MICROBIAL INTERACTIONS - EFFECT OF *Pseudomonas aeruginosa* AND PYOCYANINE ON THE GROWTH OF *Salmonella thompson*

by

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Short Title:

MICROBIAL INTERACTIONS - PSEUDOMONAS AND SALMONELLA

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A B S T R A C T

Ph.D. Malcolm S. McDonald Microbiology

MICROBIAL INTERACTIONS - EFFECT OF *Pseudomonas aeruginosa* AND PYOCYANINE ON THE GROWTH OF *Salmonella thompson*

The interactions of bacteria commonly associated with foods were investigated. Preliminary studies indicated that the growth of *Pseudomonas aeruginosa* NHW M1 and several Salmonella species was not influenced when they were grown in mixed culture. The onset of logarithmic growth of *S. thompson* MCC 608 was delayed for several hours in media containing *P. aeruginosa* culture filtrate. Addition of pseudomonad filtrate to Salmonella cultures during logarithmic growth, caused viable counts to remain constant for several hours.

Pyocyanine, a blue pigment in the filtrate, was shown to be an important factor in affecting *S. thompson* growth. Other factors in the filtrate were also involved but were not identified.

The inhibitory capacity of pyocyanine was related to the availability of oxygen. Cells grown anaerobically or under reduced oxygen conditions recovered from the inhibition more rapidly. Strict aerobes or facultative organisms growing under conditions requiring an aerobic mode of growth did not recover in the presence of pyocyanine. Pyocyanine was shown to oxidize reduced nicotine adenine dinucleotide (NADH) *in vitro* and *S. thompson* cells exposed to pyocyanine had

reduced levels of NADH as compared to control cells. The level of cytochrome b in *S. thompson* cells grown in the presence of pyocyanine was also reduced.

The immediate effect of pyocyanine addition to *S. thompson* cultures appeared to be the oxidation of reduced factors such as NADH and the long term effect was suppression of energy producing mechanisms such as the electron transport chain.

R E S U M E

Ph.D Malcolm S. McDonald Microbiologie

INTERACTIONS MICROBIONNES - EFFET DE *Pseudomonas aeruginosa* ET DE LA PYOCYANINE SUR LA CROISSANCE DE *Salmonella thompson*

Les interactions les plus fréquentes entre bactéries des aliments ont été examinés. Des études préliminaires ont indiqué que la croissance de *Pseudomonas aeruginosa* NHW M1 et celle de plusieurs espèces de *Salmonella* n'était pas influencée lorsque les bactéries étaient mises en culture mixte. Le début de la phase logarithimique de *S. thompson* MCC 608 était retardée de plusieurs heures dans les milieux contenant un filtrat de culture de *P. aeruginosa*. L'addition de ce filtrat aux cultures de *Salmonella* durant la croissance logarithimique eut pour effet de stabiliser les unités viables pour plusieurs heures.

Il a été démontré que la pyocyanine, un pigment bleu, présent dans le filtrat de culture était un facteur important qui affectait le croissance de *S. thompson*. D'autre facteurs dans le filtrat ont aussi été impliqués mais n'ont pas été identifiés.

Le pouvoir inhibiteur de la pyocyanine a été relié à la quantité d'oxygène disponible. Des cellules poussées anaérobiquement ou en condition réduite d'oxygène se sont remises de l'inhibition plus rapidement. Les organismes aérobiques stricts ou les facultatifs poussant selon les conditions exigées par un mode de croissance

aérobique ne se sont pas remises de l'inhibition en présence de la pyocyanine. Il a été démontré *in vitro* que la pyocyanine oxyde l'adénine nicotine dinucléotide et que les cellules de *S. thompson* exposées à la pyocyanine possédaient des quantités réduites de NADH comparativement aux cellules controles. Les cellules de *S. thompson* poussées en présence de pyocyanine contenaient aussi une quantité réduite de cytochrome b. L'addition de pyocyanine aux cultures de *S. thompson* semble avoir un effet immédiat, celui d'oxyder des substances réduites tel le NADH et un effet à long terme, celui d'inhiber des mécanismes producteurs d'énergie tel la chaine de transport d'électrons.

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A C K N O W L E D G E M E N T S

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I would especially like to acknowledge my wife, Linda, without whose support this work would not have been possible.

L I S T of T A B L E S

LIST of FIGURES

CLAIM of CONTRIBUTIONS to KNOWLEDGE

1. *P. aeruginosa* NHW M1 and *S. thompson* MCC 608 did not inhibit the growth of each other in mixed cultures.

2. Pseudomonad culture filtrate inhibited or delayed the growth of *S. thompson* MCC 608.

3. Pseudomonad culture filtrate may cause injury to *S. thompson* MCC 608 cells as indicated by increased sensitivity of the cells to selective media.

4. The inhibitory factors in the filtrates investigated in this study did not appear to involve "pyocyanase" as described by Waksman (1945) or the "pyo" compounds of Hayes *et al.* (1945).

5. Pyocyanine had a major role in the inhibitory activity of *P. aeruginosa* NHW M1 culture filtrates.

6. Removal of pyocyanine and the fatty acids from pseudomonad culture filtrate did not cause the filtrate to lose all of its inhibitory activity as suggested by Waksman (1945).

7. Gamma irradiation of pyocyanine solutions caused them to lose their blue colour and inhibitory activity.

8. Strict aerobes were more sensitive to pyocyanine than facultatively anaerobic or strictly anaerobic bacteria.

9. *S. thompson* MCC 608 was less sensitive to pyocyanine when grown under reduced oxygen conditions.

10. Pyocyanine at 50 µg/ml does not kill *S. thompson* MCC 608 cells but causes a shift to a slower growth rate.

11. Pyocyanine treated *S. thompson* MCC 608 cultures do continue to grow as indicated by continued sensitivity to penicillin.

12. Some *S. thompson* MCC 608 cells do become elongated when treated with pyocyanine.

13. Pyocyanine treatment does not prevent compounds such as alanine, glucose and uracil from entering *S. thompson* MCC 608 cells.

14. Pyocyanine treatment does cause decreased oxygen consumption by *S. thompson* MCC 608 cells metabolizing a variety of substrates.

15. Pyocyanine treated *S. thompson* MCC 608 cells have reduced levels of NADH as compared to control cells.

16. *S. thompson* MCC 608 cells grown in the presence of pyocyanine have reduced levels of cytochrome b.

17. The growth of *S. thompson* MCC 608 in BHI medium containing pyocyanine caused the concentration of pyocyanine to decrease with time. There was not a large decrease in concentration before the culture entered the logarithmic phase of growth.

INTRODUCTION

The microbial flora of natural systems is usually composed of a variety of microorganisms. The types of microorganisms found in any environment are determined by several factors. These factors include temperature, pH, nutrients, and the presence of toxic substances (Waksman, 1945). Microorganisms may in turn alter some of these substances either into essential nutrients for other organisms or toxic substances which may prevent or retard the growth of some life forms in the same system. Microbial interactions are therefore important in determining the resultant or predominant microbial flora in any system.

The study reported here is an investigation of the interaction of bacteria which are commonly assbciated with foods. The possible influence of a food spoilage organism, *Pseudomonas aeruginosa*, on the growth and survival of a food pathogen, *Salmonella thompson*, was studied in mixed culture, culture filtrates and culture filtrate fraction systems.

L I T E R A T U R E R E V I E W

Microbial interaction in foods:

The interaction of bacteria commonly associated with foods has been studied by several researchers. These microorganisms can be broadly divided into two groups, the food spoilage organisms and the food pathogens. Collins-Thompson *et al.* (1973) reported that the common food spoilage organism, *Pseudomonas aeruginosa,* isolated from meat, produced lytic enzyme(s) which caused decreased yields, loss of salt tolerance and reduced enterotoxin production by *Staphylococcus aureus.* Oblinger and Kraft (1970) showed that *P. aeruginosa* culture filtrates inhibited the growth of several microorganisms including members of the *Escherichia, Streptococcus, Staphylococcus* and *Salmonella* genera. These researchers suggested that the inhibition was probably not due to a protein (enzyme). Troller and Frazier (1963) thought that *P. aeruginosa* inhibited growth of *S. aureus* by out-competing it for nutrients.

Other workers on the other hand, have shown that *P. aeruginosa* can stimulate the growth of *S. aureus.* De Repentigny and Mathieu (1974) reported that both *S. aureus* cells subjected to rifamycin and *S. aureus* auxotrophic mutants requiring thiamine or tryptophan were actually stimulated when grown in mixed culture with *P. aeruginosa.* Growth stimulation of *S. aureus* by *P. aeruginosa* was also reported by Graves and Frazier (1963).

P. aeruginosa appears to be capable of both stimulating or inhibiting the growth of other bacteria found in foods. The type of influence that is exerted appears to depend on the microorganisms used and on the conditions of the experiments such as the use of mixed cultures or culture filtrates.

Antimicrobial products of *P. aeruginosa*

P. aeruginosa is known to produce substances which inhibit the growth of other microorganisms. These substances can be prepared from *Pseudomonas* cell free culture filtrate. Waksman (1945) reported that *P. aeruginosa* produced three inhibitory compounds: pyocyanase, pyocyanine and α oxyphenazine. Pyocyanase was considered to be an enzyme which caused lysis of bacterial cells. The preparation withstood heating in flow steam for 2 h. Pyocyanase therefore is probably not an "enzyme" as they are defined today. Pyocyanine is a blue pigment which is produced during the stationary phase of growth. It has been shown to inhibit the growth of a variety of microorganisms and seems to be more active against Gram-positive than against Gram-negative bacteria. α-Oxyphenazine is a yellow pigment, a derivative of pyocyanine and also has bacteriostatic activity.

A series of inhibitory "pyo" compounds were also isolated by Hayes *et al* (1945) through extraction of whole *P. aeruginosa* cells. The pyo-compounds were again generally more active against the Gram-positive bacteria. Further studies by Wells (1952) showed that some

of these compounds were quinolinols.

Collins-Thompson *et al.* (1973) demonstrated the production of a Staphylolytic enzyme(s) by *P. aeruginosa*. They found that *S. aureus* did not reach maximum population density, lost its salt tolerance, and had decreased enterotoxin production when grown in mixed culture with *P. aeruginosa*.

Several different factors, capable of inhibiting various micro-organisms, appear to be produced by *P. aeruginosa* at different times during its growth cycle. Some bacteria such as *S. aureus* appear to be more sensitive than others such as *Escherichia coli*. One of these factors, pyocyanine, is an important constituent of some inhibitory *Pseudomonas* filtrates.

Pyocyanine

Pyocyanine, a phenazine derivative, is a blue pigment which can act as a redox compound donating or accepting two electrons. The structural formulae of the three major forms are given in Figure 1. In the oxidized form the compound is blue under alkaline and red under acid conditions. The reduced form is colourless. Reduced pyocyanine is extremely unstable in air and autooxidizes (Oppenheimer and Stern, 1939). Pyocyanine can act as an indicator of both pH (Blue - red change) and redox state (coloured - colourless change).

Pyocyanine has bacteriostatic activity against a wide range of

FIGURE 1.

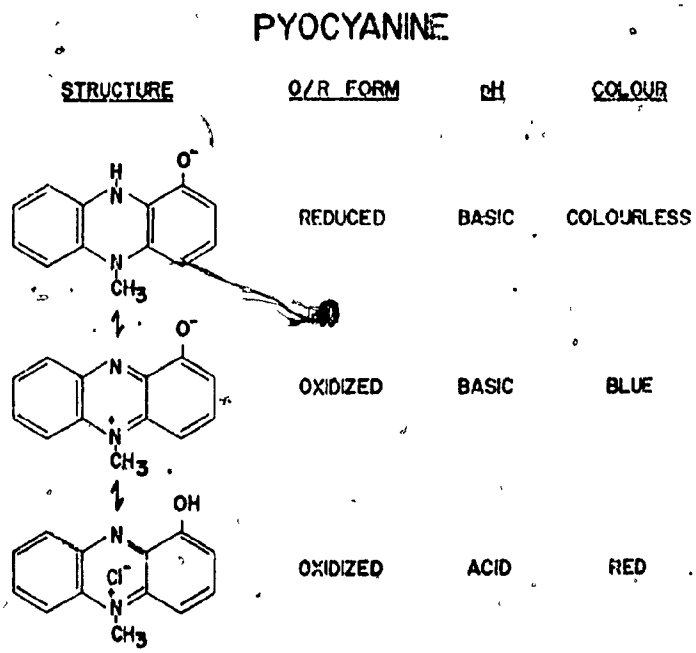


Fig. 1. The principle forms of pyocyanine (Mahler, H.R. and Cordes E.H. 1966).

microorganisms (Swan and Felton, 1957). The effect appears to be more pronounced against Gram-positive than against Gram-negative organisms (Waksman, 1945).

The mechanism by which pyocyanine influences growth is not clear and its role in pseudomonad metabolism is not known. Friedheim (1931) reported that it was a respiratory catalyst because it increased oxygen consumption in pseudomonad cells grown under conditions where little pigment was produced. Pyocyanine can cause increased oxygen consumption in both animal and bacterial systems (Caltrider, 1967). It has been reported to decrease oxygen consumption by *Vibrio cholerae* and *S. aureus* (Schoental, 1941). Oxidative phosphorylation is decreased in the presence of pyocyanine in brain tissue and liver mitochondria (Case and McIlwain, 1951; Judah and Williams-Ashman, 1951). Phosphorylation was reported increased in chloroplasts (Hill and Walker, 1959).

Landau *et al.* (1963) demonstrated inhibition by pyocyanine of substrate metabolism in both the phosphogluconate and Embden-Meyerhof pathways in liver slices. They also showed decreased incorporation of labeled substrates into glycogen and fatty acids. In a study with *Pseudomonas fluorescens* Campbell *et al.* (1957) showed that pyocyanine stopped oxidation of glucose, 2-ketogluconate, malate and lactate at the keto acid stage. They found that this was also true with *Proteus vulgaris* and extracts of *E. coli* cells but not with *E. coli* whole cells.

From the *E. coli* work they concluded that pyocyanine did not penetrate the cell membrane. Pyocyanine has also been reported to inhibit the activity of a number of enzymes including glucose-6-phosphate dehydrogenase (Campbell *et al.*, 1957), L-amino acid oxidase, succinate dehydrogenase, and staphylococcoagulase (Caltrider, 1967).

The activity of pyocyanine in cell systems is very complex. It has the ability to influence several systems and the effect on any metabolic system can be different in different organisms. The activity, however, appears to revolve around relationships with oxygen and its high affinity for flavoproteins (Caltrider, 1967). The mechanisms of action of pyocyanine would therefore depend upon the cell system in which it is used.

Salmonella cytochromes:

Salmonellae are common food pathogens and are members of the Enterobacteriaceae (Bergey, 1974). They are Gram-negative, facultatively anaerobic rods. The principle cytochromes found in the Enterobacteriaceae are b_1, a_1, d and o (Sasarman, 1972). Cytochrome b_1 always seems to be present in bacteria and it is often the only band that is readily visible in cytochrome scans (Smith, 1954). The level of cytochrome is usually higher in cells grown aerobically (Ishida and Hino, 1972). The carbon source in the medium can also effect cytochrome levels. Levels are higher in cells grown on sub-

strates such as glutamate or succinate than on glucose or mannose (Richmond and Maaloe, 1962). *Salmonella typhimurium* has been shown to have cytochromes b_1, a_1 and d (Sasarman, 1972).

MATERIALS and METHODS

1. Cultures

The cultures listed in Table 1. were used in this study. *Pseudomonas aeruginosa* NHW M1 was a meat isolate obtained from Dr. P Collins-Thompson at the National Health and Welfare Health Protection Branch, Tunney's Pasture, Ottawa, Ontario. It had been used in a study of the interaction between *P. aeruginosa* and *Staphylococcus aureus* (Collins-Thompson *et al.*, 1973) and was used in most of our preliminary experiments. *Pseudomonas aeruginosa Mac* 436 was used in later studies for the production of pyocyanine. This culture produced much larger quantities of pyocyanine than the NHW M1 culture. *Salmonella thompson* MCC 608 was used as the major test organism. The other cultures in Table 1. were used when indicated.

Stock cultures were maintained in the lyophilized state in 14% skim milk powder. In addition, *S. thompson* MCC 608 and *P. aeruginosa* NHW M1 were maintained on Bacto-Brain Heart Infusion Agar (BHIA) mini slants (2 ml in 4 ml vials). These slants were inoculated, incubated overnight at 35 C, frozen in liquid air and stored in an ultra-freezer at -80 C. These cultures were removed from the ultra-freezer bimonthly or as needed, purified and used to prepare working stock cultures on BHIA slants. Working cultures of *S. thompson* MCC 608 were stored at 4 C and *P. aeruginosa* NHW M1 cultures were stored at room temperature.

TABLE 1.

TABLE 1.

Cultures used in this study.

Culture	Source
Pseudomonas aeruginosa	NHW M1, National Health and Welfare, Ottawa
Pseudomonas aeruginosa	MCC 436, Macdonald College Culture Collection
Salmonella thompson	MCC 608
Salmonella typhimurium	ES 878, Food Microbiology Labs. Macdonald College
Salmonella heidelberg	MCC 604
Salmonella infantis	MCC 605
Salmonella enteriditis	ES 413
Salmonella tenessee	MCC 600
Staphylococcus aureus	PHL 40, Department of Microbiology, University of Alberta
Escherichia coli B	MCC 237
Bacillus megaterium	MCC 7
Clostridium perfringens	MCC 385
Streptococcus faecalis	ATCC 8034, American Type Culture Collection

2. Culture filtrate production

P. aeruginosa NHW M1 was grown under shaking conditions (150 rpm, New Brunswick Scientific Company Gyrotory Shaker Incubator Model G25) for 4 days at 35 °C in 400 ml of Bacto-Brain Heart Infusion Broth (BHI) in 1 litre Erlenmeyer flasks. The culture was then acidified with 5 N HCl to pH 2 to 3 and centrifuged (40 min 7970 x g, 4 C, Sorvall RC 2-B Centrifuge). The supernatant fluid was decanted and filtered successively through Whatman #2 filter paper and 0.45 μ membrane filters to remove the remaining cellular material. The acid filtrate was adjusted to pH 7.4 with 5 N KOH and filter sterilized (Millipore filter 0.45 μ). This filtrate was then used for inhibition studies as outlined below. Culture filtrates of other organisms were prepared in a similar manner.

3. Treatment of filtrates

a. Pyocyanine extraction

The procedures used to extract culture filtrate are outlined in Figure 2. The acid filtrate was made alkaline (pH 8-9) by addition of $NaHCO_3$ powder. The alkaline filtrate was extracted in a separatory funnel with an equal volume of chloroform. Pyocyanine is blue when alkaline and more soluble in chloroform than in water. The chloroform was removed after separation of the layers and the filtrate was extracted twice more with additional equal quantities of chloroform. The pyocyanine in the combined chloroform fractions was then extracted

FIGURE 2A.

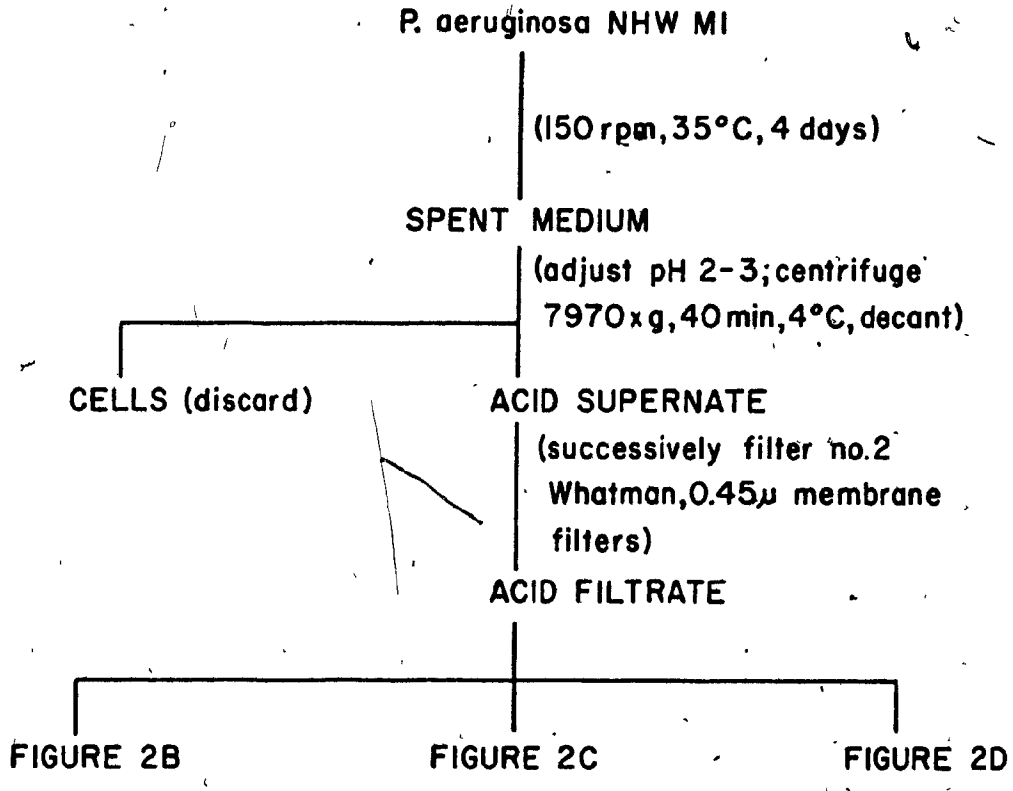


Fig. 2A. Medium extraction procedure to yield acid filtrate.

F I G U R E 2B.

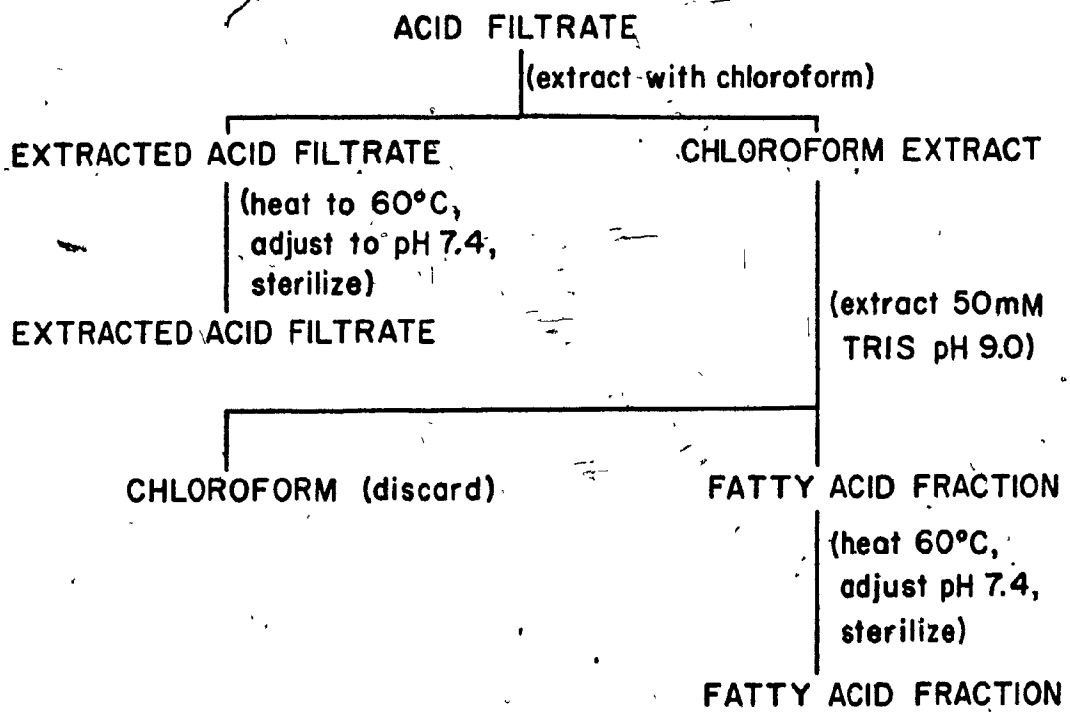


Fig. 2B. Medium extraction procedure to yield the fatty acid fraction.

