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# 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. De-

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partment of Agriculture, Peoria, Ill. Several *Pseudomonas* cultures and contaminated urine specimens were obtained from Matthew H. Fusillo, Veterans Administration Hospital, Washington, D.C. Other *Pseudomonas* cultures were isolated from soil and from meat and fish purchased locally. Still other cultures of *Pseudomonas* and all other cultures mentioned in this study were from our own collection. Cultures grown in Trypticase soy broth (TSB; BBL) were used as inocula in all studies, except in those instances in which inoculation was done directly from a contaminated sample or mixed culture. When mixed cultures were used as the inoculum, the mixtures were prepared so that 0.1 ml of the mixture resulted in an inoculum containing approximately 10<sup>4</sup> viable cells each of *E. coli*, *S. enteritidis*, and *Proteus* sp. and 10<sup>1</sup> viable cells of *P. aeruginosa*.

**Growth media.** Selenite-cystine, tetrathionate broth, triple sugar iron agar, eosin methylene blue agar, and Brilliant Green agar were obtained from Difco. In some experiments, cultures were tested for their ability to grow in TSB containing various diazenes; in other experiments, diazenes were incorporated into Trypticase soy agar (TSA; BBL) by adding them to the cooled (50 C) sterile agar before pouring plates. *Pseudomonas* cultures were isolated by streaking plates of TSA containing diamide in a final concentration of 0.05% ( $3 \times 10^{-3}$  M), and the number of positive isolations was compared to the number of positive isolations on eosin methylene blue agar. Cultures were designated positive if they conformed to the typical reactions for pseudomonads on triple sugar-iron-agar; in the indole, methyl red, Voges-Proskauer, citrate test; in the oxidase test; and in the Hugh-Leifson reaction in glucose (2).

Growth was measured by turbidity in a Beckman

TABLE 1. Samples of diazenes from the American Cyanamid Co.

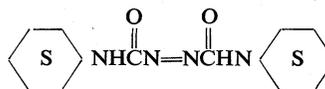
Sample	Reference	Compound
22	R-6680-22	1,1'-Azobis-(N-2-methoxyethyl)formamide $\text{CH}_3\text{OCH}_2\text{CH}_2\text{NHCN}=\text{NCHNCH}_2\text{CH}_2\text{OCH}_3$
26	R-7405-26	1,1'-Azobis-(N-n-butylformamide) $\text{nC}_4\text{H}_9\text{NHCN}=\text{NCHNH}_2\text{C}_4\text{H}_9$
146	R-7260-146	N-n-Butyl-N'-cyclohexyl-1,1'-azobisformamide $\text{nC}_4\text{H}_9\text{NHCN}=\text{NCHN}$ 
155	R-7270-155	N-n-Butyl-N'-isopropyl-1,1'-azobisformamide $\text{nC}_4\text{H}_9\text{NHCN}=\text{NCHNCH}(\text{CH}_3)_2$
171	R-6680-171	1,1'-Azobis-(N-sec-butylformamide) $\text{CH}_3\text{CH}_2\underset{\text{CH}_3}{\text{CH}}\text{NHCN}=\text{NCHNH}\underset{\text{CH}_3}{\text{C}}\text{CH}_2\text{CH}_3$
174	R-6680-174	N-Ethyl-N'-phenyl-1,1'-azobisformamide $\text{CH}_3\text{CH}_2\text{NHCN}=\text{NCHN}$ 

model B spectrophotometer at 540 nm or by the standard plate count method. All plating was done in triplicate.

**Diazenes.** 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.

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, CCl<sub>4</sub>, and petroleum ether and, consequently, were not tested (Table 2).

