Abolition of Swarming of *Proteus* by *p*-Nitrophenyl Glycerin: General Properties

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Para-nitrophenyl glycerin (PNPG) was shown to be an effective agent to abolish the swarming of *Proteus mirabilis* and *Proteus vulgaris* on predried solid culture media. The level required to abolish swarming varied with the strain of *Proteus*, the components of the medium, and also with the conditions of incubation. Generally 0.1 to 0.2 mM PNPG effectively abolished swarming for at least 24 h with aerobic incubation. Levels of PNPG that abolished swarming showed no effect upon the growth of the cells, little or no effect upon the motility characteristics of the organisms, and no effect upon the cellular morphology. PNPG was found to be freely water soluble, stable to autoclaving, and to retain biological activity for at least one month in prepared culture media stored under refrigeration.

Swarming is briefly described as the formation of concentric zones of bacterial growth (swarm bands) around a central colony on a solid culture medium. These zones of growth will eventually cover the entire surface of the solid medium. Two unique features are normally associated with the swarming phenomenon: (i) the formation of swarm bands is a periodic event, i.e., the first swarm band will appear from the edge of the inoculum and migrate outward for from 1 to 2 h, then stop for several hours prior to the appearance of the second swarm band from the outer edge of the first band and, (ii) there is a morphological change in the cells prior to, and during, swarming from normal short bacilli to greatly elongated "swarm cells." A more detailed description of swarming is contained in the article by Hoeniger (3).

Swarming of *Proteus* has been of interest to the applied microbiologist because it frequently interferes with the isolation or enumeration of other microorganisms from clinical specimens or food products, and the problem has also interested others curious about the underlying mechanism leading to this unusual form of bacterial behavior. The isolation of nonswarming variants of *Proteus* with normal growth and motility characteristics (D. L. Archer and W. M. Bain, Bacteriol. Proc., p. 23, 1970; W. C. Schmidt et al., Bacteriol. Proc., p. 28, 1971) and the reportedly successful transduction of the swarming characteristic (2) support the contention that swarming requires more than just a motile organism. What these additional requirements might be, or the underlying mechanism, and the biochemical events leading to swarming all remain unknown.

My interest was stimulated in late 1966 when a report from Germany appeared in this journal (5) suggesting that *p*-nitrophenyl glycerin (PNPG) specifically inhibited the swarming of *Proteus* without affecting growth or motility. The purpose of the experiments reported here was to carefully examine swarm inhibition by PNPG and to also determine some of the properties of this unusual anti-swarm agent.

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MATERIALS AND METHODS

**Organisms.** Cultures of *Proteus mirabilis*, *Proteus vulgaris*, *Escherichia coli* K-12, *Bacillus cereus* var. *mycoides*, *Bacillus circulans*, and *Myxococcus xanthus* were from the stock culture collection of the Department of Bacteriology, Iowa State University. Other cultures of *P. mirabilis* were obtained from M. W. Bain and C. D. Jeffries. The culture of *Bacillus alvei* was obtained from the American Type Culture Collection (ATCC no. 6344).

**Culture media.** Broth cultures of the *Proteus* species and *E. coli* were grown at 35 C in tryptose broth (Difco) consisting of tryptose, 20 g/liter, and NaCl, 15 g/liter. For the preparation of a solid medium Bacto agar (1.5%) was included. After sterilization by autoclaving, the medium was cooled to 45 C, then
dispensed into plastic petri dishes. All agar plates were predried by incubation for 16 h at 35 C before use. The predrying removed water droplets from the surface of the medium and also allowed detection of contamination. *B. cereus* and *B. alvei* were grown in Trypticase soy broth at room temperature, andpredried Tryptose soy agar (BBL) was used as a solid medium with these organisms. *M. xanthus* was grown and tested on a solid medium consisting of either a 1% peptonized milk and 1.5% agar at 25 C, or 0.8% skim milk plus 1.5% agar.

**Swarm inhibition studies.** To determine concentrations of PNPG that inhibited and abolished swarming of *Proteus*, duplicate plates were prepared containing 20 ml of tryptose agar and different levels of PNPG. Each plate was inoculated in the center with 10 filters of a 16-h broth culture. At 30-min intervals all plates were examined, and swarming was measured as the outer diameter of the swarm band. *P. mirabilis* was tested at 35 C, whereas *P. vulgaris* was tested at 28 C. These temperatures were chosen to provide maximum rates of swarming by the strains under study.

