# THE PYOCINES OF

# PSEUDOMONAS PYOCYANEA

J. R. W. Govan, B. Sc.

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University of Edinburgh.

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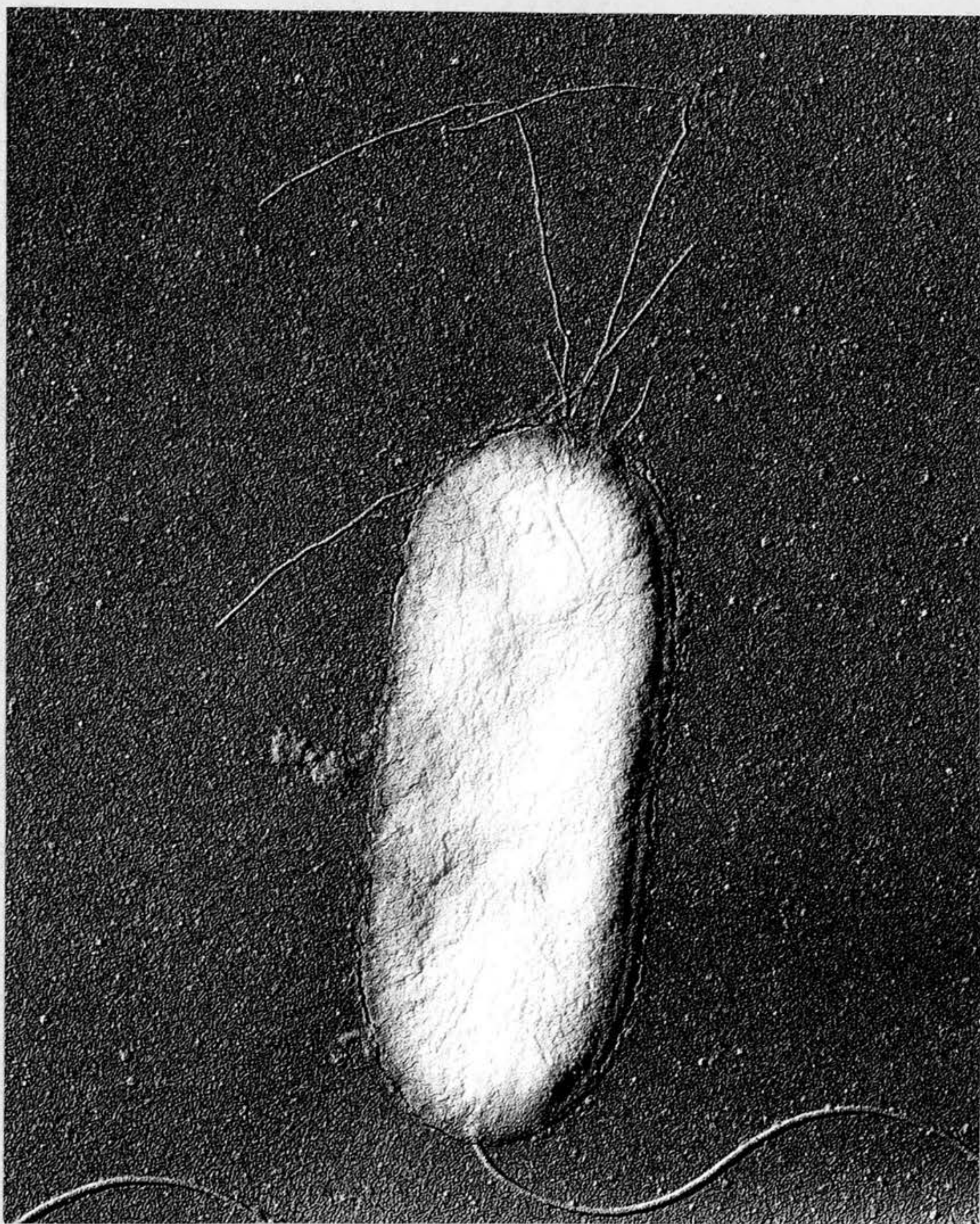

FRONTISPIECE

Electron micrograph of a fimbriate bacillus of *Pseudomonas pyocyanea* with a single polar flagellum. Gold-palladium shadowed film. X 45, 000.

# PREFACE

In the present century surgical procedures have advanced perhaps more than ever before in the history of medicine and yet along with other microbes, Pseudomonas pyocyanea continues to play a part in the defeat of such advances. Instances of open-heart surgery, transplantation of kidneys and more recently transplantation of the heart itself may be technically successful, but ultimately defeated, when following antibiotic and immunosupressive therapy, patients not infrequently become infected with Ps. pyocyanea and, all to often die.