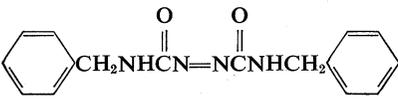
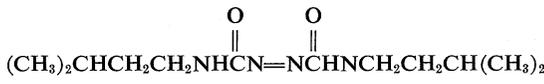
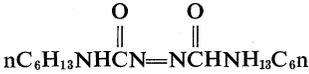
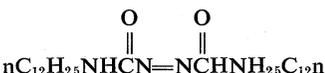
Compound R-6850-177 (American Cyanamid), 1,1'-azobis-(N-cyclohexyl-formamide),



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

TABLE 2. Samples obtained from the American Cyanamid Co. which were not used

Reference	Compound
R-6680-108	1,1'-Azobis-(N-benzylformamide) 
R-7057-167	1,1'-Azobis-(N-isopentylformamide) 
R-7057-59	1,1'-Azobis-(N-n-hexylformamide) 
R-7260-116	1,1'-Azobis-(N-n-dodecylformamide) 

trivial name for diazenedicarboxylic acid bisdimethylamide [(CH<sub>3</sub>)<sub>2</sub>NCON=NCON(CH<sub>3</sub>)<sub>2</sub>]. 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 10<sup>6</sup> 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 inhibi-

tion 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 10<sup>6</sup> 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	Diazenes						
	Diamide (0.05%)	22 (0.2%)	26 (0.5%)	146 (0.4%)	155 (0.15%)	171 (0.4%)	174 (0.1%)
<i>Escherichia coli</i> A.....	0	0	3+	2+	0	0	1+
<i>E. coli</i> B.....	0	0	0	3+	0	0	0
<i>E. coli</i> C.....	0	0	0	4+	0	0	0
<i>E. coli</i> D.....	0	0	0	3+	0	0	0
<i>E. coli</i> E.....	0	0	0	2+	0	0	0
<i>E. coli</i> CC.....	0	0	0	4+	0	0	0
<i>Salmonella derby</i> .....	0	0	2+	3+	0	0	1+
<i>S. blockley</i> .....	0	0	0	0	0	0	2+
<i>S. senftenberg</i> .....	0	0	0	0	0	0	2+
<i>S. typhimurium</i> .....	0	0	0	0	0	0	0
<i>S. oranienburg</i> .....	0	0	0	1+	0	0	0
<i>S. bredeney</i> .....	0	0	2+	1+	0	0	1+
<i>S. tennessee</i> .....	0	0	3+	3+	0	0	2+
<i>S. indiana</i> .....	0	0	3+	3+	0	4+	2+
<i>S. newport</i> .....	0	0	0	0	0	0	1+
<i>S. enteritidis</i> .....	0	0	2+	4+	0	0	0
<i>S. abortus-ovis</i> .....	0	0	0	0	0	0	0
<i>S. chester</i> .....	0	0	0	2+	0	0	2+
<i>S. heidelberg</i> .....	0	0	—	—	0	0	—
<i>Proteus</i> sp.....	0	0	0	0	0	0	0
<i>Proteus</i> sp.....	0	0	0	0	0	0	0
<i>Proteus</i> sp.....	0	0	0	0	0	0	0
<i>Arizona</i> sp.....	0	0	0	0	0	0	0
<i>Shigella</i> sp.....	0	0	0	0	0	0	0
<i>Enterobacter aerogenes</i> .....	0	0	3+	2+	0	1+	1+
<i>Klebsiella</i> sp.....	0	0	3+	3+	0	1+	0
<i>Pseudomonas ovalis</i> .....	4+	1+	0	0	0	2+	3+
<i>P. aeruginosa</i> .....	4+	4+	0	0	4+	4+	2+
<i>P. aeruginosa</i> .....	4+	4+	0	0	3+	1+	3+
<i>P. aeruginosa</i> .....	4+	4+	0	0	4+	3+	4+
<i>P. aeruginosa</i> .....	4+	4+	0	0	2+	1+	3+
<i>P. aeruginosa</i> .....	4+	4+	0	0	2+	1+	3+
<i>P. fluorescens</i> .....	4+	0	0	0	0	0	0
<i>P. fluorescens</i> .....	4+	4+	0	0	4+	2+	3+
<i>P. fluorescens</i> .....	3+	4+	0	0	0	3+	2+
<i>P. mildenbergii</i> .....	4+	4+	0	0	4+	2+	4+
<i>Pseudomonas</i> sp.....	4+	4+	0	0	4+	4+	4+
<i>Pseudomonas</i> sp.....	4+	4+	0	0	4+	4+	4+
<i>Pseudomonas</i> sp.....	4+	4+	0	0	4+	4+	4+
<i>P. putida</i> .....	4+	4+	0	0	4+	4+	4+
<i>P. putrefaciens</i> .....	4+	0	0	0	4+	4+	4+
<i>P. fluorescens</i> .....	2+	0	0	0	4+	4+	0
<i>P. convexa</i> .....	4+	4+	0	0	4+	4+	4+
<i>P. fragi</i> .....	4+	1+	0	0	4+	4+	0
<i>P. chloroaphis</i> .....	4+	3+	0	0	4+	4+	4+
<i>P. aureofaciens</i> .....	4+	3+	0	0	4+	4+	4+