F I G U R E 2C.

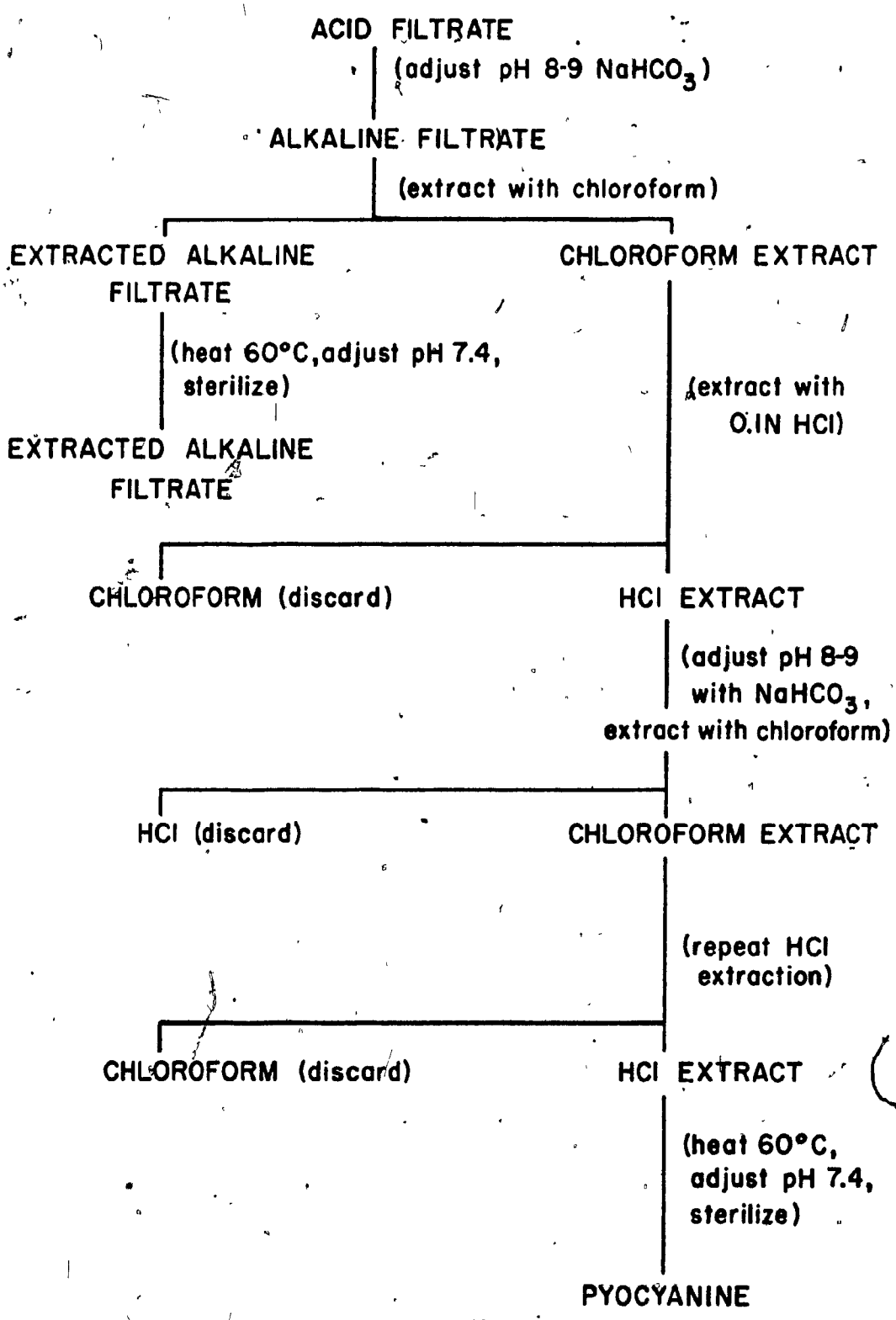


Fig. 2C. Medium extraction procedure to yield pyocyanine.

FIGURE 2D.

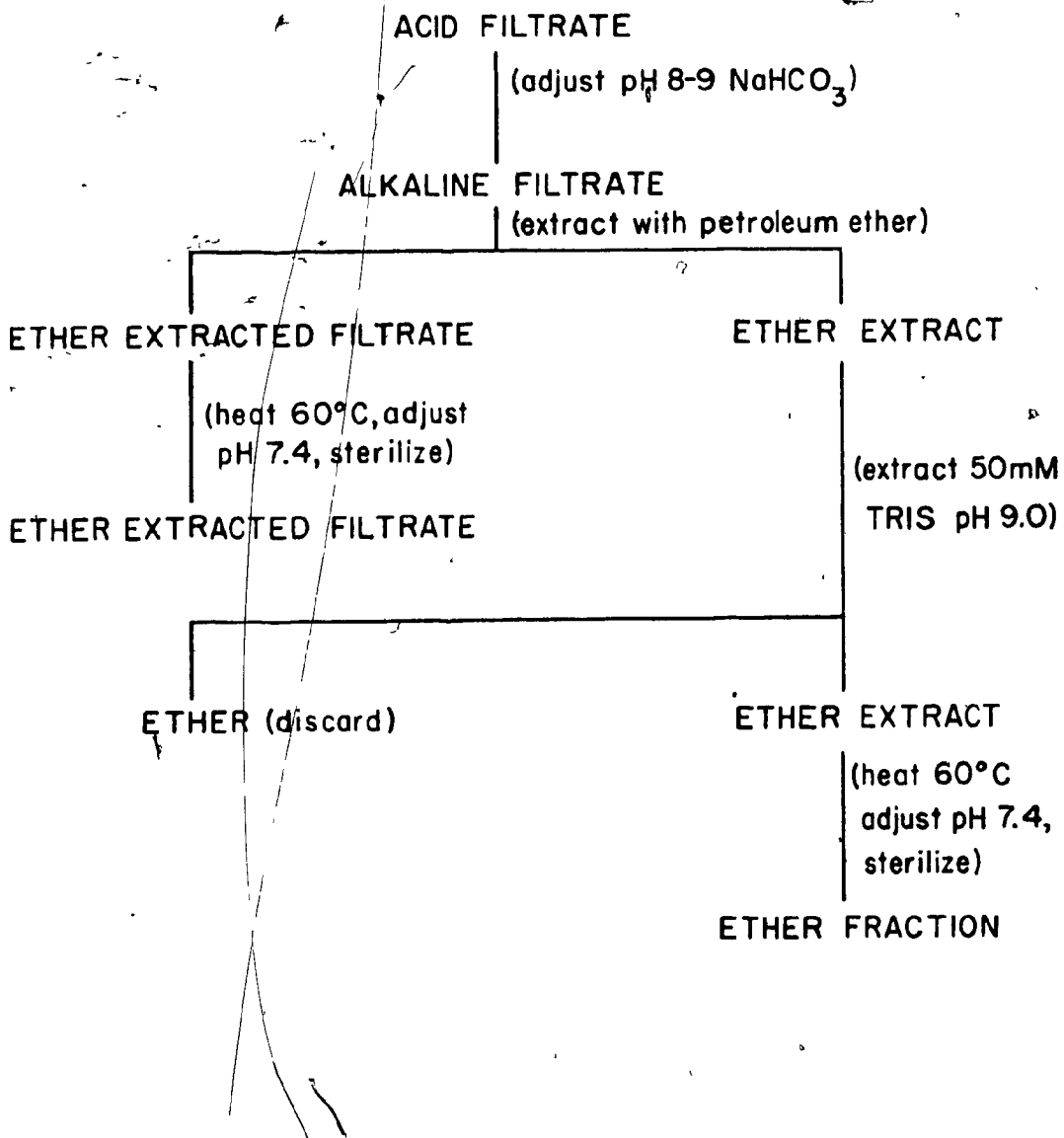


Fig. 2D. Medium extraction procedure to yield the ether fraction.

into 0.1 N HCl. This usually required only one extraction because pyocyanine is readily soluble in 0.1 N HCl. The HCl pyocyanine solution was made alkaline by addition of $NaHCO_3$ and extracted with three portions of chloroform. This procedure was repeated once more with extraction into HCl as the final step. The final volume of 0.1 N HCl could be reduced to produce a more concentrated pyocyanine solution. The pyocyanine solution was heated to 60 C to evaporate any remaining chloroform and was stored at 4 C as an acidic solution. Pyocyanine solutions used in inhibition studies were adjusted to the desired concentration with distilled water, adjusted to pH 7.4 with 5 N KOH and filter sterilized. Pyocyanine concentration in the acid solution was determined by use of the extinction coefficient:

$$E^{1\%}_{1\ cm} = 117 \text{ at } 520 \text{ nm (Macdonald, 1963)}$$

The filtrate from which pyocyanine had been extracted (extracted alkaline filtrate) was used for inhibition studies. It was heated to 60 C to remove residual chloroform, adjusted to pH 7.4 with 5 N HCL and filter sterilized.

b. Extraction of other inhibitory factors

i *Ether extraction*

Alkaline filtrate prepared as outlined above was extracted with three portions of petroleum ether. The total volume of the ether portions was equal to the volume of the spent medium being extracted.

The ether layer contained a material which was white. This white material was then extracted into 50 mM trihydroxymethylaminomethane (TRIS) buffer (pH 9.0). The TRIS buffer (ether extract) was heated to 60 C to remove residual ether, adjusted to pH 7.4, filter sterilized, and used as an additive to media for inhibition studies as described below. The extracted spent medium (ether extracted filtrate) was adjusted to pH 7.4 with 5 N HCl, heated to 60 C, filter sterilized and also used in inhibition studies.

ii *Chloroform extraction of acid filtrate*

Acid filtrate as described in Materials and Methods, section 2 was extracted three times with chloroform to give a total volume of chloroform equal to that of the extracted medium. The chloroform fraction was extracted with 50 mM TRIS buffer (pH 9.0) and the resulting solution (fatty acid fraction) was heated to 60 C, adjusted to pH 7.4 with 5 N KOH, filter sterilized and used in inhibition studies. The extracted medium (extracted acid filtrate) was also used to investigate its inhibitory activity.

c. Sephadex fractionation

A 100 ml portion of spent BHI medium was concentrated ten times in a flash evaporator (Thomas Magne-Flash Evaporator Model 38). The inhibitory activity of the distillate was investigated. The volume of the concentrated spent medium was adjusted to a x 10 concentration by addition of distilled water. A column (Pharmacia 1.8 x 30 cm)

was prepared by suspending Sephadex G-25 beads in 50 mM TRIS buffer, pH 7.4 and slowly pouring them into the column. The column was washed by allowing 250 ml of the buffer to run through. One ml of the concentrated medium was applied to the top of the column with a Pasteur pipette and carefully allowed to move below the Sephadex bead surface. Additional buffer was added and with a 400 ml reservoir in place the column was eluted with 50 mM buffer, pH 7.4. The fractions were collected in 1 ml amounts (Gilson Micro Fractionator). The pyocyanine was observed to move down the column as a distinct blue band. Addition of HCl to these fractions caused them to turn red - confirming the presence of pyocyanine. Sequential fractions that had similar colours were combined, filter sterilized and used for inhibition studies.

4. Preparation of pyocyanine

P. aeruginosa NHW M1 produced low amounts of pyocyanine in BHI. When larger quantities of pigment were required *P. aeruginosa* MCC 436 was grown in the medium of Ingram and Blackwood (1962). This medium was modified by the addition of 6.06 gm TRIS buffer per litre and adjusting the pH to 7.4 with 5 N HCl. $CaCO_3$ was omitted from the medium. One litre amounts of the medium in 2800 ml Fernbach flasks were inoculated with *P. aeruginosa* MCC 436 cells from a 16 hour BHIA slant suspended in 10 ml of the medium. The flasks were incubated with shaking (150 rpm) at 25 C for 4 days. The pyocyanine was extracted as described previously (Materials and Methods section 3a).

5. Preparation of growth flasks for inhibition studies

a. Aerobic growth

Inhibition studies were usually done in 50 ml Erlenmeyer flasks with sidearms for optical density (O.D.) measurement and were incubated at 35 C with shaking at 150 rpm. The desired medium was prepared in a concentrated form (2 or 4 times single strength). The amount of concentrated medium to give a final volume of 20 ml single strength medium in 50 ml flasks stoppered with cotton plugs was sterilized by autoclaving at 121 C for 15 min. After cooling, the desired amount of the preparation being studied was added. The flask was made up to 19 ml with sterile distilled water and 1 ml of inoculum was added. Growth control flasks contained only concentrated medium, distilled water, and inoculum. Blanks used to calibrate the spectrophotometer contained concentrated medium, water, the inhibitor under study and 1 ml of sterile BHI. The concentration of culture filtrates and pyocyanine in initial experiments are described as percentages. This indicates that the volume of the preparations were adjusted to be equal to that of the original spent medium. The percentage given is then the portion of the medium in the growth flasks that is composed of the preparation being studied.

b. Anaerobic growth

Inhibition studies under anaerobic conditions were done in 50 ml sidearm flasks prepared as described above. Flasks containing sterile

medium were stoppered with sterile rubber Suba seals. A sterile needle was then introduced through the seal and a vacuum was applied alternatively with nitrogen flushing. After three cycles of evacuation and nitrogen flushing, the nitrogen pressure in the flasks was adjusted to ± atm. The flasks were incubated for 24 h to eliminate those which were contaminated. Inoculum was added or samples removed by use of sterile 1 ml syringes. Growth was followed by either O.D. measurement or plating.

6. Inoculum preparation

Cultures to be used as inoculum were prepared by growing them through three 18-20 h serial transfers in the medium. The final culture was diluted (10^{-4} for *P. aeruginosa* and *S. aureus* and 10^{-5} for others) in the same medium equilibrated to the temperature of incubation. The inoculum level in 50 ml sidearm flasks was 5% (1 ml into 19 ml) and 1% in other cases (1 ml into 99 ml).

7. Measurement of growth

a. Optical density

Growth in 50 ml sidearm flasks was followed by measurement of O.D. at 540 nm (Coleman Junior II Spectrophotometer model 6/20). The instrument was calibrated using blank flasks prepared as described in Materials and Methods section 5a.

b. Viable count

Viable counts were determined by a drop plate method. The media were poured into plastic petri dishes and allowed to dry in a laminar flow hood (Envirogard, Envirogineering Ltd. model EVG-424) for *ca.* 2 h with the lids partially removed. Plating was done by applying drops of appropriate dilutions to the agar surface with a 1 ml biotip pipettor (Clay Adams 22-295). Two drops of each of three dilutions were applied to the agar surface in each plate. The drops were allowed to dry before inverting the plates and incubation. The drops having ten to thirty colonies were counted and used to determine the number of colony forming units (CFU) per ml according to the following formula.

CFU/ml = colony count x 43 x dilution

where 43 is a correction factor for numbers of drops/ml.

c. Slide culture technique

BHIA agar blocks were prepared on microscope slides (75 x 25 mm). The BHIA was prepared in test tubes, sterilized and cooled to 45 C in a waterbath. If desired, pyocyanine was added immediately before preparation of the slides. The sterile agar was then applied to the slide by allowing 1 to 2 ml of agar to run over the slide surface held at an angle of *ca.* 20°. The agar strip was allowed to cool and solidify. The excess agar was cut away to leave two small blocks (15 x 15 mm). The blocks were allowed to dry and one 2 to 3 mm loopful of the culture

was streaked across one block and then the other. Using this method and a culture with *ca.* 5×10^6 cells per ml, separated cells could be obtained on either the latter or both agar blocks. After drying for 10 to 20 min, coverslips (22 x 22 mm) were placed over each block. The coverslips were sealed in place by applying melted wax around the edges. This wax seal prevented the agar from drying further. The slides were incubated for 3 h and the microcolony formation by 300 cells was observed using phase microscopy (Carl Zeiss photomicroscope 63454 mag. x 800). The number of microcolonies formed by 300 cells observed was used to calculate the percentage of viability.

Sealing the agar blocks may result in reduced oxygen tension. To insure that the observations made were due to the pyocyanine alone and not due to a combined action of pyocyanine and reduced oxygen tension, microcolonies were also studied under normal oxygen tension conditions. Agar strips were prepared on sterile glass slides which were supported on a bent glass rod in a glass petri dish. The culture was streaked on the agar strip, sterile water was added to the bottom of the dish to prevent drying and the assembly was incubated at 35 C. After an appropriate incubation time, a coverslip was dropped into the agar strip and the cell growth was studied using the photomicroscope. The effect of pyocyanine appeared to be the same under both incubation conditions.

8. Resting cell suspensions

The inoculum for the desired culture was prepared as described in section 6. A dilution of the final culture was prepared to yield 2 to 6 x 10^3 CFU/ml. Four ml of the dilution was added to 400 ml of BHI in a 1 litre flask. The culture was incubated for 12 h at 35 C with shaking (150 rpm). A portion (150 ml) of the culture was centrifuged (7970 x g, 10 min, 4 C), resuspended in Straka and Stokes (1959) synthetic medium (S-medium) which was carbohydrate free (CF-S medium) (Appendix 1). The centrifugation and washing were repeated three times. The cells were resuspended in 100 ml of CF-S medium. Ten ml of the cell suspension were added to each of two 250 ml flasks containing a) 90 ml CF-S medium and b) 90 ml CF-S medium with sufficient pyocyanine to give the desired concentration. These flasks were incubated at 35 C with shaking (150 rpm). Viability was determined by plate counts at various times on BHIA and Bacto-Eosin Methylene Blue agar (EMB) supplemented with 2% sodium chloride for *S. thompson* MCC 608 and *E. coli* B MCC 237 and BHIA and Bacto-Mannitol salts agar (MSA) for *S. aureus* PHL 40.

9. Protein determination

Protein was determined by the Lowry method for bacterial cells was described by Herbert *et al.* (1971). The preparation of solution C was slightly modified because of precipitate formation upon storage. Copper sulfate and sodium potassium tartarate were prepared as 1 and

2% solutions respectively. One ml of each of these was added to 50 ml of a 5% sodium carbonate solution immediately before use (solution C).

10. Penicillin experiment

The effect of penicillin on cultures was studied by adding growing cultures to fresh medium containing sufficient penicillin G to give a final concentration of 1590 units per ml. Samples were withdrawn at various times into 0.1% peptone containing 2000 units per ml penicillinase to inactivate the penicillin G. Further dilutions were prepared in 0.1% peptone and viable counts were determined by plating on BHIA.

11. Cytochrome determination

Cells were grown in the desired medium to the stationary phase. The volume of medium used was adjusted so that a concentrated cell population could be obtained containing 5 to 25 mg protein per ml. The cells were centrifuged (7970 x g, 20 min, 4 C), washed three times in CF-S medium and resuspended in 10 ml of CF-S medium. The cells in 7 ml of this preparation were disrupted in a French Pressure cell (Power Laboratory Press, American Instrument Co., Silver Spring, Md., U.S.A. 12000 - 16000 psi) and the debris removed by centrifugation (12,000 x g. 30 min, 4 C). The supernatant fluid was used for cytochrome determination by measuring the oxidized minus the reduced difference

spectra. This was done by reducing the contents of one cuvette with a few grains of $Na_2S_2O_4$ and oxidizing the contents of the other cuvette by adding a few grains of $K_3Fe(CN)_6$. Measurements were done at room temperature with a two wavelength double beam spectrophotometer (Perkin-Elmer 356). The cuvettes had a path length of 1 cm. Cytochrome b concentration was expressed as nmole per mg protein using the standard absorption coefficient (17.5) given by Jones and Redfearn (1967).

12. Oxygen uptake

Oxygen consumption was studied in normal and in pigment treated cell preparations. *S. thompson* MCC 608 cells were grown in S-medium for 12 h, washed three times and resuspended in CF-S medium to give a final protein concentration of *ca.* 12 mg/ml. These cell preparations were incubated at 35 C with shaking (150 rpm) for 1.5 h to reduce endogenous energy supplies. Sufficient pyocyanine was added to one flask to give a final concentration of 50 µg/ml. Incubation was continued for 1 h. These cell preparations were used in an enclosed plexiglass chamber fitted with a Clark type oxygen electrode (Rank Brothers UKC B 163) connected to a linear recorder (Linear Instrument Corp. 10 MV output). CF-S medium was used as the buffer solution, the total reaction volume was 3 ml and temperature was maintained at 35 C. The oxygen uptake was calculated using the O_2 concentration value (0.41 µg atom of oxygen/ml) given by Chappell (1964).

13. Amino acid incorporation

The incorporation of ^{14}C alanine was followed by using a modification of the trichloroacetic acid (TCA) precipitation method described by Deutch and Pauling (1971). Cells were grown for 16 to 18 h at 35 C in S-medium supplemented with alanine (0.5 mM). A 1/10 dilution was prepared in fresh medium and incubated for 4 h. The culture was centrifuged (7970 x g, 20 min, 4 C) and resuspended in fresh S-medium with no alanine to give a cell concentration of 7 to 8 x 10^8 CFU/ml. Reaction vessels were 50 ml Erlenmeyer flasks containing 5 ml S-medium, 5 ml of cell suspension, 20 µl of ^{14}C alanine and 50 µg/ml pyocyanine where appropriate. Cells were incubated for 1 h with pyocyanine before addition of ^{14}C alanine. Samples of 0.1 ml were withdrawn at various times and pipetted into 5 ml of ice cold TCA. The TCA was stored overnight in a refrigerator (4 C) and filtered through 0.45 µ HA membrane filters (Millipore Corp., Bedford, Mass.). The tubes and filters were washed twice with 10 ml amounts of TCA. The filters were placed in scintillation vials, 10 ml of Aquasol counting fluid (New England Nuclear NEF-934) were added and radioactivity measured in a liquid scintillation spectrometer (Nuclear Chicago Isocap/300).

14. Transport assay

Transport of various ^{14}C labeled compounds into cells was followed in a manner similar to that described by Ames (1964). Samples of 0.5 ml were withdrawn at various times and filtered immediately. Washed

filters were placed in scintillation vials and counted as described previously.

15. Radioactive materials

Radioactive materials used in this study were purchased from New England Nuclear Corp. The specific activities (S.A.) were 50 μCi/μM for ^{14}C (u) alanine, ^{14}C (u) D-glucose, ^{14}C (u) glycerol, ^{14}C 3-0 methyl D glucose and 55 μCi/μM for uracil.

16. Nicotinamide adenine dinucleotide (NADH) determination

The internal concentration of NADH was measured in *S thompson* MCC 608 cells. A culture was grown in S medium (5.9 gm/ml succinate, 0.454 gm/l DL-alanine and 10^{-5}M $FeCl_3$) for 12 h at 35 C with shaking (150 rpm). Pyocyanine was added to one flask to give a final concentration of 50 μg/ml and an equivalent volume of 0.1 N HCl adjusted to pH 7.4 with 5 N KOH was added to the control. Incubation was continued for 1.5 h. The cultures were cooled rapidly on ice for 0.5 h, the cells were centrifuged (7970 x 9, 10 min, 4 C) and washed four times with CF-S medium at 4 C. NADH was extracted by the method of Wright and Sanwal (1969) and the concentration of NADH was measured according to the method of Klingenberg (1965). The lactic dehydrogenase used was EC 1.1.1.27 from rabbit muscle type XI (Sigma Chemical Co., St. Louis, Mo., U.S.A.).

