of diamide were kept in the dark at 6°C and used within 1 week of preparation by mixing directly with TSB or TSA in the desired concentration (final pH 7.2). This was necessary since the hydrolytic stability and the biological activity of the unbuffered reagent exposed to the air was about 2 weeks. We monitored the stability of diamide by loss of selective bactericidal activity and by the loss of the single spectral peak which diamide exhibits in the ultraviolet range at approximately 295 nm [optical density (OD) was recorded on a Cary 14 recording spectrophotometer].

RESULTS

Effect of diazenes on microbial growth in TSB. Various bacterial species were screened for ability to grow in TSB containing diazenes by inoculating tubes containing 2.5 ml of TSB and appropriate amounts of diazenes with approximately 10⁶ viable organisms from a stock TSB culture and observing for growth after 24 hr at 25 or 37°C, or at both temperatures. Table 3 summarizes the results of these experiments. Growth of all enterobacteria was inhibited by diamide and diazenes 22 and 155, whereas all of the Pseudomonas strains grew. A similar but not as inclusive inhibition was observed with diazenes 171 and 174. Sample 26 allowed the growth of several of the enterobacteria although inhibiting the growth of all of the Pseudomonas strains tested. Diazene 146 inhibited the growth of all of the Pseudomonas and Proteus strains but permitted the growth of several salmonellae and all of the E. coli strains tested. It should be noted that the final concentrations of diazenes 26 and 146 necessary to selectively inhibit the Pseudomonas cultures resulted in final dioxane concentrations of 5 and 4%, respectively, in the medium. We have found that Pseudomonas cultures grown in TSB containing dioxane in a final concentration of 4% (v/v) were inhibited by the dioxane alone. The final concentration of dioxane in TSB containing samples 155 and 174 was less than 2.0%. The final concentration of methanol in TSB containing diazene 171 was 4.0%. Control cultures demonstrated that these concentrations of dioxane and methanol in TSB were not inhibitory for any of the cultures employed in this study.

Effect of diamide on growth of enterobacteria in pure culture. The effect of one of the diazenes, diamide, on bacterial growth was studied in more detail. P. aeruginosa cultures (approximately 10⁶ viable cells per inoculum) in 10 ml of TSB containing diamide in a final concentration of 0.05% and assayed periodically by the standard plate count method showed no inhibition of growth, but diamide was bactericidal for E. coli, Proteus sp.,
Table 3. Growth in Trypticase soy broth containing various diazenes after 24 hr of incubation