**Growth inhibition studies.** Tryptose broth (100 ml) contained in 250-ml nephelometry flasks with and without selected levels of PNPG was used for the quantitative measurement of growth. All flasks were agitated during the experiments, and turbidity followed at 30-min intervals at 540 nm. From plots of log absorbancy versus time, growth rates (as mass doubling times) were determined. For these tests, an inoculum of 2.5 ml of a 16-h broth culture was employed.

**Motility studies.** The quantitative measurement of motility was achieved by using broth cultures growing under conditions identical to those used in the growth studies. At selected absorbancies a 5-ml sample was aseptically removed from the growth flask, and the percent motile cells and average rates of motility were determined at room temperature. Both determinations were completed within 5 min. When dilution was required, sterile tryptose broth preincubated at the correct growth temperature with the correct level of PNPG was used as diluent.

The percent motile cells was determined by microscopic observation at approximately \( x=1,000 \) by using a hanging-drop preparation with a bright-field microscope. The middle of the drop was selected for observation to avoid artifacts due to interfaces. The motile cells in a given field were counted followed by counting of the non-motile cells in the same field. Approximately 5 fields were counted per sample.

Measurement of average rates of motility required the use of a calibrated ocular micrometer marked in a series of grid squares. Hanging-drop preparations were employed and, as above, the middle of the drop was selected for measurement. For these studies a PDP-12 computer was employed as a timer and data storage bank. The computer also allowed the exclusion of an aborted measurement of motility. Two rocker switches are connected to the computer so that the operator has easy access to these switches while seated at the microscope. As a cell enters a grid square (perpendicular to one of the axes), the timing switch is depressed starting the computer timer. When the cell leaves the grid square the timer switch is returned to the off position, stopping the computer timer and entering the time interval into the memory bank. In the event that the organism fails to completely transverse the grid square because of spinning, diving, reversing direction, etc., the second, or abort, switch is depressed, stopping the computer from entering this time interval into the storage area. Normally 20 to 50 organisms were measured for each sample, and upon command the computer prints out the time interval for each organism and the average time interval for the sample. The operator then calculates the average rate of movement from the calibration of the ocular micrometer. The same operator was used for all measurements of average rates of motility reported in this paper.

**Examination of cell morphology.** Samples were removed from flasks of liquid growth medium and applied to the surface of Formvar-covered (lightly carbon coated) copper grids (150 mesh). The cells were allowed to settle onto the grids for a period of 2 min, then excess fluid was removed with filter paper. A solution of phosphotungstic acid (2%, pH 7.4 with KOH) was immediately applied to each grid, and staining was allowed to proceed for 2 min. Excess stain was removed with filter paper, and the grids were examined immediately in a Hitachi HU-11C electron microscope. Samples were also removed from agar surfaces by touching the coated grid surface to the region just beyond the outer edge of a swarm band after a small droplet of water had been applied to the outer edge of the band. This was followed by immediate staining.

**Chemicals.** *p*-Nitrophenyl glycerin was obtained from both Regis Chemical Co. and Sigma Chemical Co. The product of each manufacturer was found to possess equal biological activity. Initially, PNPG was sterilized by filtration through a 0.45-μm membrane filter, but when experiments revealed no loss of activity with autoclaving then PNPG was steam sterilized.

**RESULTS**

When various levels of PNPG were incorporated into tryptose agar, several effects upon swarming of *P. mirabilis* became apparent. A concentration as low as 0.02 mM showed slight swarm inhibition, but a level of 0.1 mM was required to abolish swarming for at least 24 h (Fig. 1). Similar results were obtained with *P. vulgaris*. Even at 0.1 mM PNPG *P. mirabilis* would eventually swarm, but this required about 3 days at 35 C. The eventual swarm bands that did appear were asymmetrical and suggested the selection of a spontaneous mutant resistant to the action of PNPG. Further support for this hypothesis comes from preincubating uninoculated plates for 3 days at 35 C, then inoculating the plates with a fresh culture of *P. mirabilis*. Normal swarm inhibition was ob-
Fig. 1. Inhibition of the swarming of P. mirabilis on Tryptose agar at 35 C. Measurement of swarm diameters were made at 30-min intervals, and the Iowa State University strain of P. mirabilis was used for this study.