We are indeed dealing with an organism whose repertoire of habitations is unique, scarcely anything escaping entanglement in it's web. Capable of saprophytic, commensal and pathogenic roles the widespread distribution-of Ps. pyocyanea in nature and the ability to survive in conditions of extreme hardship, in a multitude of locations, has led one bacteriologist to comment that in his opinion the organism is "the nearest thing to spontaneous generation known to man".

A satisfactory method of characterising such strains is essential if the sources and modes of spread of infections due to Ps. pyocyanea are to be studied. Previous methods of characterisation such as phage or serological typing are time-consuming and/ or produce results which are epidemiologically unreliable.

Holloway (1960) found pyocinogeny to be common in Ps. pyocyanea and suggested that this property might be useful for epidemiological studies. An investigation into the possible use of pyocine production as an epidemiological marker of Ps. pyocyanea has been undertaken as the primary aim of this work. Later, preliminary investigations into the extraction, purification and properties of pyocines were also undertaken.

Acknowledgements.

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The results of some of the work included in this thesis have already been published.

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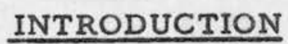


# INTRODUCTION

# CHAPTER 1

## THE GENUS PSEUDOMONAS

The term *Pseudomonas* was first introduced by Migula in 1894 to designate a new genus of rod-shaped, flagellate bacteria, One of the first species to be named was *Pseudomonas pyocyanea*, the pigment-producing organism responsible for the blue-green pus sometimes found in infected wounds and first isolated by Gessard in 1882. The Judicial Commission of the International Committee on Nomenclature of Bacteria (1952) has since recognised this organism as the types species of the genus.

In 1948, the sixth edition of Bergey's Manual of Determinative Bacteriology (Bergey's Manual) listed many additional species pathogenic for plants and at the same time 27 further species of organisms, which did not show pigmentation, were admitted to the genus. Krassilnikov (1949, quoted by Lysenko, 1961) listed more than 400 species of the genus whilst Haynes and Burkholder (1957) in the seventh edition of Bergey's Manual describe more than 140 species.

Despite the early date of it's founding the taxonomy of the genus *Pseudomonas* has not even yet been satisfactorily clarified.

It almost appears that the genus *Pseudomonas* has been used as a vacuum bag for those bacteria, which could not be allocated to more closely defined genera and have been

conveniently placed in the loosely defined Migula group.

Migula's classification was based mainly on morphology including the distribution of flagella but the introduction of sophisticated biochemical techniques in later years has resulted in a genus, containing morphologically similar organisms, whose biochemical activities are often extremely varied.

The latest edition of Bergey's Manual (1957) describes the genus Pseudomonas as follows: "Cells monotrichous, lophotrichous or non-motile. Gram-negative. Frequently develop fluorescent, diffusible pigments of a greenish, bluish, violet, rose, yellow or other color. Sometimes the pigments are bright red or yellow and non-diffusing; there are many species that fail to develop any pigmentation. The majority of species oxidise glucose to gluconic acid, 2-Ketogluconic acid or other intermediates. Usually inactive in the oxidation of lactose. Nitrates are frequently reduced either to nitrites, ammonia or to free nitrogen. Some species split fat and/or attack hydrocarbons. Many species are found in soil and water, including sea water or even heavy brines. Many are plant pathogens; very few are animal pathogens".

Klinge (1960) introduced a cytochrome oxidase reaction and this test has proved to be an extremely useful aid in the identification of those strains which do not produce the characteristic pigments.

The advent of modern sophisticated taxonomic techniques has resulted in a steady reduction in the number of groups within the genus considered to be worthy of species status.

In 1959 Rhodes, with the aid of a computer, introduced into taxonomic studies of *Pseudomonas* species Adansonian analysis; by correlating morphological, physiological and biochemical properties among a large number of strains, she concluded that many of the *Pseudomonas* species bionyms, as listed in Bergey's Manual and elsewhere, appeared to be unjustified.

In a similar fashion, Lysenko (1960) found a wide variability in carbohydrate and organic acid utilisation, as well as in proteolytic activity and thus found it impossible to divide the pseudomonads into species on the grounds of individual characters; similar difficulties had already been encountered by Seleen and Stark (1943) and Stanier (1947). This is significant since many of the species mentioned in Bergey's Manual e.g. *Pseudomonas tomato*, had been given binomial nomenclature on the grounds of a particular property or plant host. Thus the great number of different descriptions of pseudomonad species might be explained. Lysenko concluded that of the species listed only 18, including *Ps. pyocyanea*, were worthy of retention.

With few exceptions the status of strains of *Ps. pyocyanea*, as members of a distinct species, has long been accepted by

taxonomists; it's pathogenicity for man and unique production of the pigment pyocyanine have kept it aside from disputes regarding the status of other members of the genus.