| Bacterium                  | 0.05% | 0.25% | 0.5% | 0.4% | 0.15% | 0.4% | 0.1% |
|---------------------------|-------|-------|------|------|-------|------|------|
| Escherichia coli A        | 0     | 0     | 3+   | 2+   | 0     | 0    | 1+   |
| E. coli B                 | 0     | 0     | 3+   | 2+   | 0     | 0    | 0    |
| E. coli C                 | 0     | 0     | 3+   | 2+   | 0     | 0    | 0    |
| E. coli D                 | 0     | 0     | 3+   | 2+   | 0     | 0    | 0    |
| E. coli E                 | 0     | 0     | 3+   | 2+   | 0     | 0    | 0    |
| E. coli CC                | 0     | 0     | 3+   | 2+   | 0     | 0    | 0    |
| Salmonella derby          | 0     | 0     | 3+   | 2+   | 0     | 0    | 1+   |
| S. blockley               | 0     | 0     | 3+   | 2+   | 0     | 0    | 0    |
| S. senftenberg            | 0     | 0     | 3+   | 2+   | 0     | 0    | 0    |
| S. typhimurium            | 0     | 0     | 3+   | 2+   | 0     | 0    | 0    |
| S. oranienburg            | 0     | 0     | 3+   | 2+   | 0     | 0    | 0    |
| S. bredeney               | 0     | 0     | 3+   | 2+   | 0     | 0    | 0    |
| S. tennessee              | 0     | 0     | 3+   | 2+   | 0     | 0    | 2+   |
| S. indiana                | 0     | 0     | 3+   | 2+   | 0     | 0    | 2+   |
| S. newport                | 0     | 0     | 3+   | 2+   | 0     | 0    | 2+   |
| S. enteritidis            | 0     | 0     | 3+   | 2+   | 0     | 0    | 0    |
| S. abortus-ovis           | 0     | 0     | 3+   | 2+   | 0     | 0    | 0    |
| S. chester                | 0     | 0     | 3+   | 2+   | 0     | 0    | 0    |
| S. heidelberg             | 0     | 0     | 3+   | 2+   | 0     | 0    | 0    |
| Proteus sp.               | 0     | 0     | 3+   | 2+   | 0     | 0    | 0    |
| Proteus sp.               | 0     | 0     | 3+   | 2+   | 0     | 0    | 0    |
| Proteus sp.               | 0     | 0     | 3+   | 2+   | 0     | 0    | 0    |
| Arizona sp.               | 0     | 0     | 3+   | 2+   | 0     | 0    | 0    |
| Shigella sp.              | 0     | 0     | 3+   | 2+   | 0     | 0    | 0    |
| Enterobacter aerogenes     | 0     | 0     | 3+   | 2+   | 0     | 0    | 0    |
| Klebsiella sp.            | 0     | 0     | 3+   | 2+   | 0     | 0    | 0    |
| Pseudomonas ovalis        | 4+    | 1+    | 0    | 0    | 0     | 0    | 2+   |
| P. aeruginosa             | 4+    | 1+    | 0    | 0    | 0     | 0    | 2+   |
| P. aeruginosa             | 4+    | 1+    | 0    | 0    | 0     | 0    | 2+   |
| P. aeruginosa             | 4+    | 1+    | 0    | 0    | 0     | 0    | 2+   |
| P. aeruginosa             | 4+    | 1+    | 0    | 0    | 0     | 0    | 2+   |
| P. aeruginosa             | 4+    | 1+    | 0    | 0    | 0     | 0    | 2+   |
| P. fluorescens            | 4+    | 1+    | 0    | 0    | 0     | 0    | 2+   |
| P. fluorescens            | 4+    | 1+    | 0    | 0    | 0     | 0    | 2+   |
| P. fluorescens            | 4+    | 1+    | 0    | 0    | 0     | 0    | 2+   |
| P. meldenbergii           | 4+    | 1+    | 0    | 0    | 0     | 0    | 2+   |
| Pseudomonas sp.           | 4+    | 1+    | 0    | 0    | 0     | 0    | 2+   |
| Pseudomonas sp.           | 4+    | 1+    | 0    | 0    | 0     | 0    | 2+   |
| Pseudomonas sp.           | 4+    | 1+    | 0    | 0    | 0     | 0    | 2+   |
| P. putida                 | 4+    | 1+    | 0    | 0    | 0     | 0    | 2+   |
| P. putrefaciens           | 4+    | 1+    | 0    | 0    | 0     | 0    | 2+   |
| P. fluorescens            | 4+    | 1+    | 0    | 0    | 0     | 0    | 2+   |
| P. convexa                | 4+    | 1+    | 0    | 0    | 0     | 0    | 2+   |
| P. fragi                  | 4+    | 1+    | 0    | 0    | 0     | 0    | 2+   |
| P. chloroaphis            | 4+    | 1+    | 0    | 0    | 0     | 0    | 2+   |
| P. aureofaciens           | 4+    | 1+    | 0    | 0    | 0     | 0    | 2+   |

* Tubes containing 2.5 ml of Trypticase soy broth and diazene in the proper concentration were inoculated with approximately 10⁶ organisms of each culture; incubated for 24 hr at 37, 25, or 20°C, depending on the optimum growth temperature; and observed visually for growth, which was measured on a scale of 0 (no turbidity) to 4+ (maximum turbidity).