...served, indicating no inactivation of PNPG with preincubation time or temperature. Levels of PNPG higher than 0.1 mM abolished swarming of Proteus indefinitely, and not until levels approached 0.5 mM did any growth inhibition appear noticeable on the surface of plates. Electron micrographs from the edge of the central colony on plates with 0.1 mM PNPG indicated that PNPG did not abolish formation of swarm cells, but the number of long forms was reduced over the number found on plates without PNPG. No other changes were observed in number, location, nor morphology of flagella or pili when cells were inhibited by PNPG at 0.1 mM or 0.2 mM levels.

The results reported above and found in Fig. 1 were obtained by using the Iowa State Culture Collection strain of P. mirabilis. When similar swarm inhibition tests were performed with a number of other strains of P. mirabilis, 8 of the 10 strains tested showed complete swarm inhibition with 0.2 mM PNPG. The other 2 strains, however, required 0.3 mM PNPG to abolish swarming for 48 h at 35 C.

The results from studies of growth of P. mirabilis and P. vulgaris are shown in Table 1. This table also includes data from an identical study using E. coli K-12 for comparative purposes. Mass doubling times for the two species of Proteus are essentially identical with and without 0.1 mM PNPG; however this level of PNPG does slow the growth rate of the E. coli culture. When the level of PNPG was increased to 0.5 mM there was growth inhibition towards P. mirabilis. Even though there is no detectable growth inhibition at 0.1 mM PNPG with the two species of Proteus when higher levels of PNPG are used, the first sign of growth inhibition appears as a delay in onset of exponential growth rather than as a change in rate of growth during the exponential phase. When samples were removed from the growth flasks with and without 0.1 mM PNPG and examined in the electron microscope, no changes in cellular morphology were detected due to the presence of PNPG.

Table 2 contains the results from the quantitative study of motility of P. mirabilis, P. vulgaris, and E. coli K-12 in tryptose broth. This table presents average rates of motility and percent motile cells at four different times in the growth cycle. It should be noted that with P. mirabilis both the average rates of motility and the percent motile cells are unaffected by the presence of 0.1 mM PNPG, at least early in the growth cycle. When the absorbancy (growth) reaches 0.75 the cells slow their average rate of motility but remain highly motile in 0.1 mM

### Table 1. Doubling times in Tryptose broth (minutes) during exponential growth

| Organism and temperature | No PNPG | 0.1 mM PNPG |
|--------------------------|---------|-------------|
| P. mirabilis (35 C)      | 26      | 27          |
| P. vulgaris (28 C)       | 50      | 51          |
| E. coli (35 C)           | 25      | 29          |

*Doubling times calculated on the basis of time required for the optical density of the broth culture to increase by a factor of 2.

### Table 2. Average rates of motility and percent motile cells in Tryptose broth

| Organism and temperature | Growth as OD* at 540 nm | Motility in % per sec and % motile cells |
|--------------------------|-------------------------|----------------------------------------|
|                          |                         | No PNPG                                |
| P. mirabilis (35 C)      | 0.11 10.9 (20%)         | 13.4 (20%)                             |
|                          | 0.25 13.5 (25%)         | 16.5 (23%)                             |
|                          | 0.45 23.2 (42%)         | 26.4 (41%)                             |
|                          | 0.75 33.1 (65%)         | 25.4 (65%)                             |
| P. vulgaris (28 C)       | 0.10 15.8 (11%)         | 10.8 (7%)                              |
|                          | 0.22 16.2 (14%)         | 19.3 (11%)                             |
|                          | 0.44 20.8 (8%)          | 23.2 (8%)                              |
|                          | 0.80 32.9 (12%)         | 26.7 (5%)                              |
| E. coli (35 C)           | 0.10 5.8 (5%)           | 8.8 (3%)                               |
|                          | 0.22 14.7 (6%)          | 17.0 (9%)                              |
|                          | 0.43 26.8 (60%)         | 23.3 (29%)                             |
|                          | 0.75 29.5 (67%)         | 23.4 (27%)                             |

*Numbers in parentheses indicate percent motile cells.