17. Ethylene diamine tetraacetic acid (EDTA) treatment

A procedure similar to the one reported by Leive (1965) was used to treat cells with EDTA. An inoculum of 1% from an 18 to 24 h BHI culture (35 C, 150 rpm) of *S. thompson* MCC 608 was used to inoculate 200 ml of fresh BHI. This was incubated for 2 h (35 C, 150 rpm) and then washed once with 3.3×10^{-2} M TRIS (pH 8.0). The washed cells were resuspended in 7 ml of 1.0×10^{-2} M TRIS (pH 8.0). Two ml of the cell suspension were pipetted into each of three 50 ml flasks. These flasks contained 1.0×10^{-2} M TRIS (pH 8.0). One flask was a control and contained TRIS only, the second contained 5 mM EDTA, and the third 10 mM EDTA (final concentration). After 10 min incubation (35 C, 150 rpm), 10^{-2} dilutions in BHI were made from each preparation and these were used to inoculate flasks of fresh BHI (1% inoculum) with and without 50 µg/ml pyocyanine. The EDTA was diluted to 10^{-3}. These flasks were then incubated and growth was followed by plate counts at various time intervals.

18. Gamma irradiation

The solutions to be exposed to gamma irradiation were placed in 15 ml glass tubes and exposed in the irradiation chamber of a Gamma Cell 220 (283 Krad/h) for sterilization (radurization). The total dose was 3396 Krad. Sterility was tested by inoculating a portion of the preparations into BHI and incubating for 48 h at 35 C.

RESULTS and DISCUSSION

PART A *Pseudomonas* - *Salmonella* INTERACTION

1. Growth in mixed culture

The preliminary work in this study involved an investigation of the interaction of a pseudomonad with Salmonellae in mixed cultures. Inoculum cultures were prepared and diluted so that approximately the same number of each organism could be added to the growth flasks. Three 1 litre Erlenmeyer flasks, containing 400 ml of BHI, were inoculated with (1) *Salmonella* (2) *Salmonella* plus the pseudomonad and (3) the pseudomonad. These flasks were incubated at 35 C with shaking (150 rpm). Samples were withdrawn at various times and plated on BHIA. The larger white Salmonella colonies were easily distinguished from the smaller brownish pseudomonad colonies on these plates. The growth of each organism in pure culture was compared with its growth in the mixture. Figures 3 and 4 show the results of a study in which *S. thompson* MCC 608 was grown with *P. aeruginosa* NHW M1. These figures illustrated that the growth of both organisms was very similar in the pure and in the mixed culture. This experiment was repeated using several species of Salmonella including *S. heidelberg* MCC 604, *S. infantis* MCC 605, *S. enteriditis* ES 413, *S. typhimurium* ES 878, and *S. tennessee* MCC 600. The results of these trials were similar to those observed in Figures 3 and 4. The Salmonella and the pseudomonad had similar growth patterns in both the pure and mixed culture.

FIGURE 3.

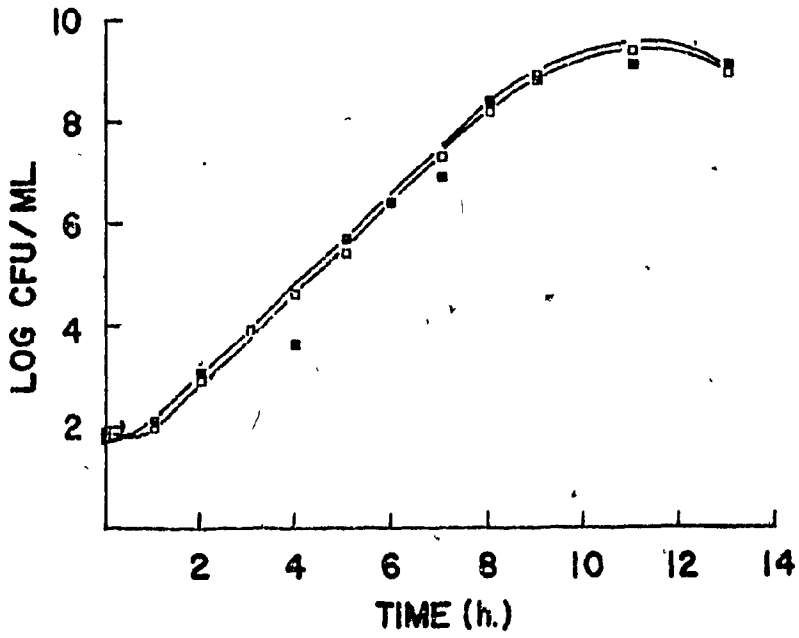


Fig. 3. The growth of *S. thompson* MCC 608 in BHI.

□ in pure culture

■ in mixed culture with *P. aeruginosa* NHW-M1

FIGURE 4.

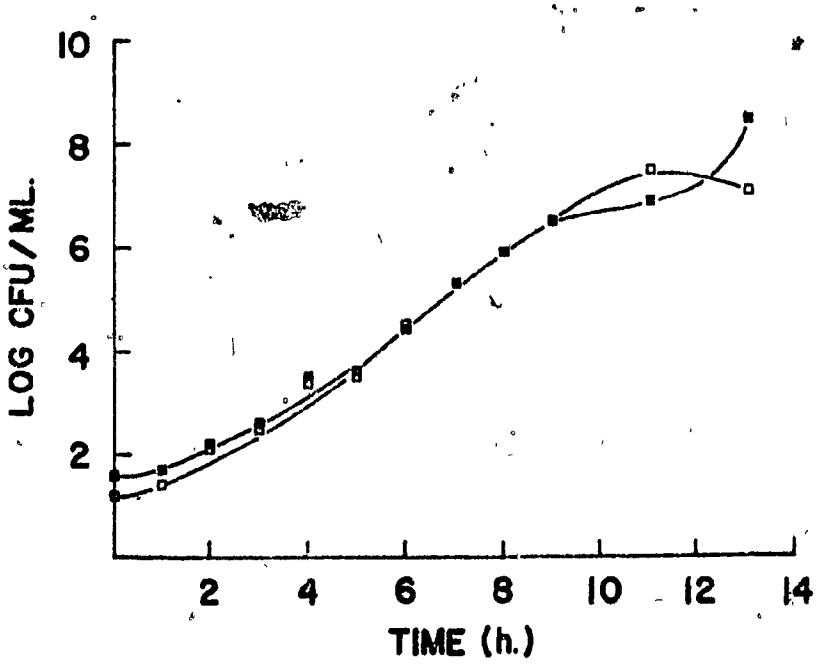


Fig. 4. The growth of *P. aeruginosa* NHW M1 in BHI.

in pure culture □

in mixed culture with *S. thompson* MCC 608 ■

2. Action of pseudomonad culture filtrate

The experiments outlined above showed that Salmonella and *P. aeruginosa* NHW M1 grew independently of each other when grown in mixed culture. However, pseudomonads are known to produce factors that inhibit the growth of microorganisms (Waksman, 1945). The effect of adding spent culture filtrates of *P. aeruginosa* NHW M1 to *S. thompson* MCC 608 cultures was investigated. The growth of *S. thompson* MCC 608 was followed in 50 ml flasks containing BHI medium with various concentrations of pseudomonad culture filtrate added (Material and Methods section 5a). Figure 5 shows that when the concentration of added pseudomonad filtrate was 10% or less there was no effect on the growth of the *S. thompson* cultures. Higher concentrations of culture filtrate caused the lag phase to increase from 2 to 5.5 h. The *P. aeruginosa* NHW M1 culture filtrate therefore contained substances that delay the onset of the log phase of growth of *S. thompson* MCC 608 cultures. The inhibitory action of the *P. aeruginosa* filtrate (P-filtrate) against other microorganisms was next assessed.

The P-filtrate at 25% concentration in BHI had no effect on the growth of *P. aeruginosa* NHI M1 up to 12 h (Figure 6). Increasing the P-filtreate concentration to 30% and recording the growth from the 11th to the 20th h resulted in greater differences between control and treated cultures (Figure 7). *P. aeruginosa* NHW M1 exposed to 30% of its own culture filtrate had reached an O.D. of 0.51 units after

FIGURE 5.

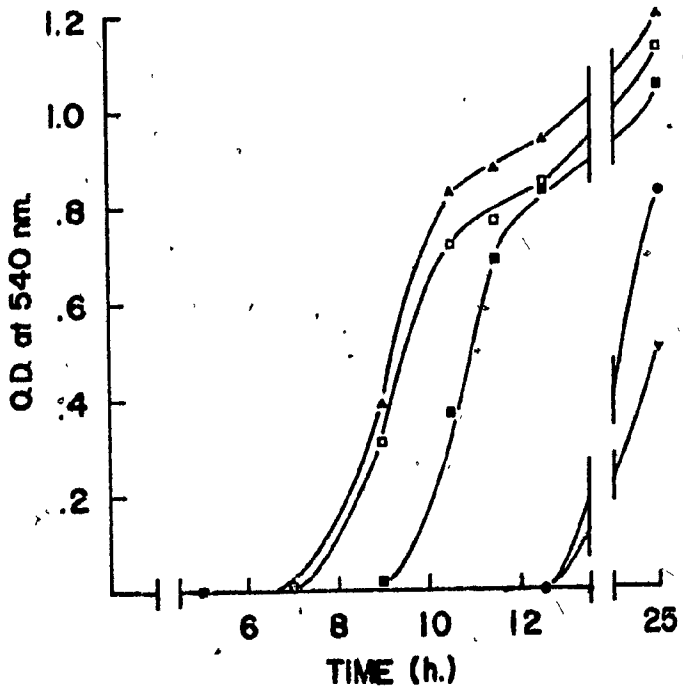


Fig. 5. The growth of *S. thompson* MCC 608 in BHI with added *P. aeruginosa* NHW M1 culture filtrate.

filtrate added	
0%	□
1%	▲
10%	■
50%	●
70%	▼

FIGURE 6.

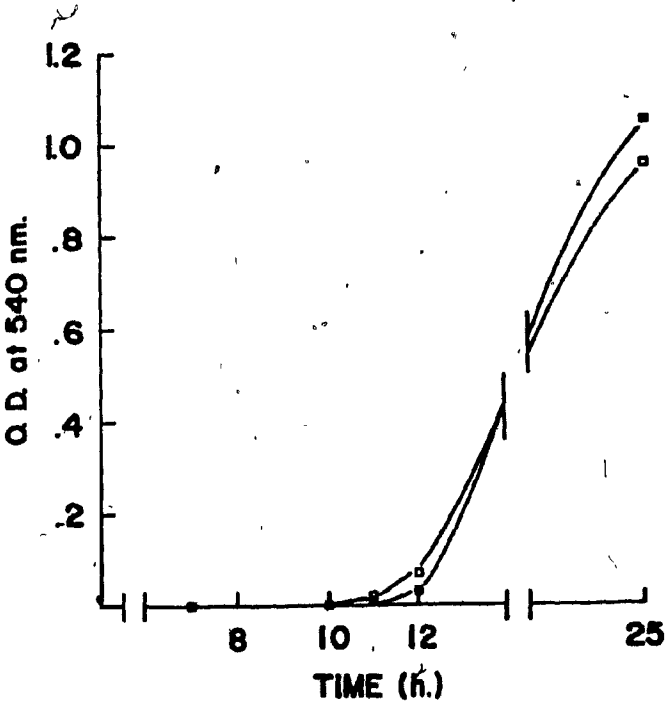


Fig. 6. The growth of *P. aeruginosa* NHW M1 in BHI with added *P. aeruginosa* NHW M1 culture filtrate.

filtrate added 0% □

25% ■

FIGURE 7.

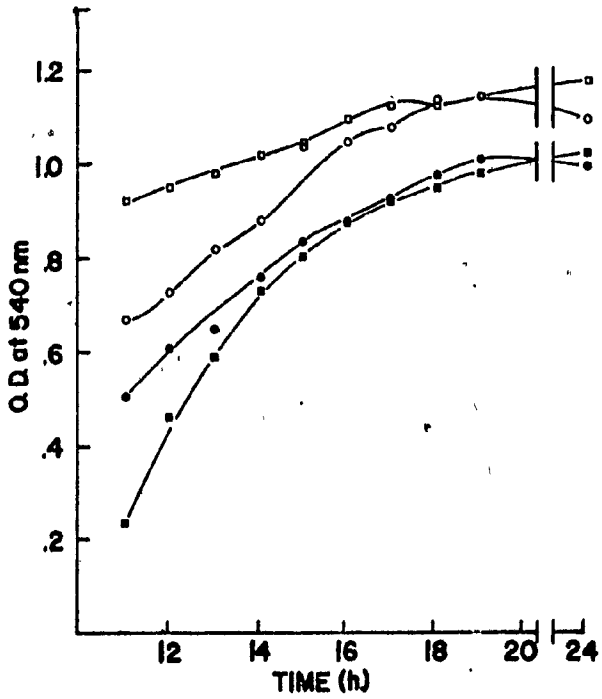


Fig. 7. The growth of *S. thompson* MCC 608 and *P. aeruginosa* NHW M1 in BHI with and without 30% added *P. aeruginosa* NHW M1 culture filtrate.

Growth of: *S. thompson* no filtrate □

S. thompson with filtrate ■

P. aeruginosa no filtrate ○

P. aeruginosa with filtrate ●

11 h, while the control culture had reached 0.67 units. The difference (0.15 O.D. units) was much less than the difference between *S. thompson* MCC 608 cultures grown under the same conditions (0.68 O.D. units). These experiments illustrated that *P. aeruginosa* NHW M1 filtrates are inhibitory to both *S. thompson* and *P. aeruginosa* NHW M1. The degree of inhibition of the pseudomonad is however much less than that observed with the Salmonella culture. *P. aeruginosa* NHW M1 therefore had some resistance to the inhibitory factor or factors that are present in its spent culture filtrate.

The P-filtrate inhibited the growth of *E. coli* B MCC 237 (Figures 8 and 9) and *S. aureus* PHL 40 (Figure 10). With *E. coli*, the lag phase was extended by more than 6 h (Figure 8) and after 12 h, incubation, the difference in the amount of growth between the control (no filtrate) and that in the presence of 30% filtrate (0.8 O.D. units) was greater than the difference observed with *S thompson* MCC 608 under similar conditions (0.68 O.D. units). *E. coli* B MCC 237 appears therefore to have greater sensitivity to the culture filtrates than *S. thompson* MCC 608. These findings are contrary to those reported by Waksman (1940) who reported that *P. aeruginosa* filtrates had no effect on *E. coli*.

The outgrowth of the Gram-positive organism *S. aureus* PHL 40 was even more affected by the pseudomonad filtrate. After 21 h incubation the culture containing 30% filtrate had reached an O.D. of 0.37

FIGURE 8.

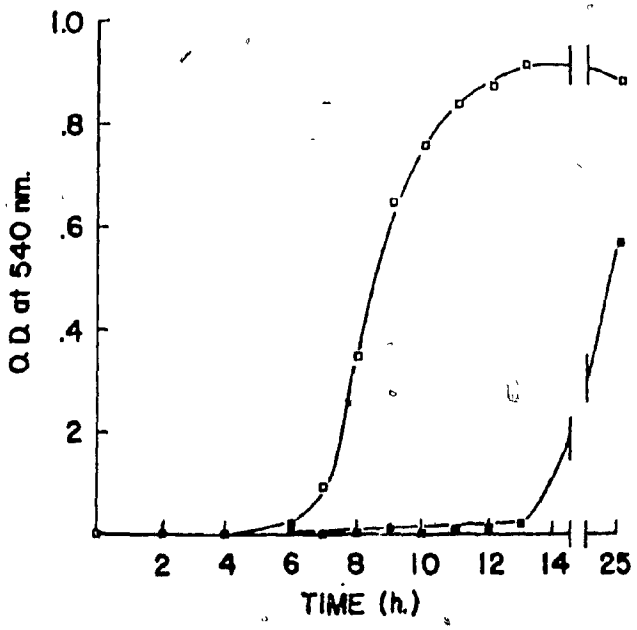


Fig. 8. The growth of *E. coli* B MCC 237 in BHI with added *P. aeruginosa* NHW M1 culture filtrate.

Filtrate added 0% □

30% ■

FIGURE 9.

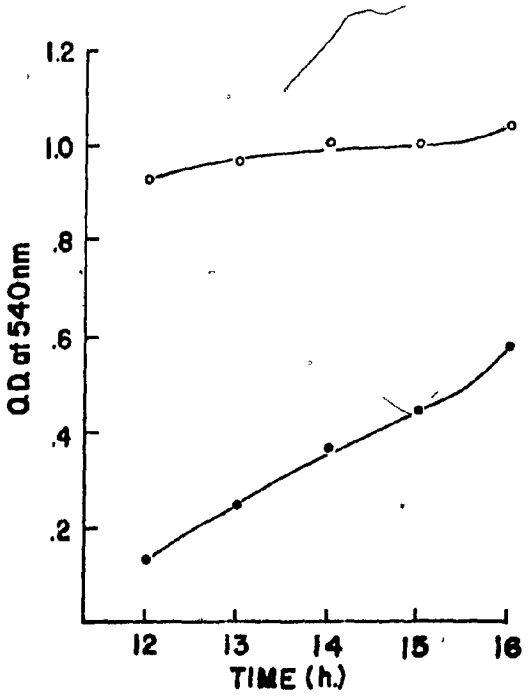


Fig. 9. The growth of *E. coli* B MCC 237 in BHI with added *P. aeruginosa* NHW M1 culture filtrate.

filtrate added 0% ○

30% ●

FIGURE 10.

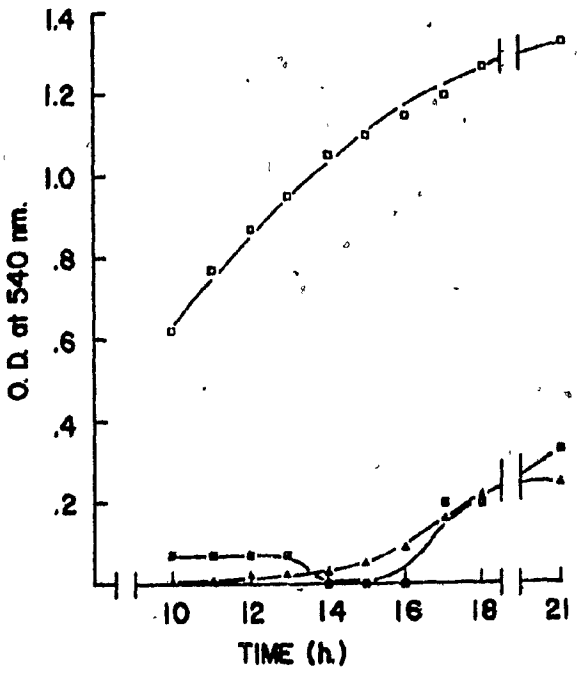


Fig. 10. The growth of *S. aureus* PHL 40 in BHI with added *P. aeruginosa* NHW M1 culture filtrate.

filtrate added	0%	□
	10%	▲
	30%	■

units while the control culture was at 1.35 units (Figure 10). This organism was even sensitive to low levels of filtrate and reached an O.D. of 0.25 units after 21 h with only 10% filtrate in the medium.

Results thus far have indicated that *P. aeruginosa* culture filtrate extended the lag phase of growth of *P. aeruginosa* NHW M1, *S. thompson* MCC 608, *E. coli* B MCC 237, and *S. aureus* PHL 40. The pseudomonad culture was shown to be the least sensitive of these bacteria while *S. aureus* PHL 40 was the most sensitive. *E. coli* B MCC 237 appeared to be more sensitive than *S. thompson* MCC 608. The time of extension of the lag phase increased with increased amounts of filtrate added to the growth medium.

3. Effect of *Salmonella* filtrate on growth

The activity of *Salmonella* filtrates was then studied. *S. thompson* MCC 608 was grown in BHI for 4 days and a filtrate was prepared as described for the pseudomonad filtrate (Materials and Methods section 2). This filtrate at 25% level did not appear to greatly extend the lag phase or to greatly inhibit the growth of *P. aeruginosa* NHW M1 (Figure 11). Addition of 70% of the *Salmonella* filtrate to an *S. thompson* MCC 608 culture did not result in a large increase in the lag time as observed with similar concentrations of pseudomonad filtrate (Figure 12). Although there was a slight inhibitory action when *Salmonella* filtrate was added to the growth medium, the effect was much less than observed when similar concentrations of pseudomonad filtrate were used.

FIGURE 11.

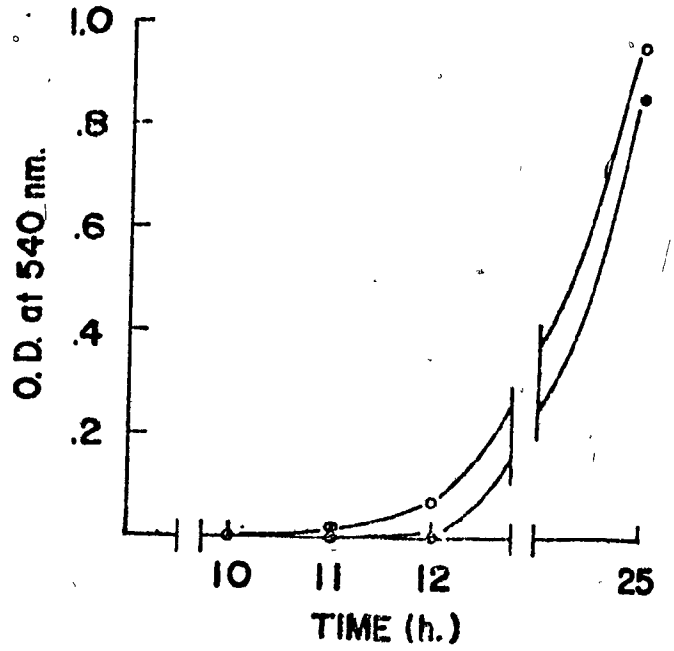


Fig. 11. The growth of *P. aeruginosa* NHW M1 in BHI with added *S. thompson* MCC 608 culture filtrate.

filtrate added 0% ○

25% ●

FIGURE 12.

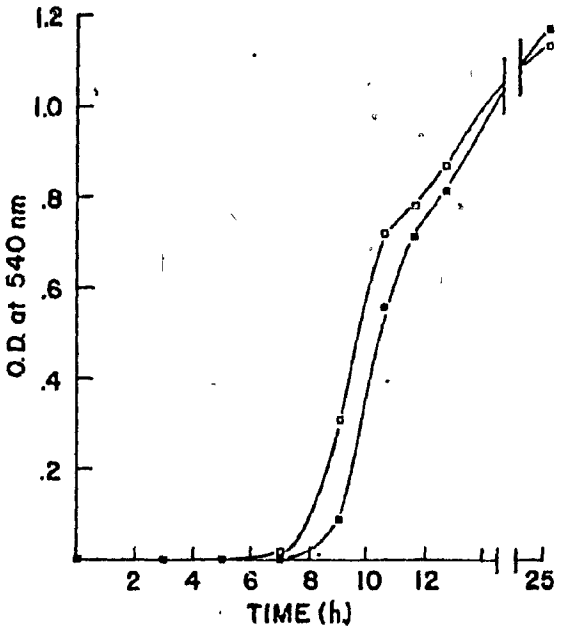


Fig. 12. The growth of *S. thompson* MCC 608 in BHI with added *S. thompson* MCC 608 culture filtrate.

filtrate added 0% □

70% ■

4. Concentration effect

The experiments outlined above involved addition of up to 70% filtrate to media which already contained the manufacturer's recommended concentration of BHI. Therefore it may be that the inhibition in growth was due to increased concentration of some component in the BHI medium. This was proved not to be the case because growth of *S. thompson* MCC 608 in single strength BHI medium (x 1) and in concentrated BHI medium (x 1.7 single strength) did not differ to any great extent (Figure 18). The inhibition caused by culture filtrates must therefore have been due to by-products of microbial growth and not due to a mere increase in the concentration of compounds such as salts when these filtrates were added to single strength BHI.