The position of Ps. pyocyanea within the genus Pseudomonas has been confirmed by recent and exhaustive taxonomic studies of the group (Jessen, 1965; Stanier et al., 1966; Mandel, 1966) viz: Using 29 diagnostic tests, Jessen found 354 strains of Ps. pyocyanea to show sufficient uniformity to justify their inclusion in a distinct biotype of the genus. Application of the same tests to 505 strains of simple fluorescent pseudomonads showed the existence of no less than 81 distinct biotypes. The author concluded, however, that the extent of variation among these pseudomonads was too great to allow the recognition of species amongst them.

The same conclusions were reached by Stanier et al., (1966). In an extensive investigation of 175 pseudomonads they found several hitherto unrecognised characters which strengthened the differentiation of Ps. pyocyanea strains from other fluorescent pseudomonads. They suggested that further studies of the remaining strains might allow their division into the two classical species Pseudomonas fluorescens and Pseudomonas putida.

De Ley (1964) made a study of deoxyribonucleic acid (DNA) base compositions in the genus Pseudomonas and related genera

which had important implications for taxonomists. Using 66 of the strains examined by Stanier _et al._, Mandel (1966) investigated their DNA base compositions and found striking differences between strains designated _Ps. pyocyanea_ and other members of the genus. The DNA base composition of _Ps. pyocyanea_ strains had a guanine + cytosine content distinctly different from that of the other pseudomonads.

The species _Ps. pyocyanea_ has in general escaped the taxonomic mêlée in which so many other pseudomonads have been embroiled. Nevertheless, disagreements have long continued regarding another aspect of this bionym, namely it's correct nomenclature.

The varied descriptions of this organism viz. _Ps. pyocyanea_, _Ps. aeruginosa_, _Bacillus pyocyaneus_ by authors of many nationalities, reveal world-wide differences in opinion. Thus, in 1968, almost 100 years after it was first isolated, there is still controversy regarding the correct nomenclature of this, the founder member of the genus.

_A problem in nomenclature._

Hugh and Lessel (1967) in a request for an opinion to the Judicial Commission of the International Committee of Nomenclature of Bacteria asked for the retention of the species nomenclature _Ps. aeruginosa_ (Schroeter) Migula. Hugh and

Lessel led evidence based on historical detail which, strangely enough, is exactly the same as that on which Véron (1965) had based a similar request for the retention of the epithet *pyocyanea.* A critical résumé of the arguments used in each of these requests now follows.

A definite opinion one way or another is certainly desirable, since apart from the confusion which such dual nomenclature arouses in the minds of those not familiar with this organism, according to the laws of nomenclature there can be only one correct name for a given taxon in a given position, i.e. the earliest one which is in accordance with the rules. However, where the correct name is a sourse of confusion or is not in common usage the rules are flexible enough to yield to arbitration and allow the use of a later synonym.

The salient historical points in this controversy are as follows:

1. Fordos (1860) described unambiguously the pigment responsible for the blue-green appearance of infected wound pus and named it pyocyanine.
2. Schroeter (1872) stated that a motile organism was responsible for production of this pigment, relying upon neither chemical nor microscopic observation for his theory. He named the organism *Bacterium aeruginosum.*

3. Gessard (1882) isolated and described an organism which produced pyocyanine but he did not propose a scientific name.

4. Migula (1894) proposed a new genus Pseudomonas and in the following year designated the organism in question Pseudomonas pyocyanea. Later, however, on discovery of Schroeter's earlier use of the epithet aeruginosum, Migula altered the name to Pseudomonas aeruginosa (1900).

Schroeter's use of the latin adjective aeruginosum was an attempt at a classical description meaning "full of copper-rust, verdigris" and hence green. The organism has since become known by several popular names, e.g. bacillus of blue-green pus, green pus bacillus and the like. Fordos, of course, had also classically defined the bacterial pigment responsible using the Greek words pyon=pus and kyanos = blue, hence pyocyanine.

Migula's first proposal of the bionym Ps. pyocyanea in 1895 followed the isolation of the organism in pure culture and yet, despite the fact that rules of bacterial nomenclature did not come into force until 1947, he later acknowledged Schroeter's previous use of the synonym Bacterium aeruginosum and suggested the new name of Ps. aeruginosa (Schroeter) Migula. This nomenclature was recognised as valid by Buchanan et al., (1966).

Much of the present controversy lies in an interpretation of

the laws of nomenclature. The pertinent rule (14a Moscow 1966 Revision) of the International Code of Nomenclature of Bacteria and Viruses states as follows: "The name of a species is validly published only when it's publication conforms to the following requirements:

(1) It must be published as a binary combination consisting of a generic name followed by a single specific epithet.

(2) It must be accompanied by a description of the species or by citation of a previously and effectively published description.

(3) The description must not be based on the properties of more than one species in mixed or impure cultures. This statement does not apply to the name of a species whose description is based on morphology or other characters and not upon growth in cultures."