*OD = optical density.
PNPG. *P. vulgaris* shows a similar picture, but always has fewer motile cells than the culture of *P. mirabilis*. In contrast to the *Proteus* species, *E. coli* shows significant inhibition of motility at an optical density of 0.43 and beyond. The cells that are motile have apparently normal rates of motion, but fewer cells are motile in the presence of PNPG.

**Properties of PNPG.** This chemical is a white powder that is freely soluble in water. Sterile solutions retain biological activity for at least one month when stored in the frozen state. Initially, PNPG was filter sterilized, but when it became commercially available it was possible to directly test the effect of autoclaving upon the biological activity of PNPG. The activity was found to be identical between autoclaved and filter-sterilized PNPG. When PNPG was added to rehydrated culture media before autoclaving, no loss in biological activity occurred with steam sterilization in the presence of organic matter. The agent seems completely stable for at least one month when present in solid media in petri plates stored at 5°C.

During the course of this study and the application of PNPG into clinical culture media it became apparent that the concentration of PNPG required to abolish swarming of *Proteus* depended upon the nature of the culture medium and also upon the conditions of incubation. In general, the richer the medium, the higher the level of PNPG required to abolish swarming. Table 3 shows the effect of the addition of yeast extract to a brain heart infusion medium supplemented with sheep blood. When petri plates were incubated under anaerobic conditions (GasPak, BBL) or in a candle jar, higher levels of PNPG were also required. Table 4 shows the effect of anaerobic and candle jar incubation upon anti-swarm activity.

Tests were undertaken to see if the CO₂ tension or reducing atmosphere inactivated the PNPG in the medium, thus effectively reducing the amount present in an active form. Quadruplicate plates were prepared with and without PNPG at 0.4 mM in an anaerobic medium consisting of brain heart infusion, yeast extract, cysteine, menadione, and sheep blood. One half of the plates were incubated after predrying in a GasPak for 22 h, whereas the other half were stored for the same period of time aerobically. After this preincubation period all plates were inoculated with a fresh culture of *P. mirabilis*, and one-half of each group of plates were incubated aerobically, whereas the other half was placed into a fresh GasPak. No major differences were observed in anti-swarm activity between plates receiving anaerobic preincubation and aerobic preincubation. Thus, the enhanced swarming of *P. mirabilis* under anaerobic incubation is due to a change in the organism's sensitivity to PNPG and not due to inactivation of the PNPG by the environmental conditions.

**Sensitivity of other organisms to PNPG.** A number of other organisms were screened for sensitivity to PNPG at 0.2 mM level in Tripticase soy agar. *B. alvei*, *B. circulans*, and *B. cereus* var. *mycoides* were all found to be sensitive to PNPG. The first two species failed to form motile colonies, but did show central colony growth. *B. cereus* var. *mycoides* showed a reduced ability to form the characteristic spreading growth in the presence of PNPG. When *M. xanthus* was tested in the presence of PNPG on skim milk medium and peptonized milk medium, it showed no sensitivity to PNPG at 0.2 mM level. Note that the tests with the

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**Table 3. Stimulation of swarming of *P. mirabilis* by yeast extract**

| Medium supplement | Incubation conditions | Duration of swarm abolation (h) |
|-------------------|-----------------------|--------------------------------|
| None              | Aerobic               | >72                            |
| 0.5% Yeast extract| Aerobic               | 30                             |
| None              | Anaerobic (GasPak)    | 38                             |
| 0.5% Yeast extract| Anaerobic (GasPak)    | 30                             |

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**Table 4. Effect of incubation conditions on anti-swarm activity of PNPG towards *P. mirabilis***

| PNPG concentration | Incubation conditions | Duration of swarm abolation (h) |
|--------------------|-----------------------|--------------------------------|
| 0.1 mM             | Aerobic               | >80                            |
| 0.1 mM             | Candle jar            | 20                             |
| 0.1 mM             | Anaerobic (GasPak)    | 25                             |
| 0.2 mM             | Aerobic               | >80                            |
| 0.2 mM             | Candle jar            | >80                            |
| 0.2 mM             | Anaerobic (GasPak)    | 30                             |

* All tests performed using the Iowa State University strain of *P. mirabilis*.
Bacillus species were of preliminary nature, and no tests were performed to determine if PNPG affected growth or motility in these organisms.