5. Action of culture filtrates

When microorganisms are subjected to inhibitory substances, the direct result can be injury to the cell. This injury may be repaired if the cells are exposed to suitable conditions post treatment. Tomlins and Ordal (1971) demonstrated that heat injured cells from cultures of *S. typhimurium* could grow readily on nonselective medium but were inhibited on Bacto-eosin methylene blue agar (EMBA) supplemented with 2% sodium chloride. The difference between viable counts on the nonselective medium and the selective medium (EMBA and 2% NaCl) was a measure of the injured portion of the population.

FIGURE 13.

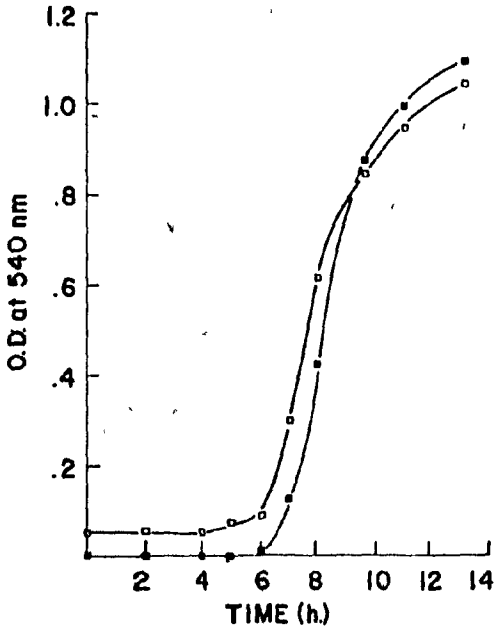


Fig. 13. The growth of *S. thompson* MCC 608 in BHI with added BHI.

BHI added 0% □

70% ■

A similar experiment was performed with *S. thompson* MCC 608 cells exposed to culture filtrate followed by plating the control and exposed cultures on BHIA and EMBAwith 2% sodium chloride. The results in Figure 14 showed that cells from control cultures developed equally well on both media. Viable counts from filtrate exposed cultures were consistently lower on the EMBAwith 2% sodium chloride as compared to counts on BHIA. Similar results were obtained by comparing viable counts on BHIA with those on Bacto Salmonella-Shigella agar (SS agar) (Figure 15). The results indicated that *S. thompson* MCC 608 may be injured when exposed to *P. aeruginosa* NHW M1 culture filtrate and that the injury makes them more sensitive to selective agents.

6. Summary

a. *Salmonella* and *P. aeruginosa* NHW M1 did not inhibit the growth of each other when grown in mixed culture from the log to the stationary phase.

b. Culture filtrates of *P. aeruginosa* NHW M1 inhibited the growth of *S. thompson* MCC 608 but the *Salmonella* filtrates did not inhibit the growth of the pseudomonad.

c. The pseudomonad filtrate caused an extension in the log phase of growth and the time of extension increased with increasing filtrate concentration.

FIGURE 14.

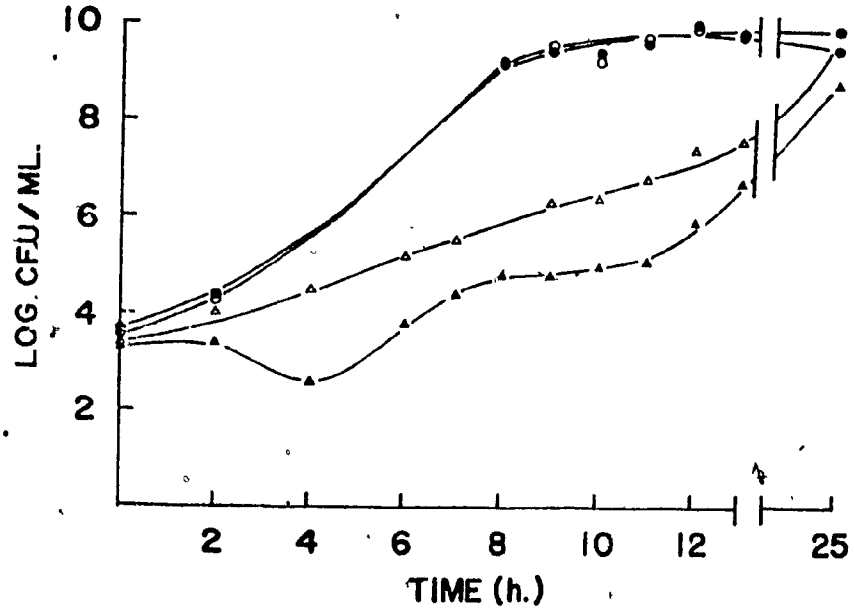


Fig. 14. The growth of *S. thompson* MCC 608 in BHI with and without 30% added *P. aeruginosa* NHW M1 culture filtrate.

Filtrate added: 0% viable count on BHIA ○

0% viable count on EMBA + 2% NaCl ●

30% viable count on BHIA △

30% viable count on EMBA + 2% NaCl ▲

FIGURE 15.

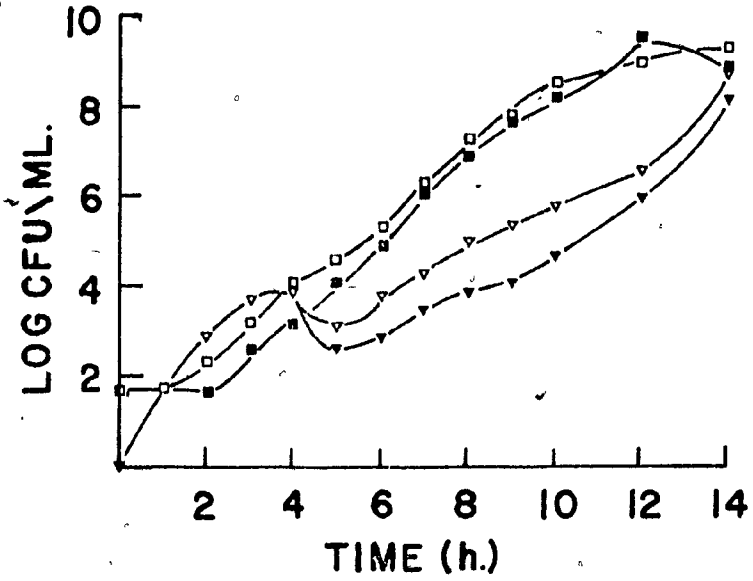


Fig. 15. The growth of *S. thompson* MCC 608 in BHI with and without 30% added *P. aeruginosa* NHW M1 culture filtrate.

filtrate added: 0% viable count on BHIA □

0% viable count on SS Agar ■

30% viable count on BHIA ▽

30% viable count on SS Agar ▼

d. Complete pseudomonad filtrate appeared to injure *S. thompson* MCC 608 cells in some way which rendered them more sensitive to selective media.

e. *S. aureus* PHL 40 and *E. coli* B MCC 237 were more sensitive to pseudomonad filtrate than *S. thompson* MCC 608. *P. aeruginosa* NHW M1 appeared to be resistant to the filtrate.

PART B INHIBITORY COMPONENTS OF *Pseudomonas aeruginosa* NHW M1 CULTURE FILTRATE

1. Pyocyanine extraction

The initial approach to the investigation of the growth inhibiting factors of the pseudomonad culture filtrate was to extract it with various solvents. The antibiotic activities of the extracted components and the remaining filtrate were then compared with the activity of the complete filtrate. In most of the subsequent experiments *S. thompson* MCC 608 was used as the test organism but in some cases other organisms were used for comparative purposes.

The first component extracted was pyocyanine. This pigment has been reported to have antibiotic properties but spent *P. aeruginosa* medium from which pyocyanine had been extracted, was said to lose very little of its inhibitory activity (Waksman, 1945). In this study, removal of pyocyanine from the spent medium filtrate resulted in a filtrate that extended the lag time of *S. thompson MCC* 608 cultures by almost one half the time observed with the complete filtrate (Figure 16). When the pyocyanine was added to the culture medium, at the concentration found in the original filtrate, it had inhibitory activity that was similar to the extracted filtrate.

2. Fatty acid extraction

Waksman (1945) also reported that pseudomonad culture filtrate

FIGURE 16.

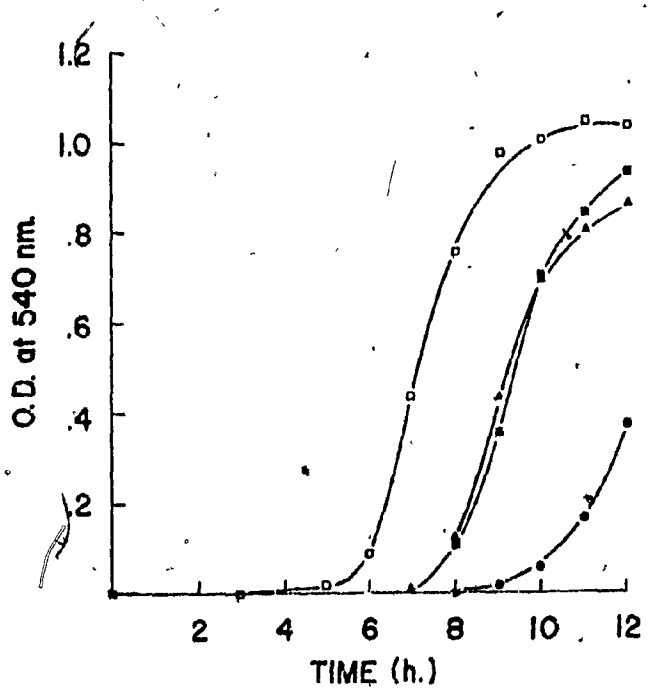


Fig. 16. The growth of *S. thompson* MCC 608 in BHI with additions prepared from *P. aeruginosa* NHW M1 culture filtrate. Additions (concentrations adjusted to be equal to the amount in the original filtrate):

extracted filtrate	30%	■
pyocyanine	30%	△
complete filtrate	30%	●
none		□

lost all of its inhibitory activity when both the pyocyanine and fatty acids were removed. With the removal of either pigment or pigment and fatty acid (Materials and Methods section 3b ii), residual antimicrobial activity was still evident in the filtrate (Figure 17). This observation was contrary to the information reported by Waksman (1945). He also stated that the fatty acid fraction had no inhibitory activity and that was true of the fatty acid fraction prepared in this study.

3. Residual pyocyanine

The inhibitory activity in the extracted culture filtrates could have been due to residual pyocyanine. This possibility was investigated by studying the inhibitory activity of several preparations. Pyocyanine was added to fresh BHI medium and then removed by the extraction procedure (Materials and Methods section 3a). The addition of 70% of this extracted BHI to BHI cultures caused an extension in the lag phase of growth of *S. thompson* MCC 608 of 2 h (Figure 18). This extension is less than the 5 h extension observed when extracted spent medium was added to a culture (Figure 17). The difference in the amount of pigment added (44 µg/ml) and the amount recovered (30.0 µg/ml) was 13.2 µg/ml. A growth flask was prepared containing pyocyanine at a concentration equivalent to the unremoved amount that would be present in the above flask that contained 70% extracted BHI. In this case the onset of log phase of growth was delayed by 1 h. Incorporation of 70% of a BHI medium preparation which was subjected to the extraction pro-

FIGURE 17.

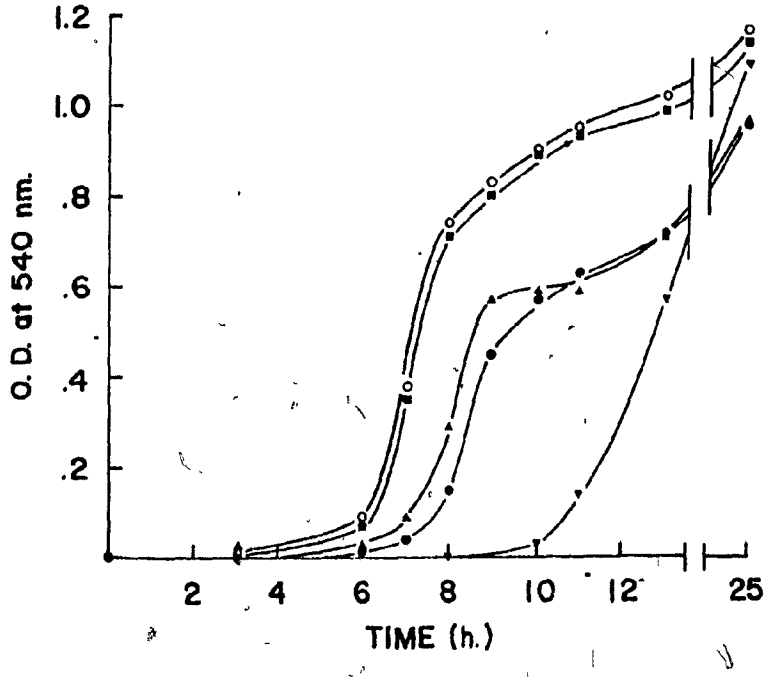


Fig. 17. The growth of *S. thompson* MCO 608 in BHI with additions prepared from *P. aeruginosa* NHW M1 culture filtrate. Additions (concentrations adjusted to be equal to the original filtrate).

none ○

fatty acid fraction ■

extracted filtrate 1 (pigment removed) ▲

extracted filtrate 2 (pigment and fatty acid removed) ●

pyocyanine ▼

F I G U R E 18.

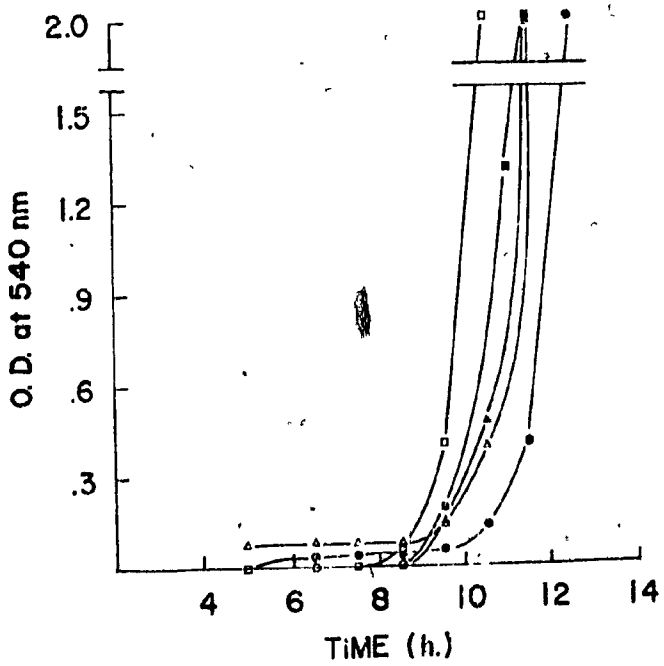


Fig. 18. The growth of *S. thompson* MCC 608 in BHI with various additions.

Additions:

none □

70% addition BHI ■

pyocyanine (concentration equal to the amount not recovered after extraction procedure) △

70% pyocyanine free BHI which had been extracted with chloroform ▲

70% BHI to which 44 µg/ml pyocyanine had been added and then removed by chloroform extraction. ●

cedure, but to which pyocyanine had not been added, also caused the lag phase to be extended by 1 h. When 70% additional fresh BHI was incorporated in the medium in this experiment no lag phase extension was evident but O.D. readings were slightly lower than the control (single strength BHI). The residual inhibitory activity could not be attributed solely to any of the factors examined in this experiment. It appears that spent pseudomonad medium must contain factors other than pyocyanine which are inhibitory and these are not removed by chloroform extraction.

4. Ether extraction

There have been reports in the literature concerning the preparation of antimicrobial substances from ether extracts of spent pseudomonad medium. Waksman (1945) reported the isolation of pyocyanase which had lytic activity. Hayes *et al.* (1945) also reported that ether extraction of pseudomonad cultures yielded inhibitory substances which they classified as PYO I, II, III and IV. Ether extracts were prepared from the *P. aeruginosa* NHW M1 filtrates and were found to have no detectable inhibitory activity (Figure 19).

5. Sephadex fractionation

A further attempt was made to isolate inhibitory fractions by use of a Sephadex G-25 column (Materials and Methods section 3c). The pyocyanine containing fraction was the only one which had measurable

FIGURE 19.

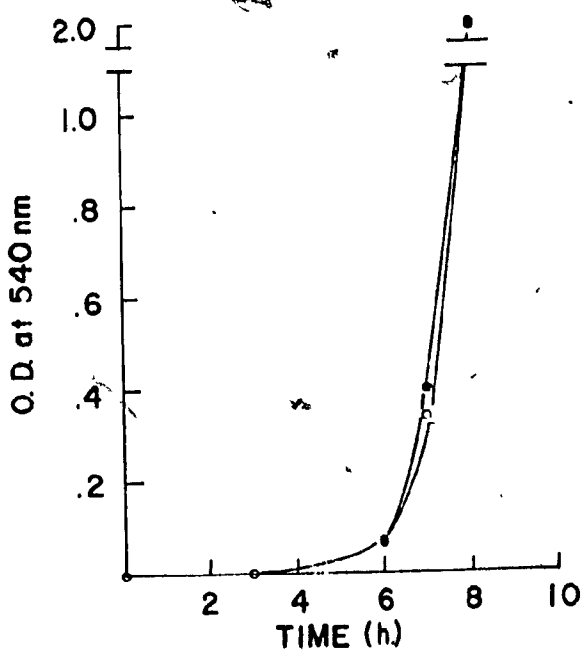


Fig. 19. The growth of *S. thompson* MCC 608 in BHI with 50% added ether extract of a *P. aeruginosa* NHW M1 culture filtrate.

no addition ⊙

ether extract 30% ●

inhibitory activity (Figure 20). The growth of cultures exposed to other fractions were similar to that of the control. Since *E. coli* B MCC 237 was more sensitive than *S. thompson* MCC 608, this experiment was repeated using *E. coli* B MCC 237 as the test organism. Again the pyocyanine fraction was the only one that had antimicrobial activity (Figure 21).

Cultures which had been extracted with chloroform were also fractionated on the Sephadex column. There was no pyocyanine visible in any fraction and the fractions corresponding in elution time to the pigment fractions from complete filtrate showed no measureable O.D. reading at 520 nm, indicating the total absence of pyocyanine. All the fractions collected under these conditions had no inhibitory activity. The Sephadex fractionation was not helpful in isolating inhibitory factors other than pyocyanine from the culture filtrates. The other factors may have been diluted too much during separation to retain measureable inhibitory action or they may have been lost through binding to the column. If the factors were protein or lipid they should be eluted from the column before the pyocyanine which is a relatively low molecular weight molecule. A brown residue was retained on the top portion of the column, and it is possible that the other inhibitory factors reside in this fraction.

6. Effect of heating

Some early researchers (Waksman, 1945) have suggested that *P. aeru-*

FIGURE 20.

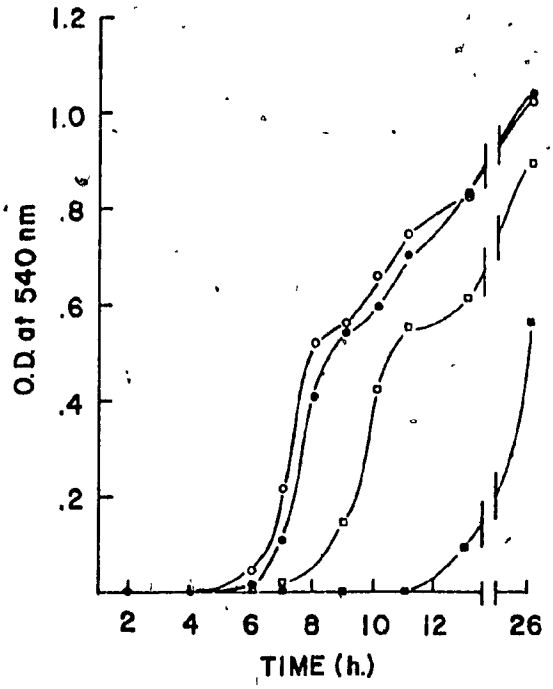


Fig. 20. The growth of *S. thompson* MCC 608 in BHI with additions of Sephadex fractions of a *P. aeruginosa* NHW M1 culture filtrate prepared with a G-25 column.

no addition ○

pyocyanine fraction □

fraction eluted before pyocyanine ●

complete culture filtrate ■

FIGURE 21.

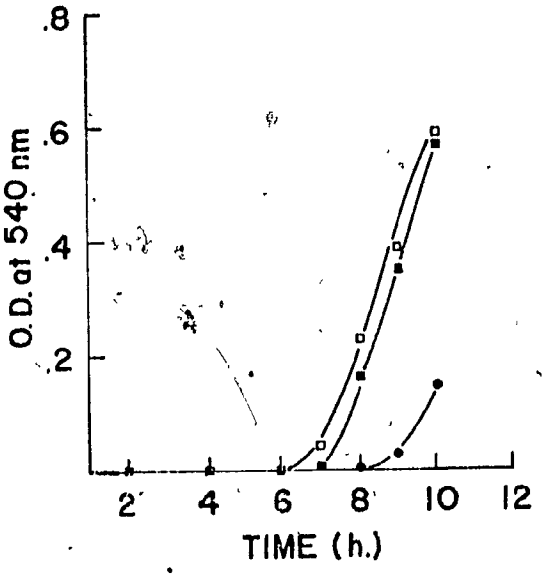


Fig. 21. The growth of *E. coli* B'MCC 237 in BHI with additions of Sephadex fractions of a *P. aeruginosa* NHW M1 culture filtrate prepared with a G-25 column.

□ no addition

● pyocyanine fraction

■ a slightly inhibitory fraction eluted after pyocyanine

ginosa produced pyocyanase which inhibited growth. There was some controversy about this material because it was considered to be an enzyme and therefore according to definition would be a protein. It was also reported to be soluble in ether. Autoclaving of our culture filtrate at 121 C for 15 min did not reduce its antimicrobial activity (Figure 22). It is unlikely therefore, that the other factor in this filtrate is a protein. The exact nature of pyocyanase as described by Waksman (1945) has not been clearly defined and the results of this study indicate that it was probably not an important component of the inhibitory factors because heating did not inactivate it and the ether extracts prepared showed no inhibitory activity.

7. Gamma irradiation

Gamma irradiation was considered at one stage of this work as a possible method of filtrate sterilization. As a result of irradiation the pyocyanine changed colour from blue to colourless. The resulting solution had no antimicrobial activity (Figure 23) and such treatment could therefore be used to eliminate the antimicrobial activity due to pyocyanine without subjecting the filtrate to the extraction procedures. Exposure of complete filtrate to gamma irradiation resulted in a large decrease in its inhibitory activity. The irradiated filtrate caused a delay of 1 h in the onset of log phase growth of *S. thompson* MCC 608 while filter sterilized filtrate caused a delay of more than 3 h (Figure 24). Growth of *S. thompson* in filtrate from which pyocyanine had been

FIGURE 22.