and S. enteritidis cultured similarly (Fig. 1). That there was some effect of diamide on P. aeruginosa is demonstrated in Fig. 2. When growth of P. aeruginosa at 37°C in TSB and TSB containing 0.05% diamide was measured hourly by means of OD, a brief bacteriostatic effect was noted, as evidenced by a lag of about 1 hr in the growth of the diamide culture. In the same experiment, cultures of E. coli, Proteus sp., and S. enteritidis in TSB alone increased in OD from less than 0.01 to
mixture was inoculated into several tubes each of selenite-cystine broth, tetrathionate broth, and TSB containing 0.05% diamide. The cultures were incubated at 37°C for 24 hr, and surviving organisms were identified by streaking onto Brilliant Green agar and triple sugar-iron-agar slants and, in the case of the salmonellae, by serology. Selective growth of organisms in selenite-cystine and tetrathionate broths followed the expected pattern for these media; however, in every instance, only *P. aeruginosa* grew in and was isolated from the diamide broth. Similar results were obtained when mixed cultures were streaked onto TSA containing 0.05% diamide; only *P. aeruginosa* grew.

**Isolation of pseudomonads from contaminated samples.** Samples (100 g) of ground beef, ground veal, ground pork, and flounder were obtained locally, mixed with 250 ml of sterile TSB in a Waring Blender for 1 min, and incubated at 25°C overnight. A soil sample was mixed with TSB and similarly incubated. These cultures, urine samples known to be contaminated with pseudomonads (Veterans Administration Hospital), and broth suspensions of 32 clinical isolates of *Pseudomonas* sp. (Veterans Administration Hospital) were inoculated directly onto eosin methylene blue agar and TSA containing 0.05% diamide. Of the 32 clinical isolates, 29 grew on both media, one grew on neither medium, and two grew on eosin methylene blue agar but not on TSA-diamide medium. *Pseudomonas* was isolated from all of the other samples on both media. Although only a small number of samples have been tested thus far, TSA containing diamide appears to be effective for the isolation of pseudomonads.

**DISCUSSION**

The results of studies designed to determine the effect of diamide upon the growth and survival of pure cultures demonstrated that it exerted a bactericidal effect upon *E. coli* (Fig. 1). This finding would seem to be in conflict with the report of Wax et al. (8) that the same concentration of diamide (3 × 10^{-4} M) was bacteriostatic but not bactericidal for *E. coli* B. They found no death of *E. coli* B after 15-min treatments with diamide; however, the results summarized in Fig. 1 demonstrated that it takes several hours before the bactericidal effect of diamide upon the *E. coli* strain employed in our studies becomes significant. The outgrowth of *P. aeruginosa* inoculated in very low numbers as part of a mixed culture into diamide-TSB becomes even more impressive when one considers the much slower growth rate exhibited by *P. aeruginosa* as compared to the other organisms, *E.*
coli, Proteus sp., and S. enteritidis, that were present in the mixture in much greater numbers.

The possible usefulness of several diazene-type compounds as selective agents for the growth of pseudomonads has been demonstrated. Four of the diazenes tested (diamide, 22, 155, and 171) permitted growth of pseudomonads but inhibited the growth of the enterobacteria tested (Table 3). Although samples 26 and 146 selectively inhibited several of the pseudomonads listed in Table 3, most of them were inhibited by the high concentration of dioxane (4.0%) which was also present in these cultures. Perhaps dioxane itself can be employed as a selective inhibitor of Pseudomonas cultures. The effect of diazenes 26, 146, and 174 within the several genera appears to be variable so that their usefulness as selective agents for the growth of microorganisms is questionable. It is possible that combinations of the diazenes or use of some basal media other than TSB, or both, might result in a superior selective medium.

Many questions have yet to be answered concerning the mechanism of action of the diazenes. Future work should be centered in this area. For example, since both selenite and diamide are known to be thiol-oxidizing agents, it would be useful to know why selenite permits the growth of salmonellae but not pseudomonads whereas diamide exhibits an opposite pattern.

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