**DISCUSSION**

It must be emphasized that all of the solid media used in this study were predried for a 16-h period to remove water droplets from the agar surface. As it turned out, this step is essential for reproducible results with anti-swarm agents such as PNPG. This anti-swarm agent does not inhibit cell motility; thus if a water droplet or a film of water is present the organisms will cover the plate by motility rather than by true swarming. These predried plates support excellent growth of all but the most moisture-loving organisms.

From these studies it is apparent that PNPG is an anti-swarm agent with unusual properties, in that its action cannot be explained by inhibition of growth or cell motility. These findings are in agreement with those of Kopp et al. (5), although their evidence for ruling out growth and motility inhibition was not conclusive. It is also apparent that at higher levels with Proteus (0.5 mM and above) and with 0.1 mM levels with E. coli, PNPG has other physiological effects besides swarm inhibition. Fortunately low levels of the anti-swarm agent are fully effective in controlling the swarming of Proteus in most culture media. When PNPG is considered with other commonly used anti-swarm agents such as phenylethanol or sodium azide, the specificity of swarm inhibition becomes more noticeable. These agents act by interference with motility in the case of phenylethanol (5) and as a general growth inhibitor with azide (6), yet these agents are commonly used to aid in the isolation of clinically important bacteria contaminated with Proteus. One can only speculate as to the number of significant organisms that fail to grow on the isolation media supplemented with these agents. The next paper in this series will examine the viability of streptococci and staphylococci with PNPG as an anti-swarm agent compared to azide and phenylethanol (8).

The stimulatory effect of yeast extract in the culture medium was observed by Jones and Park (4) and more recently by Brogen et al. (1). My studies also show that yeast extract has some stimulatory effect upon swarming, but this was shown in an indirect manner, in that higher concentrations of PNPG were required to control swarming on a medium containing yeast extract than the same medium without yeast extract. The effect of the CO₂ atmosphere or the GasPak incubation also suggests that these environmental conditions also stimulate the swarming of Proteus. Apparently the swarming characteristic is closely linked to the overall metabolism of the cell, and any number of chemical or physical agents should have an effect upon swarming. It is unfortunate that so little is known about the mechanism that leads to this phenomenon. Lominski and Lendrum (7) interpreted the results of their studies to indicate that swarming of Proteus might best be explained as a form of negative chemotaxis. According to this hypothesis, when species of Proteus that are capable of swarming are growing on the surface of a solid medium they produce one or more toxic waste products from metabolism. These waste products diffuse into the solid medium and away from the area of growth producing a gradient. These toxic products cause the morphological change in the cells and lead to movement away from the inoculum (negative chemotaxis). When the swarm cells reach an environment free of the toxic material, or at least below the detectable threshold concentration, they divide, and swarming stops until the level of the waste product is again increased due to cell growth and metabolism. With this working hypothesis the effect of yeast extract and the conditions of incubation could either affect the rate of formation of a toxic product or the sensitivity of the organism to the toxic material. PNPG might interfere with either the production of the toxic product or the sensitivity of the organism to this material.

That P. mirabilis will eventually swarm on 0.1 PNPG after 30 to 72 h suggests the eventual development of resistance to the action of this agent. It is noteworthy that cells grown in broth containing PNPG do not develop this resistance in the broth environment, but cells picked from the edge of a swarm band after such a band has formed show less sensitivity to 0.1 mM PNPG as they will swarm sooner than an inoculum previously unexposed to PNPG (unpublished observations). These facts argue in favor of the specific action of PNPG upon swarming and not upon growth, because no selective pressure was detected by this agent in a broth system where swarming does not occur. When the selective pressure is applied to a swarming system on a solid medium, it is simple to select a variant of the wild-type strain that is less sensitive to the PNPG.

Of a more concrete nature are the findings that PNPG has numerous properties that make it a potentially superior anti-swarm agent for the isolation of clinically important bacteria, or to simplify viable cell number determinations or pure culture isolation with Proteus species. This
compound can be sterilized in the culture medium by conventional methods, and it will retain biological activity permitting storage of the prepared medium. It is active at low concentrations, thereby reducing the price of its use to acceptable levels. Finally, it is commercially available. The accompanying article (8) deals with the application of PNPG in blood agar for the isolation of staphylococci and streptococci.

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