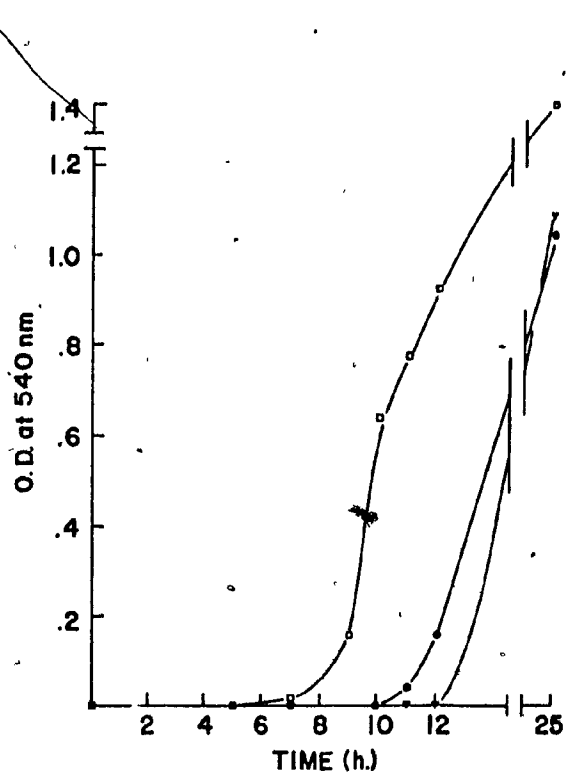


Fig. 22. The growth of *S. thompson* MCC 608 in BHI with additions of *P. aeruginosa* NHW M1 culture filtrate sterilized by filtration or autoclaving.

no addition □

filtrate (filter sterilized) 25% ▽

filtrate (autoclaved) 25% ●

FIGURE 23.

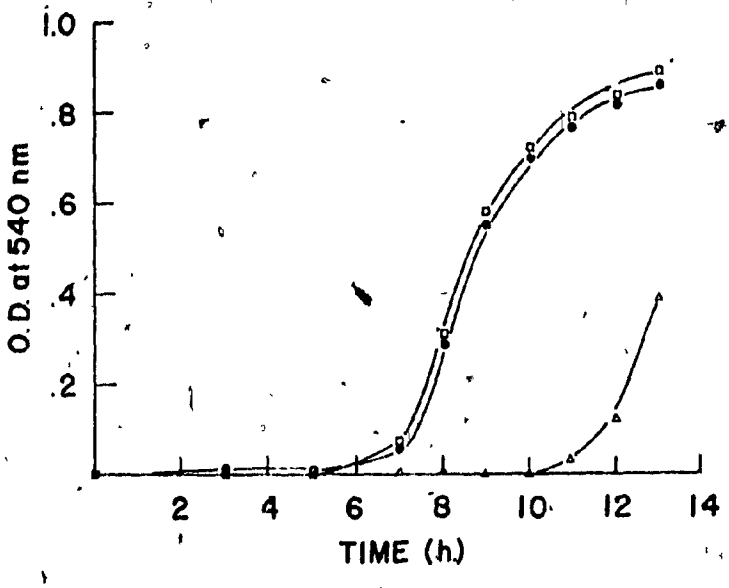


Fig. 23. The growth of *S. thompson* MCC 608 in BHI with additions of pyocyanine (23 µg/ml) sterilized by filtration or gamma irradiation.

no addition □

pyocyanine (filter sterilized) △

pyocyanine (irradiated) ●

FIGURE 24.

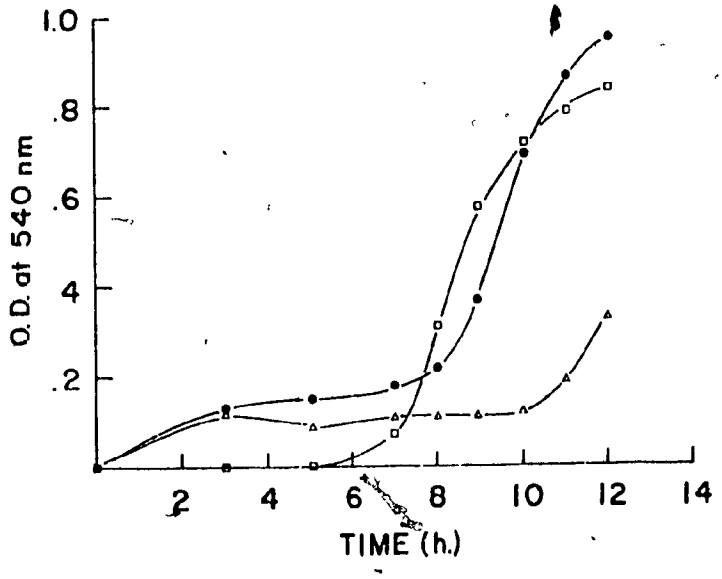


Fig. 24. The growth of *S. thompson* MCC 608 in BHI with additions of *P. aeruginosa* NHW M1 culture filtrate sterilized by filtration or gamma irradiation.

no addition □

filtrate (filter sterilized) 50% △

filtrate (irradiated) 50% ●

extracted resulted in a similar decrease in its inhibitory activity (Figure 25). These experiments again substantiate the fact that the major inhibitory component in this pseudomonad filtrate was in fact pyocyanine.

8. Effect of pyocyanine concentration

BHI was used as the growth medium for preparation of culture filtrates. There were variations in pyocyanine production between the various lot numbers of the BHI used. Some lot numbers resulted in filtrates that contained no detectable pyocyanine. In these cases the antimicrobial activity should be mainly due to the "other factors" alone since the pyocyanine level would be very low if present at all. The outgrowth of *S. thompson* MCC 608 was followed in two filtrates one of which contained no pyocyanine. The pigment deficient filtrate delayed the onset of log phase growth by 1 h while a similar filtrate containing pyocyanine caused a delay of more than 3 h (Figure 26). Since the pyocyanine free filtrate should contain the "other factors", this experiment would again suggest that pyocyanine played the most important role in the antimicrobial activity of the filtrate prepared in this study.

9. Summary

a. *P. aeruginosa* NHW M1 culture filtrate contained two or more factors that caused inhibition of growth. These did not appear to involve pyocyanase as described by Waksman (1945) or the pyo compounds

FIGURE 25.

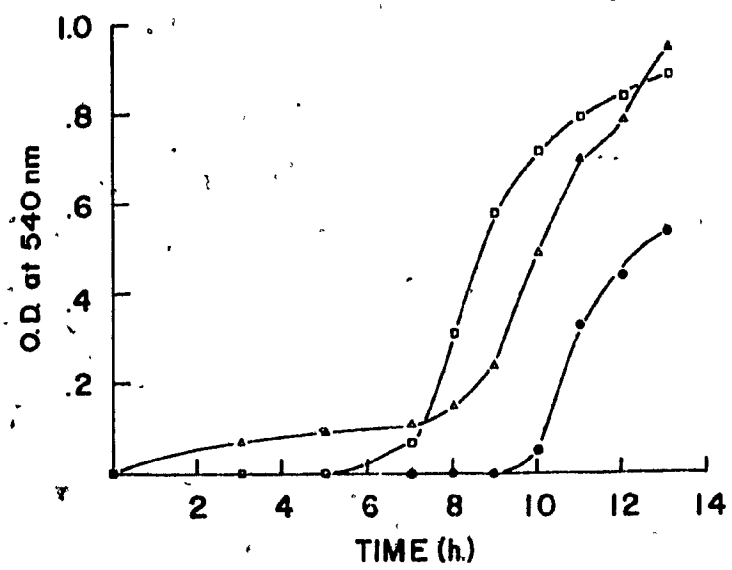


Fig. 25. The growth of *S. thompson* MCC 608 in BHI with additions of a *P. aeruginosa* NHW M1 culture filtrate from which pyocyanine had been extracted. Extracted filtrate sterilized by filtration or gamma irradiation.

no addition □

filtrate (filter sterilized) 50% ●

filtrate (irradiated) 50% △

FIGURE 26.

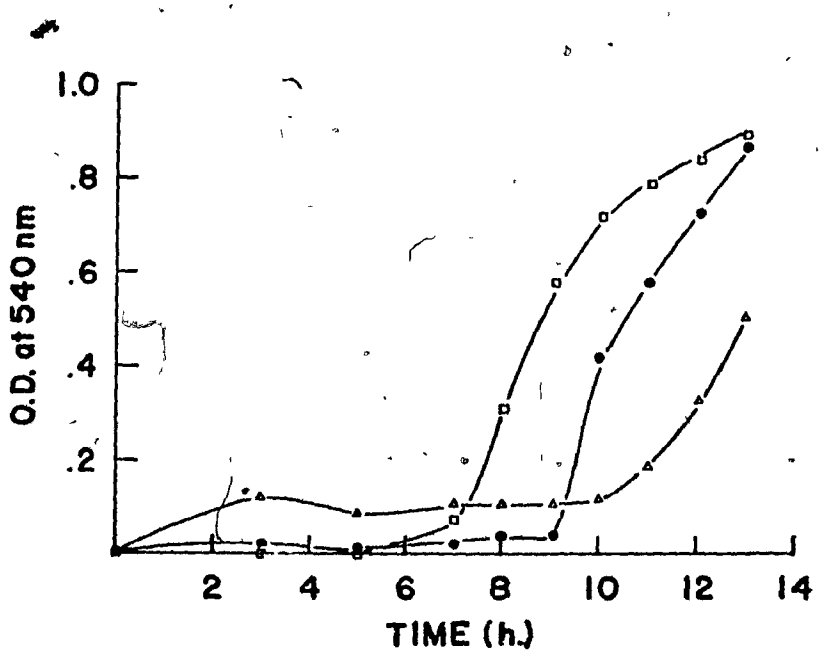


Fig. 26. The growth of *S. thompson* MCC 608 in BHI with added *P. aeruginosa* NHW M1 culture filtrate.

Additions:

no addition □

filtrate (containing detectable pyocyanine) 50% △

filtrate (not containing detectable pyocyanine) 50% ●

of Hayes *et al.* (1945).

b. The major inhibitory factor in this study was the blue pigment pyocyanine.

c. Gamma irradiation of pyocyanine solutions resulted in a loss of the blue colour and all of its inhibitory activity.

d. The factors other than pyocyanine that inhibited growth were not identified.

PART C INHIBITORY ACTIVITY OF PYOCYANINE

1. Antimicrobial activity of pyocyanine

The addition of pyocyanine to a culture of *S. thompson* MCC 608 in BHI medium caused inhibition of growth. When the pyocyanine was added prior to the inoculum, a concentration dependent extension of the lag phase of growth resulted (Figure 27). As shown in Figure 27, the higher the concentration of pyocyanine added the greater the extension of the lag phase of growth. Addition of pyocyanine to *S. thompson* cultures during log phase of growth caused the viable count to remain stable for several hours (Figure 28). This was followed by an increase in viable numbers and the logarithmic phase was reached.

2. Antimicrobial effect of pyocyanine on other organisms

a. *Effect on S. aureus (a Gram-positive) and on E. coli (a Gram-negative)*

The results reported in PART A showed that both Gram-positive and Gram-negative organisms were sensitive to the *Pseudomonas* culture filtrate. The Gram-positive *S. aureus* PHL 40 was more sensitive to the complete filtrate than were several Gram-negative organisms. Similar observations were found when pyocyanine alone was used as the inhibitory substance. *S. aureus* was extremely sensitive to pyocyanine when it was added to an actively growing culture in BHI medium at 35 C (Figure 29). There was no recovery as was observed with *S. thompson*

FIGURE 27.

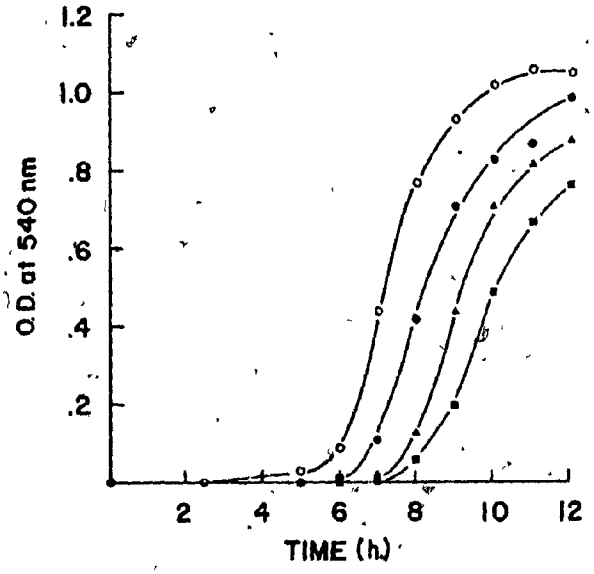


Fig. 27. The effect of pyocyanine on the growth at 35 C of *S. thompson* MCC 608 in BHI medium. The pyocyanine solution was prepared by chloroform extraction and the concentration was adjusted to be equal to that of the original filtrate.

Additions:

no addition ○

pyocyanine 15% ●

pyocyanine 30% ▲

pyocyanine 45% ■

FIGURE 28.

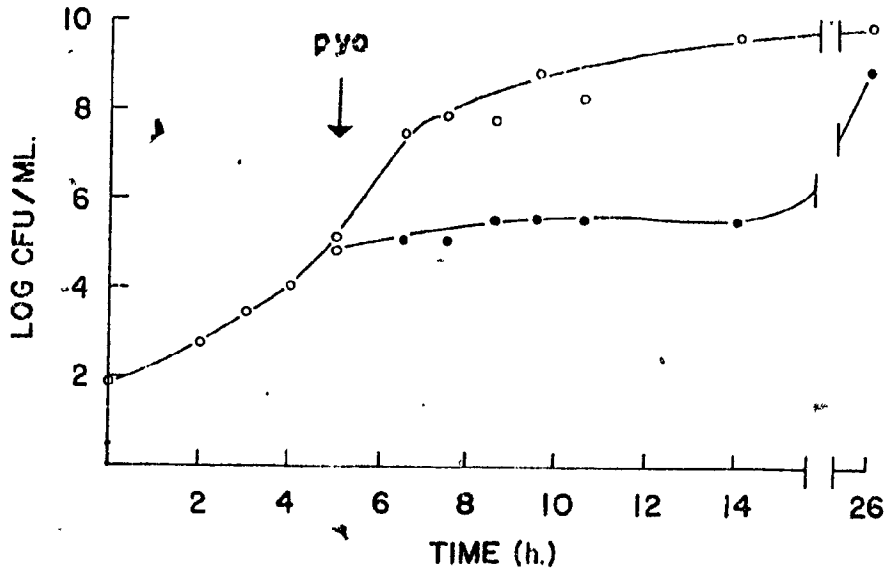


Fig. 28. The effect of pyocyanine on the growth of *S. thompson* MCC 608 in BHI medium at 35 C. The culture was grown for 5 h prior to the addition of pyocyanine.

Additions:

none o

pyocyanine 50 µg/ml added after 5 h growth ●

FIGURE 29.

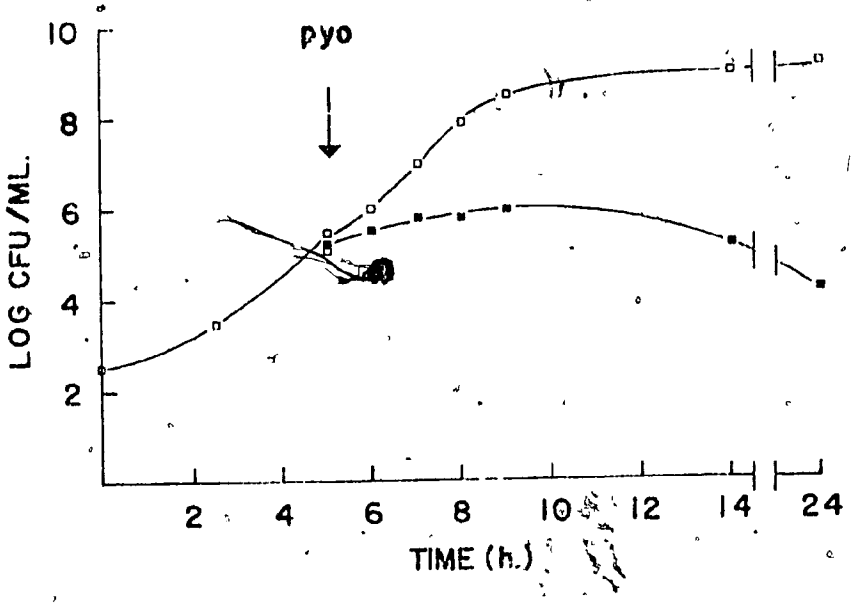


Fig. 29. The effect of pyocyanine on the growth of *S. aureus* PHL 40 in BHI at 35 C. *S. aureus* was grown for 5 h prior to the addition of pyocyanine.

Additions:

none □

pyocyanine 50 µg/ml after 5 h growth ■

(Figure 28). The control culture had reached stationary phase (> 10^9 CFU/ml) after 24 h while the pyocyanine treated culture had declined to 10^4 CFU/ml. *E. coli* B MCC 237 was also sensitive to pyocyanine but unlike *S. aureus* was able to recover in the pyocyanine containing system. The extent of the inhibition of *E. coli* B was dependent on the concentration of pyocyanine used (Figure 30).

Pyocyanine was capable of inhibiting the growth of a Gram-positive and a Gram-negative organism. The Gram-positive organism, *S. aureus* appeared to be more sensitive than the Gram-negative organisms, *S. thompson* and *E. coli* B.

b. *Effect of pyocyanine on the growth of B. megaterium (A strict aerobe) and on Cl. perfringens (A strict anaerobe)*

Thus far, the effect of pyocyanine on facultative (with respect to oxygen) organisms was tested. The work was extended to include a strict aerobe and a strict anaerobe. The anaerobe, *Cl. perfringens* MCC 387 was sensitive to pyocyanine (Figure 31). Although the culture recovered and grew after 24 h it did not attain the maximum viable count observed with the control. The aerobe, *B. megaterium* MCC 7 was very sensitive to pyocyanine and no growth was measureable in the culture up to 25 h after inoculation (Figure 32).

c. *Effect of pyocyanine on the growth of P. aeruginosa NHW M1*

The addition of pyocyanine to a *P. aeruginosa NHW M1* culture which had been grown for 5 h in BHI at 35 C had little effect (Figure 33).

FIGURE 30.

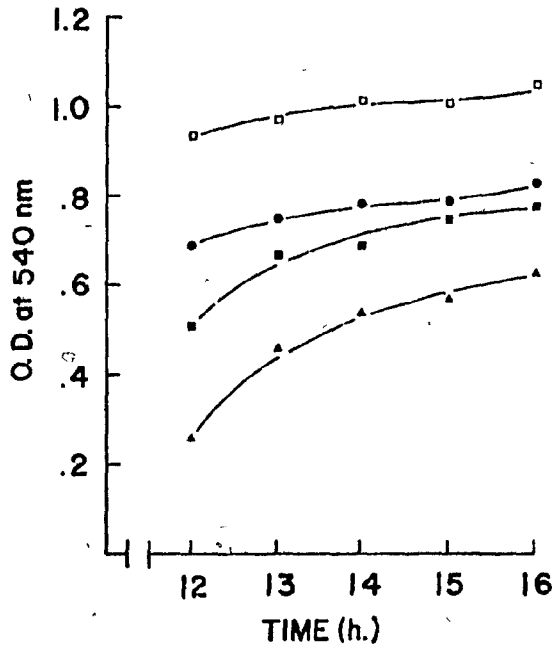


Fig. 30. The effect of pyocyanine on the growth of *E. coli* B MCC 237 in BHI at 35 C. The figure shows growth curves after 12 h because in previous experiments inhibition was complete up to 12 h. Pyocyanine concentration was adjusted to be the same as that in the original filtrate used.

Additions:

none □

pyocyanine 15% ●

pyocyanine 30% ■

pyocyanine 45% ▲

FIGURE 31.

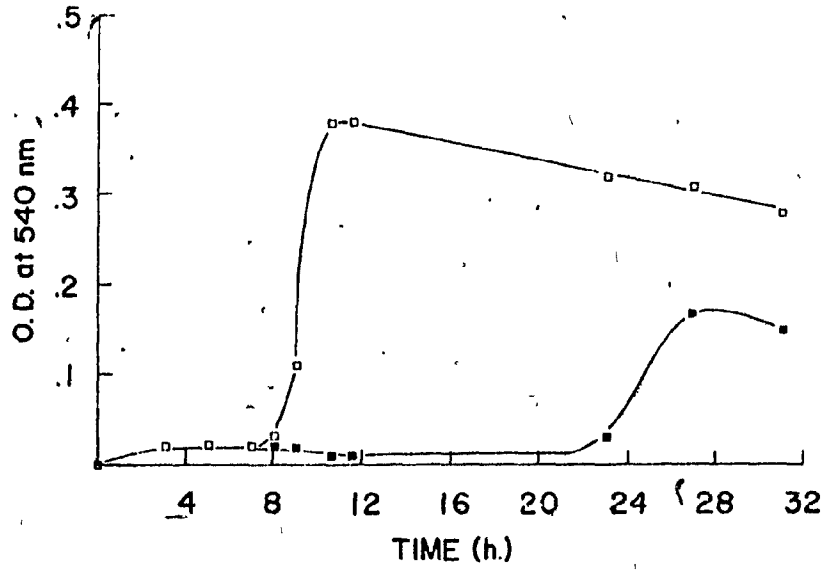


Fig. 31. The effect of pyocyanine on the growth of *Cl. perfringens* MCC 385 in BHI at 35 C.

Additions:

none □

pyocyanine 50 µg/ml ■

FIGURE 32.

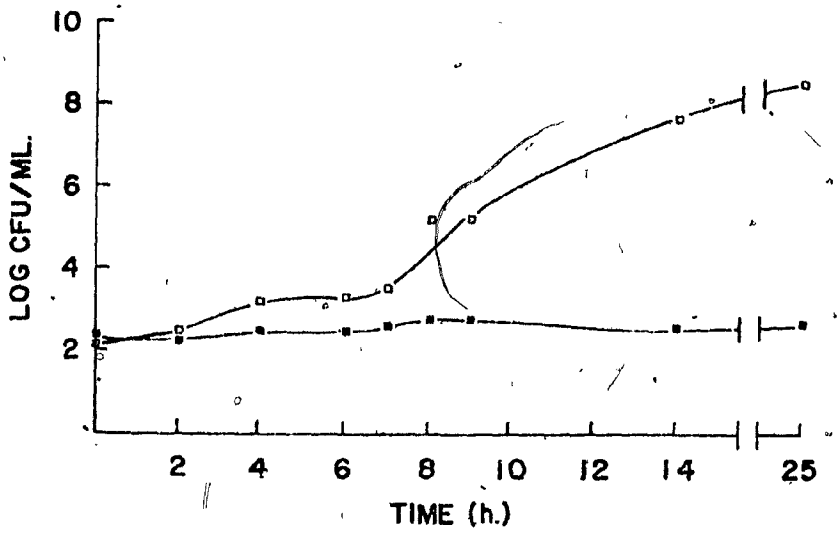


Fig. 32. The effect of pyocyanine on the growth of *B. megaterium* MCC 7 in BHI at 35 C.

Additions:

none □

pyocyanine 50 µg/ml ■

FIGURE 33.

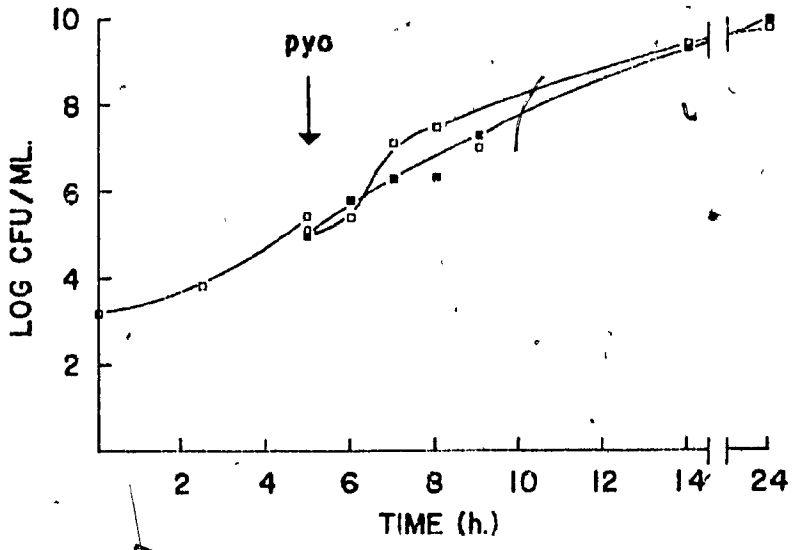


Fig. 33. The effect of pyocyanine on the growth of *P. aeruginosa* NHW M1 in BHI at 35 C.