\* Tubes containing 2.5 ml of Trypticase soy broth and diazene in the proper concentration were inoculated with approximately 10<sup>6</sup> 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

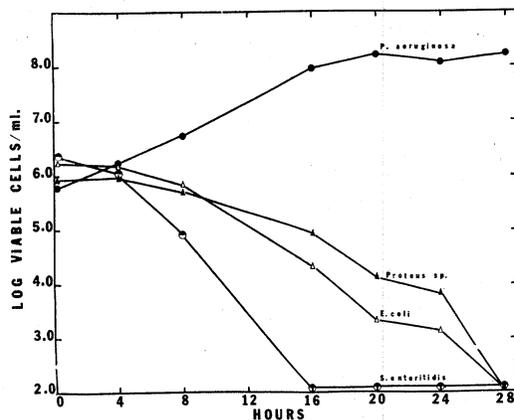


FIG. 1. Effect of diamide on growth and survival of selected cultures. Symbols: ●, *Pseudomonas aeruginosa*; ▲, *Proteus sp.*; △, *Escherichia coli*; ●, *Salmonella enteritidis*.

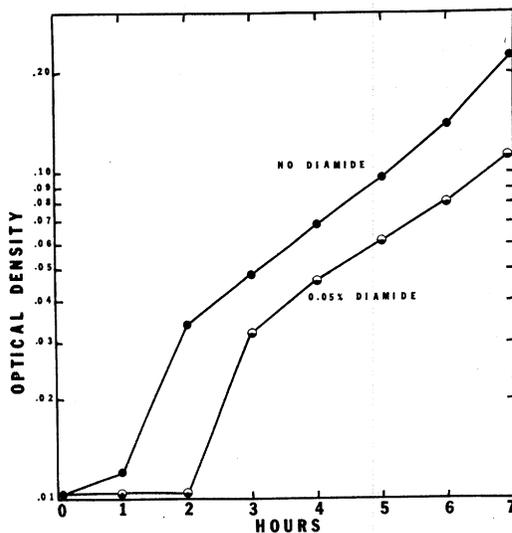


FIG. 2. Effect of diamide on growth of *Pseudomonas aeruginosa*.

greater than 1.0 in 7 hr, whereas similar cultures in TSB containing diamide in a final concentration of 0.05% showed no increase in OD during the same time period. Similar results were obtained when pure cultures of these four organisms were streaked onto plates of TSA containing 0.05% diamide; only *P. aeruginosa* grew.

**Effect of diamide on growth and survival of mixed cultures.** A mixed culture was prepared from stock broth cultures so that it would contain numbers of *E. coli*, *Proteus sp.*, and *S. enteritidis* in relatively high concentrations with respect to *P. aeruginosa*. A 0.1-ml amount of this

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 Blendor 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 ( $3 \times 10^{-3}$  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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