Additions:

none □

pyocyanine 50 µg/ml ■

Pyocyanine inhibited both Gram-positive and Gram-negative organisms. The Gram-positive organisms, *B. megaterium* MCC 7 and *S. aureus* PHL 40 were more sensitive than the Gram-negative organisms *S. thompson* MCC 608, *E. coli* B MCC 237 and *P. aeruginosa* NHW M1. The strict aerobe, *B. megaterium* MCC 7 was totally arrested, whereas the strict anaerobe *Cl. perfringens* MCC 387 was inhibited for some time but eventually returned to normal within 24 h. *P. aeruginosa* NHW M1 possessed some mechanism of resistance to the antimicrobial activity of pyocyanine. This resistance aspect was not further investigated.

3. Effect of reduced oxygen tension on pyocyanine activity

The marked difference in response to pyocyanine by the aerobic organism, *B. megaterium* and the anaerobic organism *Cl. perfringens*, suggested a comparison of the effects of pyocyanine on *S. thompson* grown under both aerobic and anaerobic conditions. The organism was grown aerobically and anaerobically for 5 h. Each culture was then divided into two equal portions. Fresh BHI alone was added to two portions (one aerobic and one anaerobic). An equal volume of BHI containing 100 µg/ml pyocyanine was added to the remaining two flasks. The final concentration of pyocyanine in the treated flasks was thus 50 µg/ml. Care was taken to minimize the amount of air added to the anaerobic flasks. The fresh BHI and BHI with pyocyanine were prepared in sealed flasks, flushed with nitrogen, and added aseptically through the scuba seal with a syringe.

Viable counts were determined at various time intervals. As shown in Figure 34 the growth of both the aerobic and anaerobic cultures was affected by the pyocyanine. However, the extent of inhibition was less under anaerobic conditions. After 24 h, the aerobic control had reached 8.4×10^9 CFU/ml whereas the number of the pigment treated cells remained static at 1.7×10^5 CFU/ml. Under anaerobic conditions the control reached 1.7×10^9 CFU/ml while the pigment treated anaerobic culture was only slightly lower in number (8.2×10^8). The inhibitory effect of pyocyanine was therefore more pronounced under aerobic than under anaerobic conditions. The recovery in the anaerobic culture was almost complete after 24 h while growth appeared to have stopped in the pigment treated aerobic culture. This experiment indicated that oxygen must play a role in enabling pyocyanine to inhibit growth of *S. thompson* MCC 608. When available oxygen was reduced the inhibitory effect of pyocyanine was lessened.

4. Effect of pyocyanine on the growth of *S. thompson* in BHI medium containing a reducing compound.

Sodium thioglycolate, a strong reducing agent, was added to BHI medium (pH 6.5) to lower the available oxygen content of the medium. The control flasks, one containing BHI alone and the other with BHI and 5% sodium thioglycolate, followed very similar growth patterns (Figure 35). Pyocyanine inhibited growth in both the normal (BHI and pyocyanine) and the reduced medium (BHI and 5% sodium thioglycolate

FIGURE 34.

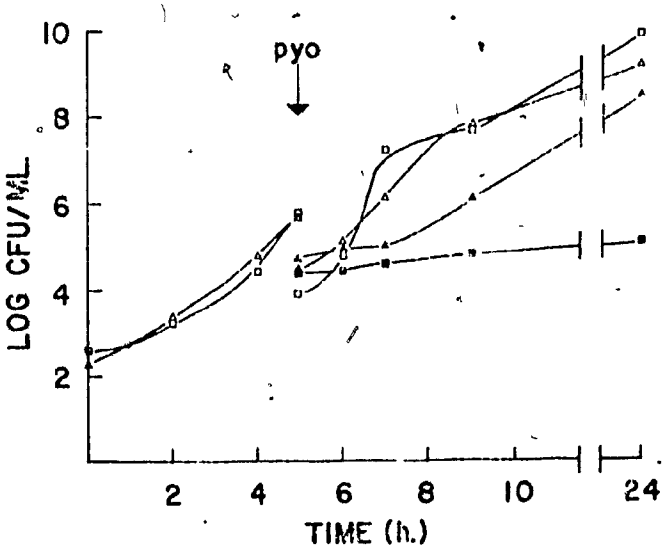


Fig. 34. The effect of pyocyanine on the aerobic and anaerobic growth of *S. thompson* MCC 608 at 35 C in BHI. Pyocyanine was added after 5 h incubation.

aerobic	no pyocyanine	□
aerobic	50 µg/ml pyocyanine	■
anaerobic	no pyocyanine	△
anaerobic	50 µg/ml pyocyanine	▲

FIGURE 35.

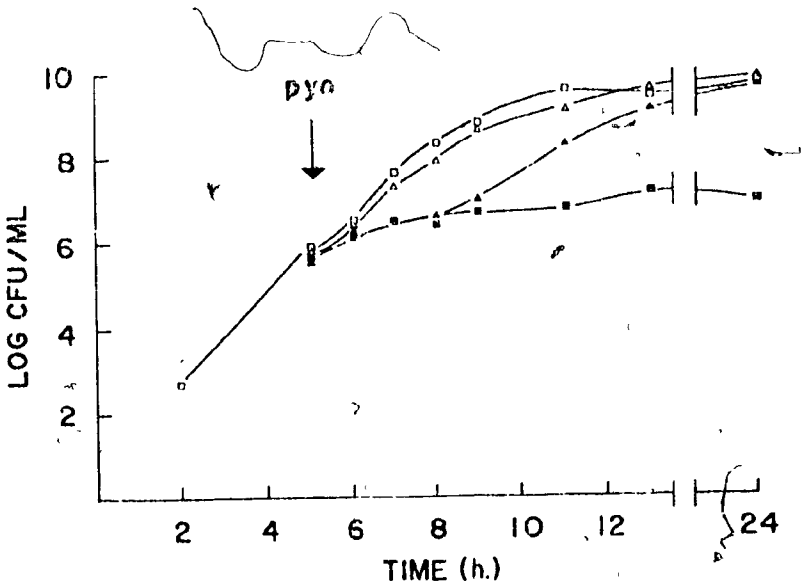


Fig. 35. The effect of pyocyanine on the growth of *S. thompson* MCC 608 at 35 C in BHI with and without 5% sodium thioglycolate added. Pyocyanine was added after 5 h growth.

Additions:

no pyocyanine and no thioglycolate □

pyocyanine 50 µg/ml and no thioglycolate ■

no pyocyanine and 5% thioglycolate △

pyocyanine 50 µg/ml and 5% thioglycolate ▲

and pyocyanine). Recovery was more rapid in the reduced medium. Limiting the amount of available oxygen enabled *S. thompson* MCC 608 cultures to more rapidly overcome the inhibitory activity of pyocyanine.

5. Summary

a. Pyocyanine inhibited or delayed the growth of a variety of microorganisms.

b. Gram-positive organisms were more sensitive than Gram-negative organisms.

c. Both strict aerobes and anaerobes were affected but the aerobic strain was more sensitive.

d. *P. aeruginosa* NHW M1 was resistant to the inhibitory effect of pyocyanine.

e. Pyocyanine inhibition of *S. thompson* MCC 608 decreased in anaerobic systems and in reduced medium.

f. Oxygen appeared to play an important role in the ability of pyocyanine to inhibit growth.

P A R T D ACTION OF PYOCYANINE ON *S. thompson* MCC 608

1. Injury

The addition of pyocyanine to a log phase culture of *S. thompson* MCC 608 caused the number of viable cells in the culture to remain constant for several hours. This was followed by recovery and normal cell multiplication (Figure 28). This change could reflect a change in growth rate or the death of a portion of the cell population. A change in the growth rate might be the result of injury to a majority of the cells. A period of time might then be required for the injury to be repaired before the normal logarithmic growth could resume. If, on the other hand, some cells were sensitive and killed, the resistant cells would have to continue to grow at a rate sufficient to replace those killed by the pigment treatment.

The results in Part A, Figures 14 and 15, suggested that *S. thompson* MCC 608 might be injured by exposure to complete culture filtrate. Therefore, attempts were made to demonstrate injury to *S. thompson* cultures exposed to pyocyanine alone. Control and pyocyanine treated samples (see Figure 28) were plated on EMBA and 2% NaCl as well as on the normal BHIA. The viable counts were similar for both pigment treated and control cells on both media and therefore indicated no injury. Injury could not be demonstrated when other selective media such as brilliant green agar, Salmonella Shigella agar or Synthesis S medium counts were compared with those on BHIA. These results indi-

cated that if *S. thompson* MCC 608 cells were injured by exposure to pyocyanine, the injury could not be detected through the differential plating methods used.

2. Effect of pyocyanine on resting cell suspensions

The possibility that pyocyanine had a lethal effect was investigated by studying the actions of pyocyanine on cells maintained under resting conditions. The cell suspensions were prepared as described in the Materials and Methods section 8. CF-S medium was used as the suspending fluid so that cell division should not occur. If some cells were killed by addition of pyocyanine, the viable counts should decrease with time. As shown in Table 2, the number of viable cells remained relatively constant, when *S. thompson* MCC 608 was exposed for 8 h to 150 µg/ml pyocyanine. In growing cultures and at lower pigment concentrations (50 µg/ml), the effect of the pyocyanine is detectable within an hour of pigment addition. Similar experiments with the more sensitive organisms *E. coli* B MCC 237 or *S. aureus* PHL 40 indicated that cell death after addition of pyocyanine could not be detected up to 5 h after pigment addition. There were decreases in viable cell numbers after 24 h with *S. thompson* MCC 608 and *E. coli* B MCC 237 but no decrease was detected with the more sensitive *S. aureus* PHL 40. These experiments indicated that pyocyanine does not kill cells.

TABLE 2.

TABLE 2.

Viable counts in resting cell suspensions of bacteria in CF-S medium with and without pyocyanine

Organism	Pyocyanine concentration µg/ml	Time h	Viability Log CFU/ml	
			Control	Pigment exposed
S. thompson MCC 608	150	0	7.24	7.33
		2.0	7.41	7.41
		6.0	7.41	6.86
		8.0	7.48	7.41
		11.5	7.41	6.67
		24.0	7.24	6.93
E. coli B MCC 237	50	0	8.67	8.84
		2.5	8.81	8.67
		5.0	8.98	8.67
		24.0	8.89	7.59
S. aureus PHL 40	50	0	8.42	8.42
		2.5	8.23	8.42
		5.0	8.34	8.67
		24.0	8.53	8.42

3. Effect of penicillin on pyocyanine treated *S. thompson* MCC 608

Penicillin is known to act on actively growing and dividing bacterial cells by disrupting cell wall synthesis (Blumberg and Strominger, 1974). The killing action of penicillin on an actively growing culture is therefore more rapid than on a culture multiplying at a much slower rate. A culture of *S. thompson* MCC 608 was grown in BHI for 5 h and one half was exposed to pyocyanine (50 µg/ml) and the other half was maintained as a control in BHI alone. These two cultures were incubated for 1.5 h and again divided into two portions. One half of each culture was exposed to penicillin (1590 units/ml). The pyocyanine concentration was maintained at 50 µg/ml in the pigment treated cultures. Viable counts were determined by plating on BHIA. The results of this experiment are illustrated in Figure 36. As observed previously, addition of pyocyanine caused the viable count in the treated culture to remain constant. When penicillin was added to the portion of the control there was a rapid decline in the viable count and viable cell numbers dropped below the detectable level within 2 h. Penicillin also caused a decrease in the viable count of the pyocyanine treated culture but the decline was not as rapid as observed with the control culture and minimal levels were only reached after 6.5 h. The results of this experiment indicated that pyocyanine treated *S. thompson* MCC 608 cultures do continue to grow but at a slower rate. One would therefore have expected an increase in the viable count in the pyocyanine treated penicillin free system

FIGURE 36.

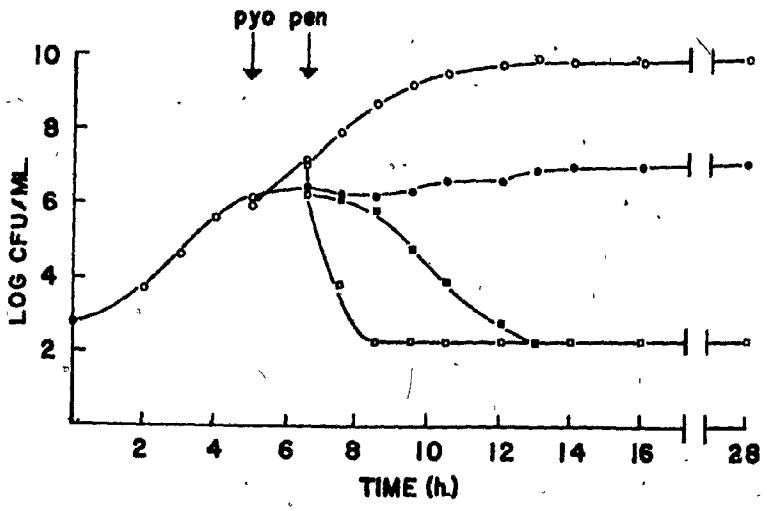


Fig. 36. The effect of penicillin on the growth of control and pyocyanine treated *S. thompson* MCC 608 in BHI at 35 C. Pyocyanine was added after 5 h growth followed by penicillin G after an additional 1.5 h.

Additions:		
none		○
pyocyanine	50 µg/ml	●
penicillin G	1590 units/ml	□
pyocyanine penicillin G	50 µg/ml 1590 units/ml	■

but this did not occur. This might indicate that the cells are not actually dividing but that some wall synthesis does take place and that this renders the cells susceptible to the penicillin or that the increase in cell numbers in the pyocyanine treated penicillin free system may be too small to be detectable by the plating method used. These results might also suggest that some process other than division such as cell elongation, was taking place which would also not be detected by the viability measurements.

4. Slide culture

The fate of log phase cells streaked on BHIA with and without pyocyanine was followed using the slide culture technique described in Materials and Methods, section 7C. Log phase cells of *S. thompson* MCC 608 were streaked on BHIA and BHIA containing 50 µg/ml pyocyanine and observed under the phase microscope (Figure 37a). After incubation for 2 h at 35 C cells on BHIA produced colonies (99.5% frequency Figure 37b), whereas cells on BHIA plus pyocyanine usually did not (2.6% frequency Figure 37c). After 24 h incubation control slides were completely overgrown while in the pigment treated slides there was limited microcolony formation and some cells were elongated to as much as 10 x normal cell length (Figure 38A). The numbers of elongated cells were small as compared with the number of cells of normal size. There was a large increase in the proportion of elongated cells on pyocyanine containing agar if the pH was adjusted to

FIGURE 37.

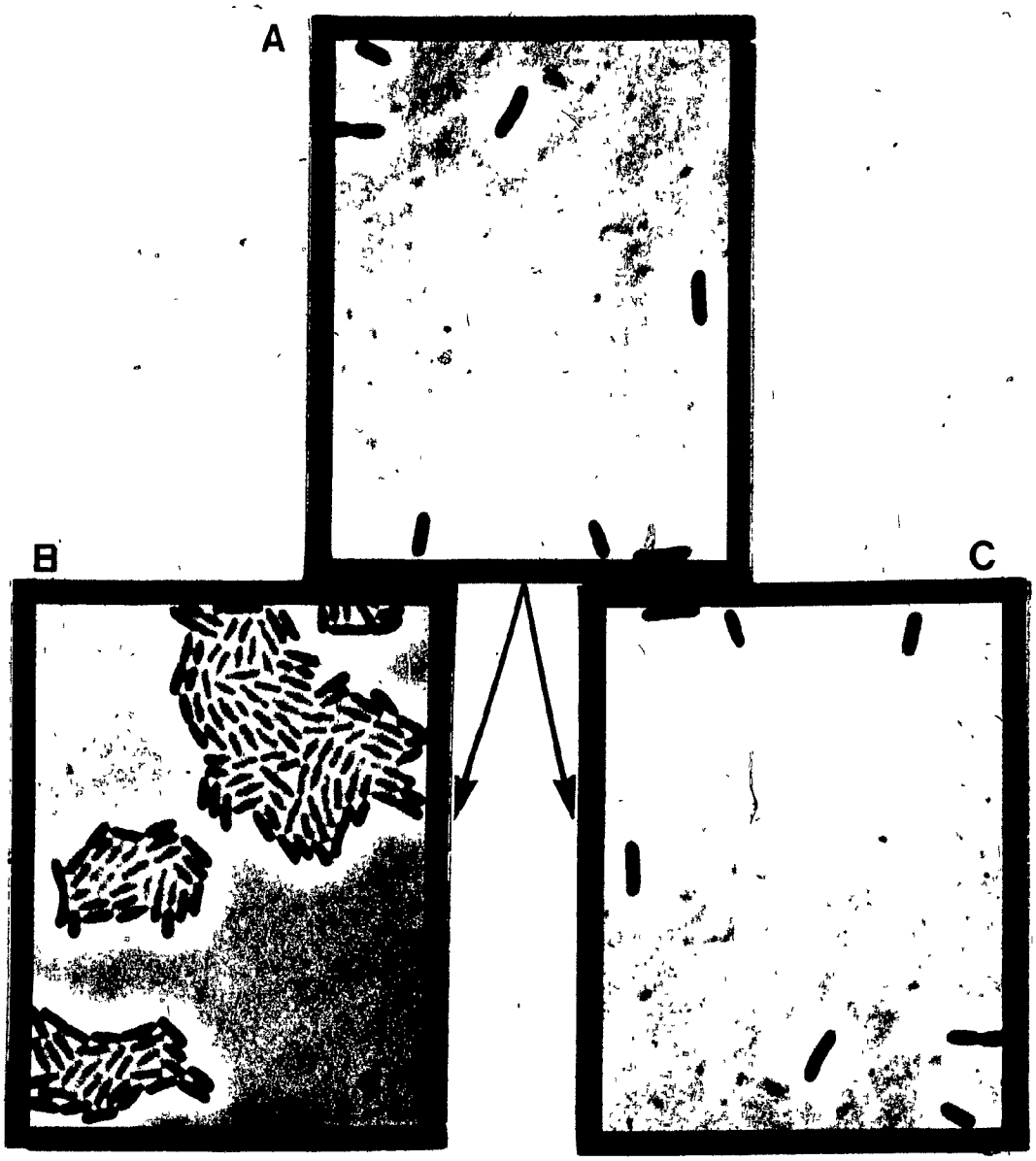


Fig. 37. The effect of pyocyanine on the growth of *S. thompson* MCC 608 in slide culture on BHIA at 35 C (mag. x 1600).

A. Slide culture on BHIA at time zero.

B. Slide culture on BHIA at time 3 h.

C. Slide culture on BHI with 50 µg/ml pyocyanine at time 3 h.

FIGURE 38.

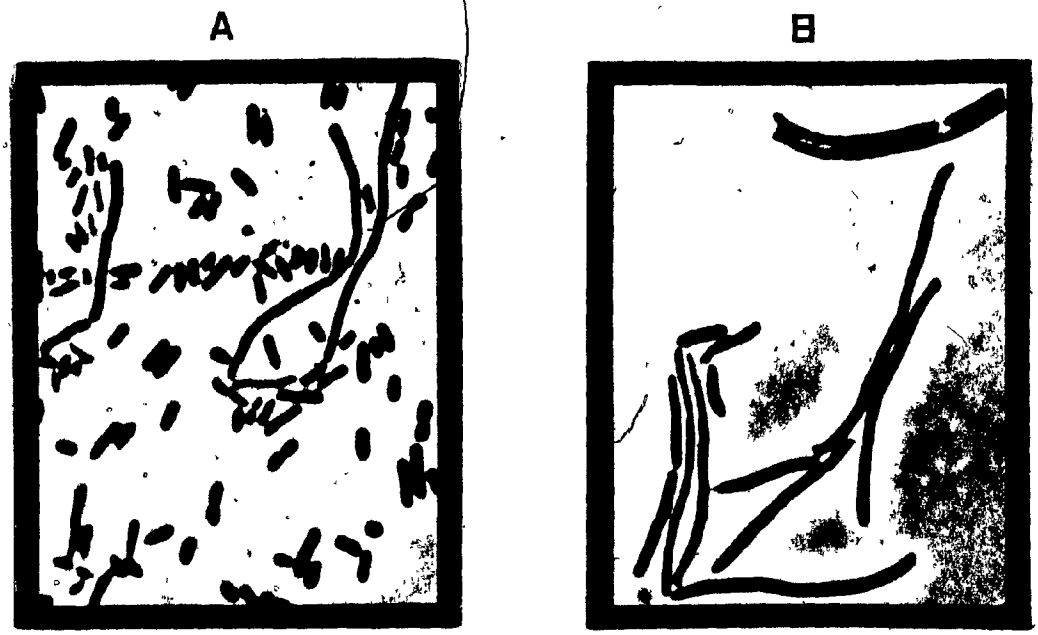


Fig. 38. The effect of pyocyanine on the growth of *S. thompson* MCC 608 in slide culture on BHIA containing 50 µg/ml pyocyanine at pH 7.4 and 5.5 at 35 C. (mag. x 1600)

A. Slide culture BHIA with 50 µg/ml pyocyanine at pH 7.4 after 24 h incubation.

B. Slide culture BHIA with 50 µg/ml pyocyanine at pH 5.5 after 24 h incubation.

5.5 rather than 7.4. A typical field is shown in Figure 38B. To insure that the elongation was due to the pyocyanine and not to some other factor such as reduced oxygen tension under the cover slips, cultures grown with shaking in liquid medium, on agar plate surfaces and on slide culture slides with no coverslips in place were examined for elongated cells. The same proportions of elongated cells were also found under these three different conditions. Cell elongation was considered to be due to pyocyanine exposure.

5. Effect of pyocyanine on protein synthesis

A culture of *S. thompson* MCC 608 was grown in BHI for 5 h and then divided into two parts. One part was maintained as a control and the other half was exposed to 50 µg/ml pyocyanine. Incubation was continued and samples were withdrawn at various times for viable counts and protein content determination. Figure 39 shows that addition of pigment caused the viable counts to increase at a slower rate for several hours. The protein concentration also showed very little increase until after 24 h. This suggested that cell elongation did not happen to a large proportion of the population when *S. thompson* MCC 608 cells were exposed to pyocyanine. Growth must have simply shifted to a slower rate. Final yields were also lower as reflected by lower protein concentration in the treated culture.

FIGURE 39.

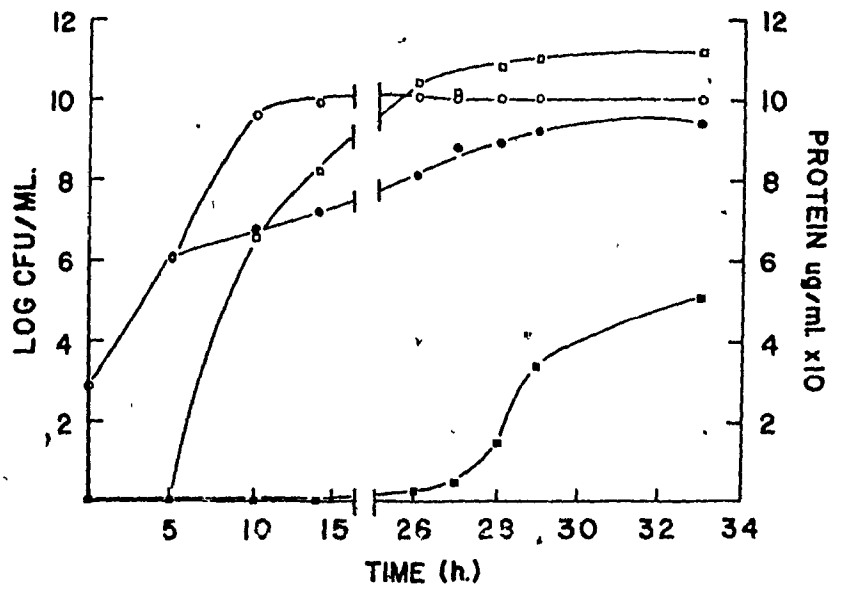


Fig. 39. The effect of pyocyanine (50 µg/ml) on the growth of and protein concentration in *S. thompson* MCC 608 cultures in BHI at 35 C.

Additions:

none	viable count on BHIA	○
	culture protein concentration	□
pyocyanine 50 µg/ml		
	viable count on BHIA	●
	culture protein concentration	■

6. Summary

a. Pyocyanine probably acts on *S. thompson* MCC 608 to cause a shift to a slower growth rate.

b. Cell injury caused by pyocyanine could not be demonstrated by differential plating methods.

c. Sensitivity of pyocyanine treated cells to penicillin suggested there was some cell wall biosynthetic activity.

d. Pyocyanine did not kill cells as indicated by constant viable numbers in pyocyanine treated resting cell suspensions.

e. Cell elongation did happen in pyocyanine treated cultures but this did not appear to be a general phenomenon because there was no detectable increase in protein content of pyocyanine treated cultures during the inhibtory period.

f. There was a decrease in final yield in pyocyanine treated cultures as reflected by decreased final protein concentration in pyocyanine treated cultures.

P A R T E SITE OF ACTION OF PYOCYANINE

The work thus far suggests that pyocyanine inhibits the growth of *S. thompson* MCC 608. Experiments with resting cell suspensions suggested that cells are not killed by exposure to pyocyanine. There appeared merely to be a decrease in the rate of growth. The following section of this investigation was an attempt to show what the mechanism of inhibition was and to confirm that cells were not actually killed by pyocyanine.

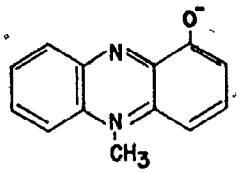
1. Mutation and selection

The structural formula of pyocyanine is very similar to that of acridine orange (AO) (Figure 40). Acridine orange at a concentration of 100 µg/ml caused lethal mutations of *E. coli* (Southwick *et al.*, 1972). It seemed possible therefore that pyocyanine might act in a similar manner and cause lethal mutations of *S. thompson* MCC 608. The action of AO and pyocyanine on slide cultures of this organism was studied.

Acridine orange at concentrations of 100 to 200 µg/ml had little effect on microcolony formation on BHIA after 3 h incubation (Table 3). In all cases viability was greater than 96%. The molar concentration of AO that was equivalent to 50 µg/ml pyocyanine was 70 µg/ml. 200 µg/ml, twice that used by Southinck *et al.* (1972) had little effect on the growth of *S. thompson* MCC 608.

FIGURE 40.

PYOCYANINE



ACRIDINE ORANGE

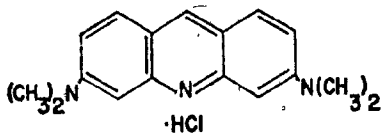


Fig. 40. The structural formulae of pyocyanine and acridine orange, (Southwick *et al.* (1972) J. Bacteriol. 110 (1): 439)

TABLE 3.

TABLE 3.

The effect of acridine orange on microcolony formation by *S. thompson* MCC 608

Concentration of acridine orange in medium µg/ml	Viability after 3 h %
0	99
100	96
150	99
250	100

Experiments reported earlier in which the effect of pyocyanine on resting cells suspensions was studied (Table 2), suggested that pyocyanine did not kill cells. This possibility was further investigated by studying the effect of pyocyanine on *S. thompson* MCC 608 growing on BHI. In this experiment an attempt was made to show that pyocyanine did not cause viable counts to remain constant by killing a proportion of the population and thus selecting the resistant cells. Two cultures were grown in BHI for 24 h. One culture was grown in BHI alone and the second in BHI supplemented with 50 µg/ml pyocyanine. At the end of the growth period a portion of each culture was diluted and plated on BHIA alone and on BHIA containing 50 µg/ml pyocyanine. If a resistant population were selected, a higher proportion of the cells from the pyocyanine grown culture would be expected to form colonies on the pyocyanine containing BHIA plates. Results in Table 4 show the differences in colony formation between BHIA and BHIA with pyocyanine under aerobic and anaerobic conditions. The per cent difference in the counts were lower on the pyocyanine grown culture. However, if pyocyanine actually killed a sufficiently large proportion of the cells to cause the growth to become stationary, 90% or more of the cells would have to be killed. The highest difference was only 35%. Therefore, this experiment again suggests that pyocyanine did not kill *S. thompson* MCC 608 cells or select a resistant population. These results suggest that pyocyanine does not act by causing lethal mutations or by selecting a resistant population.

TABLE 4.

TABLE 4.

Potential development and selection of a pyocyanine resistant population of *S. thompson* MCC 608.

Cells initially grown in		Plating medium		CFU/ml	% difference
BHI	BHI + pyocyanine	BHIA	BHIA + pyocyanine		
		Aerobic plate incubation			
+		+		6.5×10^9	20
+			+	5.2×10^9	
	+	+		3.0×10^8	13
	+		+	2.6×10^8	
		Anaerobic plate incubation			
+		+		6.0×10^9	35
+			+	3.9×10^9	
	+	+		3.4×10^8	12
	+		+	3.0×10^8	

2. Pyocyanine uptake by *S. thompson* MCC 608

The extent to which pyocyanine was taken up by *S. thompson* MCC 608 was studied by preparing ^{14}C labelled pyocyanine. This was done by growing *P. aeruginosa* MCC 436 in the modified medium of Ingram and Blackwood (1962) (Materials and Methods, section 4). This medium was supplemented with ^{14}C glycerol (9.6 mc/mM). The pyocyanine extracted had a specific activity of 2×10^{-3} mc/mM. This preparation was used to prepare a pyocyanine solution containing 500 µg/ml.

A suspension of cells was exposed to 50 µg/ml of ^{14}C pyocyanine for 1.5 h. Samples were taken in triplicate and averaged results are reported in Table 5. These results indicate that uptake or adsorption of pyocyanine by *S. thompson* MCC 608 was negligible.

Ethylenediaminetetraacetic acid (EDTA) can render cells more permeable and thus more sensitive to antibiotics. Leive (1965) reported that *E. coli* cells pretreated with 1 mM EDTA became permeable and sensitive to actinomycin. *S. thompson* cells pretreated with 5 mM EDTA prior to addition of pyocyanine were not more sensitive to pyocyanine (Figure 41). Increasing the EDTA concentration to 10 mM had no effect.

These results suggest that pyocyanine acts in some way that does not necessitate pyocyanine uptake by the cells. It appears therefore to act at the periphery of the cell.

TABLE 5.

TABLE 5.

^{14}C-Pyocyanine uptake by log phase cells of *S. thompson* MCC 608

Sample description	CPM[1]	CPM corrected for background	% of total
0.5 ml reaction mixture	349	321	100
0.5 ml supernate from centrifuged cells	297	269	84
0.5 ml cells filtered (filters counted)	33	5	2
0.5 ml into 50 ml S-medium with 50 µg/ml cold pyocyanine filtered after 1 h (filters counted)	27	-1	-
0.5 ml S-medium filtered (filters counted)	26	-2	-
0.5 ml cold pyocyanine solution filtered (filters counted)	33	5	2
0.5 ml S-medium counted directly (used as background)	28	0	-

[1] Average of triplicate observations

FIGURE 41.

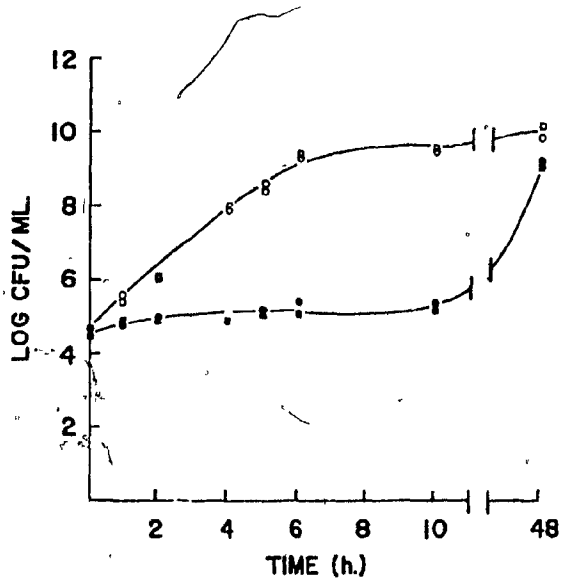


Fig. 41. The effect of EDTA pretreated on the inhibition of growth of *S. thompson* MCC 608 by pyocyanine.

Treatment:

none □

Pretreatment with 5 mM EDTA ○

Treated with 50 µg/ml pyocyanine ■

Pretreatment with 5 mM EDTA and 50 µg/ml pyocyanine added ●

3. Biosynthesis

The results reported in Figure 39 showed that pyocyanine treated cells had a decreased level of protein per ml of culture. Pyocyanine might therefore act by disrupting protein synthesis. The effect of the pigment on protein synthesis was studied in *S. thompson* MCC 608 by following the incorporation of ^{14}C alanine for 1 h in a suspension of log phase cells. The suspension was then divided into two portions and one half was maintained in the S-medium and the other in S-medium and 50 µg/ml pyocyanine. As shown in Figure 42 alanine incorporation stopped within 20 min after the addition of pyocyanine. One possible method by which pyocyanine might act to inhibit protein biosynthesis could involve disruption of transport systems so that essential nutrients would not be available to the cells. This was investigated by following the transport of various ^{14}C labelled compounds into pigment treated and control cells.

4. Transport

a. Alanine

The transport of ^{14}C alanine into pigment treated and control cells was followed using the procedure described in Materials and Methods, section 14. The cells were exposed to 50 µg/ml pyocyanine before the beginning of the uptake. Figure 43 shows that uptake appeared to be similar in both control and treated cell preparations.

FIGURE 42.

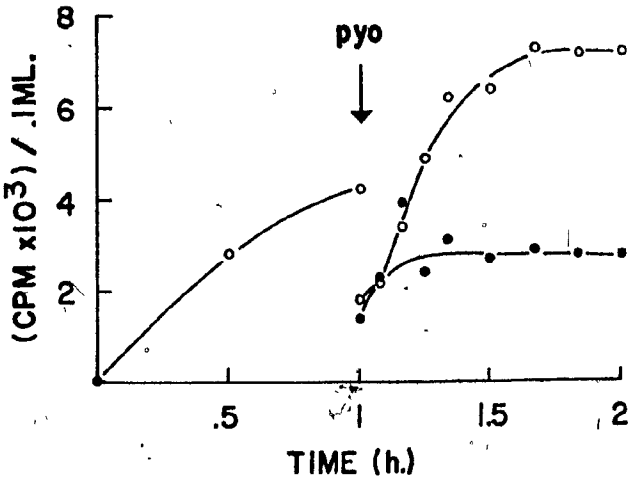


Fig. 42. The effect of pyocyanine on the incorporation of ^{14}C alanine into cells of *S. thompson* MCC 608 grown in S-medium at 35 C.

Additions:

none ○

pyocyanine 50 µg/ml ●

FIGURE 43.

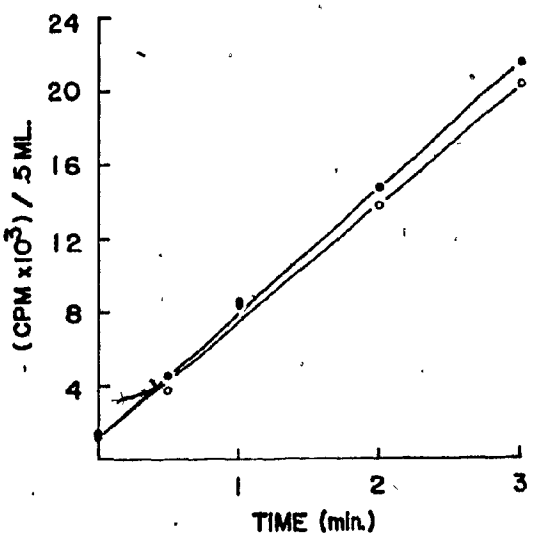


Fig. 43. The effect of pyocyanine on the uptake of ^{14}C alanine by *S. thompson* MCC 608 cells grown in S- medium at 35 C.

Pretreatment:

none ○

pretreated with pyocyanine 50 µg/ml. ●

Pyocyanine therefore did not appear to affect alanine uptake.

b. Uracil

Uptake of ^{14}C uracil was followed in a manner similar to that for alanine. Although the amount of uptake appeared to be low, transport in the pyocyanine treated cells appeared to be similar or slightly greater than in the control cells (Figure 44).

c. Glucose and α-methyl glucose uptake

Preliminary studies with ^{14}C glucose indicated that glucose uptake was greater in control than in treated cells (Figure 45). However, when uptake was followed using the nonmetabolizable α-methyl derivative of glucose, the results (Figure 46) showed greater uptake in the pyocyanine exposed cells. The higher levels of counts observed in control cells when ^{14}C glucose was used was probably due to glucose incorporation.

This series of transport experiments indicated that the action of pyocyanine probably did not involve disruption of transport systems. The pigment exposed cells transported the amino acid, carbohydrate and nucleotide studied, as readily or at a faster rate than the normal untreated cells. The pyocyanine treated cells therefore appeared to have nutrients available for biosynthesis. The pigment must therefore inhibit growth by acting at some other site.

FIGURE 44.

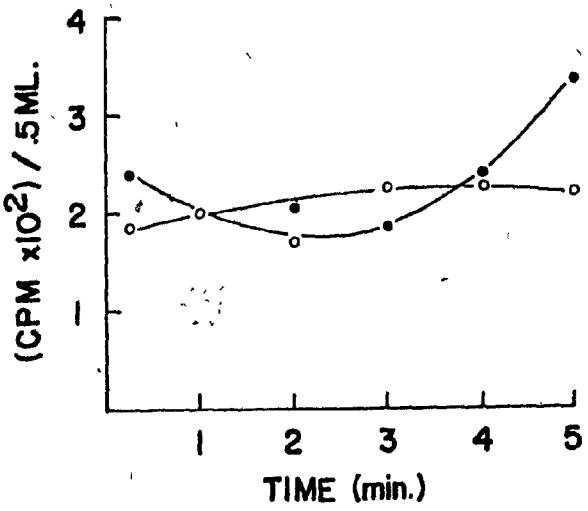


Fig. 44. The effect of pyocyanine on the uptake of ^{14}C uracil by *S. thompson* MCC 608 cells grown in S- medium at 35 C.

Treatment:

none ○

pretreatment with pyocyanine

50 µg/ml ●

FIGURE 45.

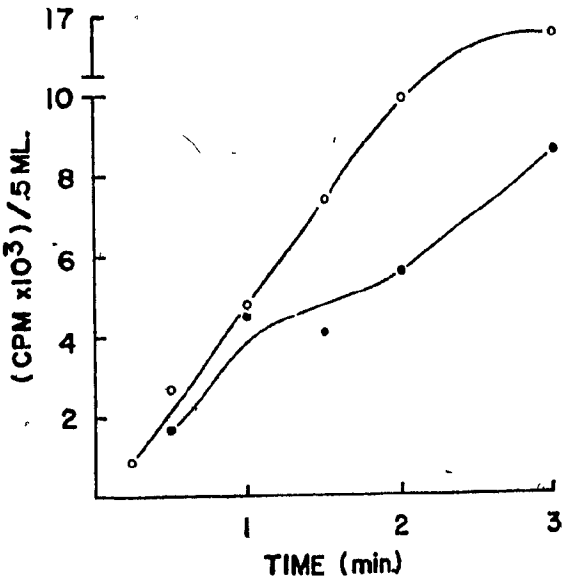


Fig. 45. The effect of pyocyanine on the uptake of ^{14}C glucose by *S. thompson* MCC 608 cells grown in S- medium at 35 C.

Treatment:

none ⊙

pretreatment with pyocyanine,

50 µg/ml ●

FIGURE 46.

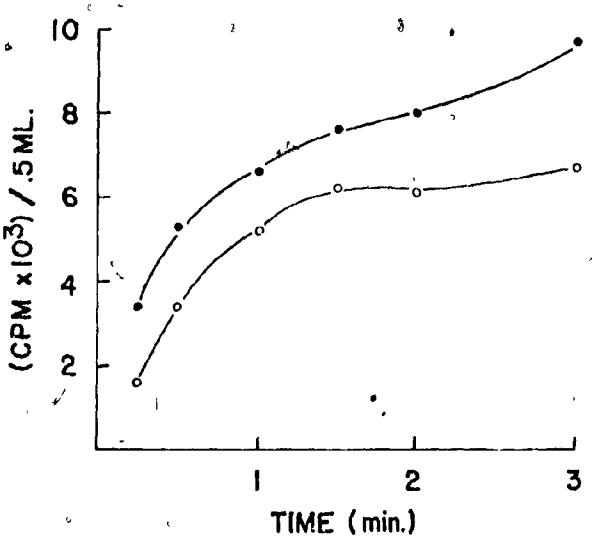


Fig. 46. The effect of pyocyanine on the uptake of ^{14}C α methyl glucose by *S. thompson* MCC 608 cells grown in S- medium at 35 C.

Treatment:

none

pretreatment with pyocyanine

50 µg/ml

5. Oxygen consumption

Cells must have a system of generating energy to drive various cell processes. When growth is aerobic and energy is derived from oxidative processes, the degree of activity can be estimated by measuring the rate of oxygen consumption. Preliminary experiments demonstrated that oxygen consumption in control and pyocyanine treated cultures were similar when the pigment was added to the treated culture immediately prior to measurement. It was necessary therefore to incubate the cell suspension in pyocyanine for more than 1 h before differences in oxygen consumption could be detected.

A cell suspension was prepared from washed log phase *S. thompson* MCC 608 cells. One portion was incubated in 50 µg/ml pyocyanine and the other in CF-S medium alone for 1.5 h. The oxygen consumed during the oxidation of various substrates was then estimated using an oxygen electrode. As shown in Table 6 the oxygen consumption was lower in the treated culture than in the control with most substrates. The notable exception to this was NADH. However, when the treated cells were washed free of pyocyanine before measurement, the oxygen consumption was lower than observed with the control cells (Table 6).

These experiments demonstrated that exposure of *S. thompson* MCC 608 to pyocyanine resulted in reduced levels of oxidation of a variety of substrates. This would then result in reduced energy production and would necessitate the treated cell preparations having reduced growth rates.

TABLE 6.

TABLE 6.

The effect of pyocyanine on oxygen consumption by starved *S. thompson* MCC 608 cells metabolizing a variety of substrates

Substrate	Concentration mM	O_2 uptake n atoms/min/mg protein	
		Control cells[4]	Pigment[4] treated cells
Glucose	10.0	359[1]	222
	10.0[2]	233	161
Succinate	10.0	44	37
Pyruvate	10.0	37	25
NADH	1.4	25	102
	1.4[2]	23	10
Lactate	10.0	21	17
Nicotinate	10.0	ND[3]	ND
Isocitrate	10.0	19	18
Galactose	10.0	328	190
Acetate	10.0	ND	ND
Glycero phosphate	10.0	ND	ND

[1] Average of duplicate result.
[2] Treated cells washed free of pyocyanine prior to oxygen consumption measurement.
[3] ND = no measureable oxygen consumption.
[4] Protein concentrations –

	mg protein/ml control	treated
no washing	12.3	12.5
pigment removed	6.6	7.6

6. *In vitro* Activity of pyocyanine

Previous results indicated that pyocyanine may react directly with NADH resulting in measureable oxygen consumption. Since pyocyanine is autooxidizable (Mahler and Cordes, 1966), it could possibly oxidize the NADH and then itself be reoxidized by any available oxygen.

Four flasks were prepared containing 0, 10, 25 and 50 µg/ml pyocyanine. A known quantity of NADH was then added to each and these were incubated at 35 C with shaking (150 rpm) for 1.5 h. The level of NADH remaining was then determined using the lactic dehydrogenase method (Klingenberg, 1965). Table 7 shows that the only flask with measureable residual NADH was the one which had no pyocyanine added. The level of recovery was low however, indicating that NADH was oxidized in the CF-S medium alone. The possibility that the CF-S medium or pyocyanine inhibited the enzyme reaction directly was investigated by adding an additional quantity of NADH to each of the reaction mixtures just prior to making NADH measurements. The NADH recovered in the pyocyanine free system was greater than the amount added the second time. The per cent recovery in the pyocyanine treatments decreased with increasing pyocyanine concentration. The results indicated a direct relationship between NADH and pyocyanine. Since NADH is an important component of the electron transport chain (ETC), it was of interest then to ascertain what effect pyocyanine might have on the ETC of *S. thompson* MCC 608.

TABLE 7.

T A B L E 7.

The interaction between pyocyanine and NADH[1] in CF-S medium

Pyocyanine concentration µg/ml	NADH % recovery
0[2]	17.5
10	0
25	0
50	0
0[3]	50.7
10	16.5
25	7.4
50	0

[1] NADH - 0.0846 µmol.
[2] Recovery after 1.5 h incubation.
[3] Same as [1] but an additional 0.0846 µmol NADH added just prior to absorbance measurement.

7. Electron transport chain

Pyocyanine is known to influence the ETC by reaction with flavoproteins (Caltrider 1967). However, the exact mechanism of reaction is not clear. In this work it has been observed that pyocyanine could react directly with NADH. The redox potential of pyocyanine (-0.34 volts) is close to that of NADH (-0.32 volts).(Figure 47). It seemed possible therefore that the inhibitory effects of pyocyanine might be bypassed by using a carbon source other than glucose. The metabolism of succinate for example, does not involve the NADH portion of the ETC.

An inoculum culture of *S. thompson* MCC 608 was prepared in S- medium with succinate as the carbon source. When the cells were streaked on slide culture agar blocks, the control cultures produced 93% microcolony formation within 3 h. When the S- medium agar blocks contained 50 µg/ml pyocyanine microcolony formation after 3 h was only 0.2%.

Richmond and Maaloe (1962) demonstrated that a functional ETC was necessary when *S. typhimurium* was growing on minimal medium with succinate as a carbon source. Glucose grown cells can develop without a high level of ETC activity. The effect of the addition of pyocyanine to a *S. thompson* MCC 608 culture growing in succinate S- medium was studied. Figure 48 shows that addition of pyocyanine to this culture caused growth to stop and no recovery followed within 24.5 h.

FIGURE 47.

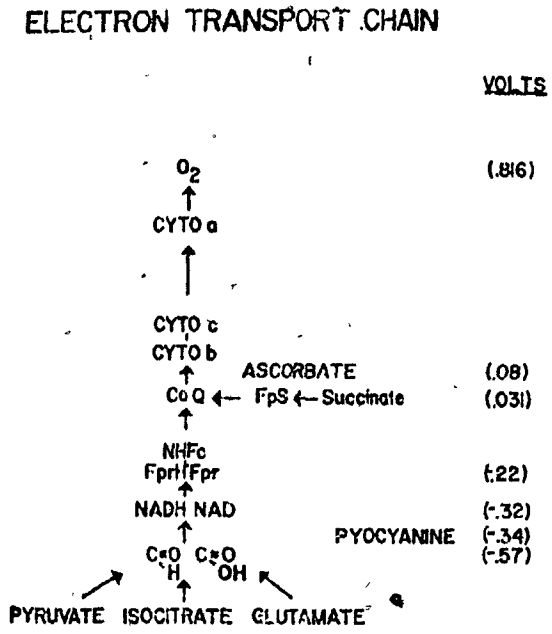


Fig. 47. A diagram of an electron transport chain showing the redox potential of pyocyanine as compared with other commonly found components.

FIGURE 48.

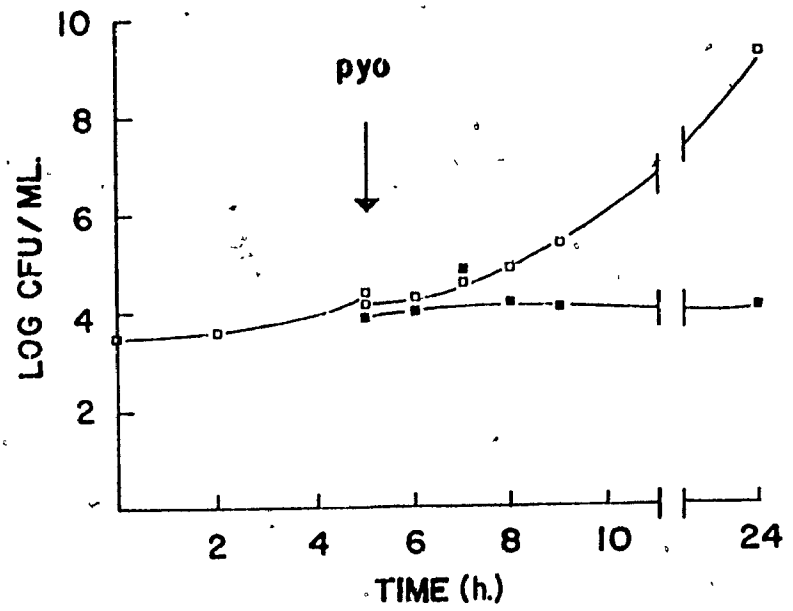


Fig. 48. The effect of pyocyanine on the growth of *S. thompson* MCC 608 in S- medium with succinate as the carbon source.

Additions:

none □

pyocyanine 50 µg/ml ■

Recovery usually happens within 24 h when glucose is the C source.

An experiment was then done to compare recovery of *S. thompson* MCC 608 in S- medium with glucose or succinate as the carbon source with various levels of pyocyanine. The medium had to be supplemented with 0.2% vitamin free casamino acids to get recovery in the pyocyanine containing cultures. Table 8 shows that *S. thompson* MCC 608 could only recover in glucose medium if the pyocyanine concentration was 20 µg/ml or less. Cells in succinate S- medium did not recover in any medium containing pyocyanine.

These experiments indicated that pyocyanine activity could not be bypassed by using substrates such as succinate which does not involve NADH in its metabolism through the ETC. This might indicate that the activity of pyocyanine is not restricted to the ETC but may involve several sites in the bacterial cell.

Pyocyanine at 5 µg/ml caused oxygen consumption of 100 n mol/min in a solution containing 3 m mol/ascorbate. Since ascorbate has a redox potential of 0.08 V, pyocyanine seemed capable of reacting with compounds with different redox potentials. The pigment may disrupt the ETC by reaction with a variety of compounds in the chain.

8. Cytochrome levels

Richmond and Maaloe (1962) demonstrated that cells grown in succinate contained high levels of cytochrome. *S. thompson* MCC 608

TABLE 8.

T A B L E 8.

Growth of *S. thompson* MCC 608 in S-medium with glucose or succinate as the carbon source and various concentrations of pyocyanine.

Carbon source	Pyocyanine concentration µg/ml	Growth at 48 h
Glucose	0	+
	10	+
	20	+
	30	-
	40	-
	50	-
Succinate	0	+
	10	-
	20	-
	30	-
	40	-
	50	-

grown in BHI contained 0.14 to 0.15 n mol. cytochrome b per mg protein (Table 9). Cytochrome b could not be detected in cells grown in BHI containing 50 µg/ml pyocyanine. Under anaerobic conditions recovery from pyocyanine is more rapid (Figure 49) and the level of cytochrome b, with or without pyocyanine, was 0.05 n mol/mg protein. Glucose is the carbon source in BHI. Growth in minimal media [S-medium or Richmond Maaloe (1962) medium (R-M medium)] with glucose as the carbon source yielded cytochrome b levels of 0.11 and 0.08 n mol/mg protein, respectively. If succinate is substituted for glucose in these media the cytochrome b level is higher, being 0.17 n mol and 0.19 n mol/mg protein in S-medium and R-M medium, respectively. Inclusion of pyocyanine in the minimal media resulted in complete inhibition of growth if succinate was the carbon source. Recovery in the minimal media with glucose as carbon source only occurred if the concentration of pyocyanine was 20 µg/ml or lower. Cells grown on glucose S- medium with 20 µg/ml pyocyanine produced 0.09 n mol cytochrome b/mg protein. The same concentration of pigment in BHI resulted in 0.07 n mol cytochrome b/mg protein.

Cells grown in the presence of pyocyanine produce reduced amounts of cytochrome b. This might also reflect a general decrease in the level of cytochrome or ETC function in these cells. Cells growing with a functional ETC can produce much more energy than cells which are dependent on solely glycolytic processes. This decreased level of

TABLE 9.

T A B L E 9.

Cytochrome b levels in *S. thompson* MCC 608 cells grown under various conditions

Growth conditions medium	pyocyanine (µg/ml)	cell protein mg/ml	cytochrome b n mol/mg protein
		aerobic incubation	
BHI[1]	0	25.5	0.14
	0	6.9	0.15
	20	31.3	0.07
	50	13.5	ND[3]
S-medium			
- glucose[2]	0	15.8	0.11
	20	18.1	0.09
- succinate	0	18.9	0.17
RM-medium			
- glucose	0	20.6	0.08
- succinate	0	20.1	0.19
		anaerobic incubation	
BHI	0	16.9	0.06
	50	15.0	0.05

[1] BHI = Brain heart infusion

[2] S-medium) glucose or succinate concentration 22 m mol.
RM-medium)

[3] ND = not detected

FIGURE 49.

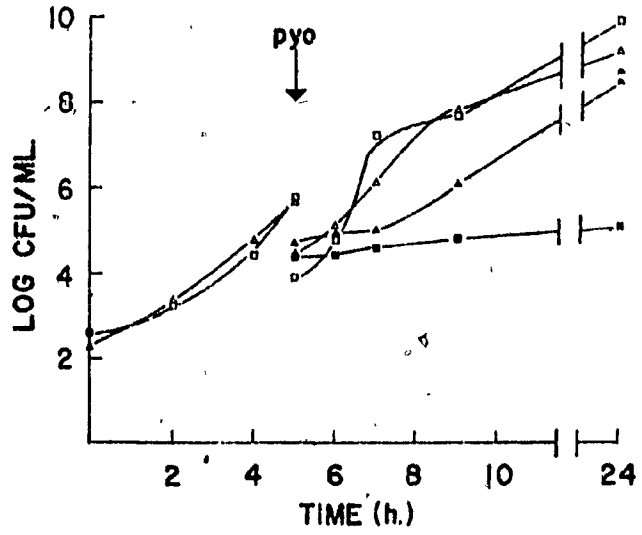


Fig. 49. Repeat of Fig. 34. The effect of pyocyanine on the aerobic and anaerobic growth of *S. thompson* MCC 608 at 35 C in BHI. Pyocyanine was added after 5 h incubation.

aerobic	no pyocyanine	□
aerobic	50 µg/ml pyocyanine	■
anaerobic	no pyocyanine	△
anaerobic	50 µg/ml pyocyanine	▲

energy production is generally reflected in decreased growth rates. The observations made here of low levels of cytochrome b and reduced growth rates in the presence of pyocyanine may indicate that the pigment causes a shift from an oxidative pathway to a predominantly glycolytic type of metabolism. This shift would result in less energy being available for various cell processes and the change would be reflected by a decrease in the rate of growth as observed in this study.

9. Effect of pyocyanine on "cytochromeless growth"

Pyocyanine has been shown to cause a reduction of the level of cytochrome b produced by cultures of *S. thompson* MCC 608. If the inhibitory action of the pigment was confined solely to suppression of cytochrome biosynthesis or disruption of the ETC, organisms which had no functional ETC should be resistant to pyocyanine. This hypothesis was tested in two ways.

Potassium cyanide (KCN) is known to inactivate the terminal oxidase of ETC (Cox *et al.*, 1970 and Kaback and Barnes, 1971). *S. thompson* MCC 608 was grown in BHI with 5 mM KCN and in BHI with 5 mM KCN and 50 µg/ml pyocyanine. The control tubes (BHI and KCN) produced visible growth within 31 h while no growth was observed in the KCN-pyocyanine tubes up to 72 h. The second experiment involved a study of the effect of pyocyanine on *S. faecalis* ATCC 8034 which has no detectable cytochromes. Inclusion of pyocyanine in a BHI culture of this organism resulted in no growth after 72 h.

These experiments demonstrated that while pyocyanine may act on some bacteria such as *S. thompson* MCC 608 to inhibit cytochrome b biosynthesis or disrupt the ETC its activity is not limited to these areas. The fact that the pigment is capable of oxidizing NADH suggests that pyocyanine may influence a wide range of cell functions.

10. *In vivo* NADH levels

We have previously shown that NADH is readily oxidized *in vitro* by pyocyanine. NADH can play an important energy yielding role in cells with an active ETC. It seemed possible therefore that pyocyanine might be capable of causing the oxidation of reduced (energy yielding) compounds, such as NADH, in bacterial cells. This loss of energy could possibly account for, at least in part, the observed decrease in growth rate.

A culture of *S. thompson* MCC 608 was grown in S- medium with succinate as carbon source. The culture was grown in 1.5 litre of medium in a 2.8 litre Fernbach flask at 35 C with shaking (150 rpm). After 24 h incubation sufficient pyocyanine was added to give a final concentration of 50 µg/ml. Incubation was continued for 1.5 h. The internal cell concentration of NADH was then determined as described in Materials and Methods, section 15. The NADH extraction was done in triplicate and the NADH determination in duplicate.

The average NADH concentration in control cells was 1.29 µmol/g

dry weight as compared to 0.10 μmol/g in pigment exposed cells (Appendix II). There was a significant difference at the .001 level. This experiment clearly shows that pyocyanine can act on *S. thompson* MCC 608 cells to reduce the internal level of NADH. This loss of energy yielding reduced compounds could account for the observed inhibitory action.

11. Summary

a. Pyocyanine probably does not inhibit growth of *S. thompson* MCC 608 by causing lethal mutations and/or selection of resistant populations.

b. Pyocyanine does not appear to be taken up by or adhere to *S. thompson* MCC 608 cells. EDTA treatment does not render *S. thompson* cells sensitive to pyocyanine.

c. Pyocyanine causes disruption of protein biosynthesis when added to growing culture of *S. thompson* MCC 608.

d. Pyocyanine does not appear to disrupt transport mechanisms of *S. thompson* MCC 608.

e. Pyocyanine causes reduced oxygen consumption by *S. thompson* MCC 608 cells metabolizing a variety of substrates.

f. Pyocyanine can oxidize some compounds such as NADH and ascorbate *in vitro*.

g. Inhibition of growth of *S. thompson* MCC 608 cannot be bypassed by growing cells on succinate which does not involve NADH in its metabolism.

h. Cytochrome b levels in *S. thompson* cells are reduced when grown in media containing pyocyanine.

i. Pyocyanine inhibits the growth of *S. thompson* MCC 608 under conditions where there is no functional ETC and the growth of *S. faecalis* ATCC 8034 which has no detectable cytochromes.

j. *S. thompson* MCC 608 cells exposed to pyocyanine have a reduced internal NADH level as compared to control cells.

PART F RECOVERY IN THE PRESENCE OF PYOCYANINE

The growth of *S. thompson* MCC 608 is inhibited in the presence of pyocyanine. When added to a culture before inoculation, the pigment causes an extension of the lag phase. Viable cell numbers remain constant for several hours if pyocyanine is added to cultures during the log phase of growth. In most cases the cultures recover and eventually reach stationary phase. This section of the study reports some of the factors that influence recovery of *S. thompson* MCC 608 in the presence of pyocyanine.

1. Effect of cell numbers

A culture of *S. thompson* MCC 608 log phase cells grown in BHI was washed three times and resuspended in CF-S medium to a concentration of 7.0×10^5 CFU/ml. The cell suspension was diluted and the dilutions were used to inoculate 50 ml sidearm flasks containing BHI or BHI with 50 µg/ml pyocyanine. As shown in Table 10 all cultures were capable of recovery in BHI-pyocyanine medium. The time required to reach stationary phase of growth decreased with increased numbers of cells added as inoculum.

2. Effect of pyocyanine concentration

A standard washed inoculum (to give 3×10^5 CFU/ml)was added to

T A B L E 10.

TABLE 10.

The effect of cell density of log phase cells of *S. thompson* MCC 608 on the recovery time in BHI containing pyocyanine[1].

Inoculum CFU/ml	h to visible growth BHI	BHI + pyocyanine
1.8×10^5	24[2]	48
9.0×10^4	24	48
1.8×10^4	24	48
9.0×10^3	24	48
1.8×10^3	24	72
9.0×10^2	24	48
1.8×10^2	24	72
9.0×10^1	24	72
1.8×10^1	24	72

[1] 50 µg/ml pyocyanine

[2] 24 h or less.

BHI medium containing various concentrations of pyocyanine (0 to 250 µg/ml). Increasing the pyocyanine concentration caused an increase in the time required for the culture to show visible growth (approximately 1 x 10^7 CFU/ml). At concentrations of 80 µg/ml and higher, recovery was not observed within 46 h (Table 11). Recovery is therefore dependent on pigment concentration and increased pyocyanine concentration causes increased time of inhibition.

3. Fate of pyocyanine

The growth of *S. thompson* MCC 608 in BHI containing pyocyanine results in a decrease in the amount of recoverable pigment (Figure 50). However, the times at which minimal pyocyanine concentration and active growth occurs do not seem to correspond (Figure 51). The pyocyanine level remained relatively constant (> 20 µg/ml) while the viable cell numbers had reached > 1.6 x 10^9 CFU/ml).

4. Oxidation state of pyocyanine

Experiments reported previously (Figures 34 and 35) showed that in a more reduced environment (anaerobiosis or chemically reduced medium) *S. thompson* MCC 608 could more rapidly overcome the inhibitory effect of pyocyanine. This organism appeared to be less sensitive to the reduced form of pyocyanine. An experiment was done to study the capacity of *S. thompson* MCC 608 to reduce pyocyanine. Washed cells were suspended in CF-S medium in test tubes so that

TABLE 11.

TABLE 11.

The effect of increased concentration of pyocyanine on the time required for *S. thompson* MCC 608 to show visible growth in BHI.

Pyocyanine concentration µg/ml	Visible growth h
0	13
10	13
20	13
30	24
40	30
50	32
60	48
70	48
80	--[1]
90	--
100	--
125	--
150	--
175	--
200	--
250	--

[1]At concentrations greater than 80 µg/ml no visible growth was observed at 48 h.

FIGURE 50.

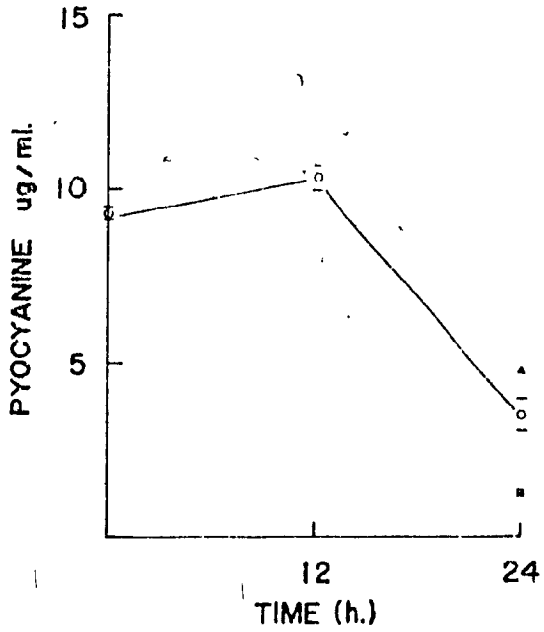


Fig. 50. Recoverable pyocyanine in a pyocyanine containing BHI culture of *S. thompson* MCC 608 grown at 35 C.

pyocyanine concentration in culture fluid ○

pyocyanine concentration in the cells ■

total final pyocyanine concentration ▲

FIGURE 51.

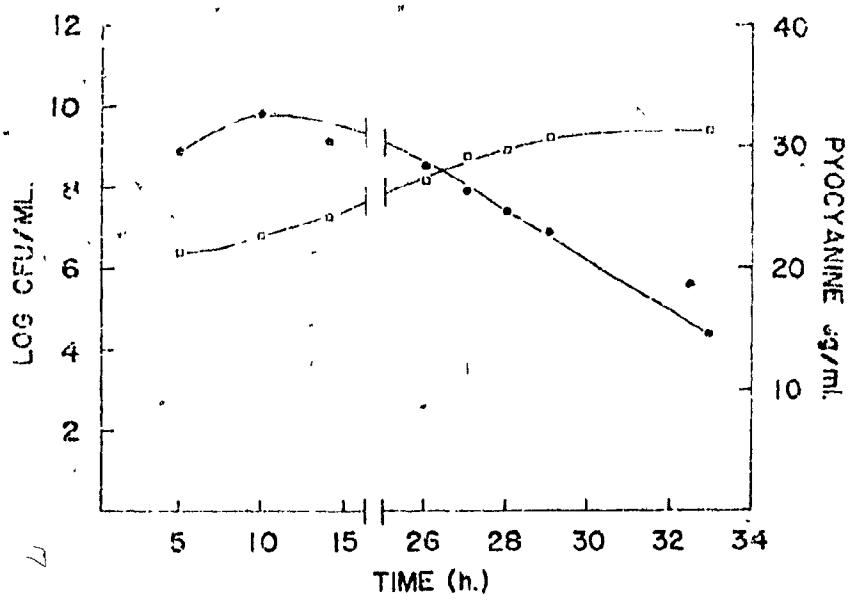


Fig. 51. Viable counts and the culture fluid pyocyanine concentration of a *S. thompson* MCC 608 culture grown at 35 C in BHI containing an initial concentration of 50 µg/ml pyocyanine.

Viable counts □

Pyocyanine concentration ●

they contained 50 µg/ml pyocyanine. The pyocyanine was completely reduced (colourless) within 3 h with 7 x 10^8 CFU/ml. When glucose was included in the medium reduction was complete after 3 h with 7 x 10^7 CFU/ml. The tubes were incubated at 35 C with no shaking. This indicated that *S. thompson* MCC 608 cells were capable of pyocyanine reduction and this mechanism may be a part of the process by which the organism can overcome the inhibitory action of the pyocyanine.

5. Summary

a. Increases in cell numbers decreases the time of inhibition of *S. thompson* MCC 608 by pyocyanine.

b. Recovery of *S. thompson* MCC 608 is more rapid with decreased concentration of pypcyanine.

c. The amount of recoverable pyocyanine is decreased in the medium in which *S. thompson* MCC 608 is able to grow.

d. Pyocyanine does not appear to reach minimal levels before or during the onset of rapid *S. thompson* MCC 608 cell multiplication.

e. *S. thompson* MCC 608 is capable of converting pyocyanine to its reduced colourless form.

GENERAL SUMMARY

The principle interaction between *P. aeruginosa* NHW M1 and *S. thompson* MCC 608 involved inhibition of *S. thompson* functions by compounds produced by the pseudomonad. Products of *S. thompson* possessed none or very little antimicrobial activity. The pseudomonad filtrate caused an extension of the lag phase of not only *S. thompson* but also of *S. aureus* and *E. coli*. The latter two organisms were more sensitive. *P. aeruginosa*, itself was resistant.

The ability of *S. thompson* to recover in the presence of pyocyanine was dependent on the cell population at the time of addition of pigment and on the concentration of pigment. The higher the cell numbers and the lower the concentration of pyocyanine, the more rapid is the recovery. High numbers of cells were capable of reducing pyocyanine to the colourless form. Pyocyanine concentrations in the cultures decreased with time. The culture did, however, appear to reach the logarithmic phase of growth before there was a large measureable decrease in the pigment concentration.

Reports in the literature indicated that pseudomonads produced a variety of compounds that caused growth inhibition of other organisms. In this study, pyocyanine appeared to play the major role in causing decreases in the growth rate of *S. thompson* cultures. Irradiation of the pyocyanine solutions resulted in the loss of the blue

colour and antimicrobial activity. Filtrates from which pyocyanine had been extracted retained some antimicrobial activity indicating that some other inhibitory factors were present. The latter were not identified.

Pyocyanine was also observed to be active against a variety of microorganisms. Some were more sensitive than others. Gram-positive bacteria and those that are strict aerobes were the most sensitive. The facultatively anaerobic organism, *S. thompson*, which was capable of recovery in the presence of pyocyanine, did so more rapidly under reduced oxygen conditions. The availability of oxygen was therefore considered to be an important factor in enabling pyocyanine to inhibit growth.

Pyocyanine appeared to act on *S. thompson* by causing a shift to a slower growth rate rather than by destroying a segment of the population. This was substantiated by the fact that viable counts remained constant during exposure to pyocyanine and the fact that viable counts were reduced more rapidly in control cultures exposed to penicillin than in pyocyanine treated cultures exposed to penicillin. Although cell elongation was observed in pigment treated slide cultures, this did not appear to be a general phenomenon as there was not an accompanying increase in culture cell protein concentration.

The exact mechanism by which pyocyanine acts to cause a shift in growth rate was not established. Pyocyanine does not appear to

be taken up by or bound to the cells. EDTA treatment of *S. thompson* cells did not render them more sensitive to the pigment. Studies with several compounds showed that the pigment treatment did not prevent them from moving into the cells. Pyocyanine did, however, disrupt protein synthesis and caused reduced oxygen consumption when the organism was metabolizing a variety of substrates. Pyocyanine oxidized NADH *in vitro* and cells exposed to pyocyanine had reduced internal levels of NADH. The level of cytochrome b in pigment grown *S. thompson* cells was also lower than those observed in control cultures. This inhibition caused by the pigment could not be overcome by growing the culture with a carbon source such as succinate which supplies electrons to the electron transport chain at a site other than NADH. Pyocyanine also inhibited the growth of other organisms that had no functional or detectable cytochrome system. Pyocyanine therefore appeared to act on *S. thompson* by oxidizing reduced intermediates such as NADH and by suppression of energy producing processes such as electron transport. The capacity of pyocyanine to cause rapid oxidation of NADH would indicate that it could influence a wide variety of cell processes.

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A P P E N D I X I

STRAKES AND STOKES MEDIUM (1959)

Chemicals	g/l
Basic composition:	
K_2HPO_4	7.0
KH_2PO_4	3.0
Sodium citrate	0.1
$MgSO_4.7H_2O$	0.1
$(NH_4)_2\ SO_4$	1.0
Additions:	
Glucose	2.0 or 4.0
Succinate	3.0 or 5.9
DL Alanine	0.445
Caseamino Acids (Vitamin free)	2.0
Agar	15.0

The basic salts solution without sodium citrate was used as a wash solution. The additions such as glucose were autoclaved separately and added to the salts solution after cooling. All preparations were adjusted to pH 7.4 with 5 N NaOH or HCl before autoclaving.

APPENDIX II

The effect of pyocyanine on the concentration of NADH in *S. thompson* MCC 608 cells treated with pyocyanine[1]

Sample[2]	NADH µmol/g Dry weight	
	Treatment	
	none	pyocyanine
1. A	1.22	0.11
B	1.12	0.15
2. A.	1.67	0.20
B	1.75	0.11
3. A	1.02	ND[3]
B	0.98	0.01
Average	1.29	0.10

[1] 50 µg/ml pyocyanine

[2] A and B are duplicate measurements

[3] ND = none detected