Certainly Schroeter's bionym Bacterium aeruginosum was a correctly formed binary combination but does his brief description and lack of even microscopic observation merit the recognition of a distinct species ? The fact that it is the only known organism which is motile and produces pyocyanine is perhaps due to chance and hardly constitutes a scientific approach. Véron (1965) argues for the retention of the name Ps. pyocyanea on the grounds that Schroeter neither isolated the organism nor even made microscopic observations "... und unter Umständen, die mir eine

mikroskopische und chemische Prufüng nicht möglich machten" does not conform to the laws of nomenclature. Certainly, in regard to section 3 of the rule, it would seem difficult to ensure that a description involved a single species when both microscopic and cultural evidence of purity was lacking.

Regarding the publication of names, rule 12c of the International Code of Nomenclature of Bacteria (1966) states that "a name which is not accepted by the author who published it ..... is not validly published". If one applies this rule to Migula's statements of 1900 then we must accept as valid his second choice, _Ps. aeruginosa_. This would seem, however, to be subject to the validity of Schroeter's designation.

Even if we accept Schroeter's description as a valid criterion of the bacterium in question we are nevertheless left with the acceptance of a bacterial cause without even microscopic evidence. If the epithet _aeruginosum_ is invalid then Migula's rejection of the alternative adjective _pyocyanea_ was unnecessary.

Whilst it confirmed the generic title _Pseudomonas_ as a _nomina generica conservanda_ Opinion No. 5 of the Judicial Commission of Bacterial Nomenclature and Taxonomy (1952) ensured the _status quo_ regarding the nomenclature of this particular species by stating that:

"The type species of the genus _Pseudomonas_ Migula 1894 is

Pseudomonas aeruginosa (Schroeter) Migula 1900 (4) Bacterium aeruginosum Schroeter 1872 (5), Bacillus pyocyaneus Gessard 1882 (6), Pseudomonas pyocyanea Migula 1895 (3)".

Hugh and Lessel (1967), after investigating a large volume of the relevant literature, concluded that the bionym Ps. aeruginosa enjoyed the greater usage by the world's microbiologists, though neither epithet enjoyed absolute usage in any one country. In this context it is worth bearing in mind rule 23 of the laws of nomenclature which states that:

"A legitimate name or epithet must not be rejected .... because another is preferred or better known, .... ".

Much of this controversy is no doubt due to the application of present laws of nomenclature to work carried out many years before such laws were formalised. An authorative statement supporting the adoption of either epithet, though desirable, must now be of academic interest only, since in the words Joseph Addison "much might be said on both sides". Perhaps in the future the situation will be resolved by the use of a specific epithet e.g. schroeter, gessard or migula, though on reflection, this would in all probability lead to further arguments over the choice of the scientist so honoured.

In the light of such dubiety Christie's approach (1948) though not the complete answer seems quite logical. He suggests that since we are dealing with an organism which is capable of producing blue-green pus in infected wounds it would seem reasonable to describe it as pyocyanea; an adjective describing both it's pyogenic and pigment producing abilities. Saprophytic members of the species could then be described as aeruginosa in keeping with their pigment producing characteristic. Pigment production is a variable property, however, and on descriptive grounds, strictly speaking neither epithet is correct. Would such a terminology infer separate species status or are we faced with a dual description of a single multitalented species ?

For the present the controversy continues. In order to avoid repitition throughout this thesis and since the majority of strains examined were of pathological origin Christie's suggestion has been followed and the name Ps. pyocyanea retained.

# CHAPTER II

"Students of hospital cross-infection, those insatiable fans of the micromystery and the bijou blood-and-thunder, are well acquanted with the versatility of *Pseudomonas aeruginosa*, the elusive pyocyanea which lurks unseen and often unsuspected in places likely and unlikely, awaiting yet another opportunity to attack those who, by reason of age, or illness are least able to resist. His lean and blue-green (or, if you prefer him gram-stained, pink) form wrapped in his polar flagella may be found in sinks, drains, or taps. He may hide in emulsion floor-polish, the lubricating oil in engineering workshops, and, with a fine bravado, on cakes of soap medicated with antimicrobial agents."

"DEATH BY SUCTION"

The LANCET, April, 1965.

## PS. PYOCYANEA:

## IT'S ROLE AS A PATHOGEN AND CONTAMINANT

Since it's first isolation from the blue-green pus of infected wounds the role of Ps. pyocyanea as a contaminant has become well known to the clinician, pathologist and bacteriologist alike. Severe and sometimes fatal infections, however, are not unknown and today almost eighty years after Charrin's (1890) first description of it's pathogenic effect in fatal septicaemia it's catalogue of destruction is a lengthy one. To the perennial query of the medical student "What does it cause?" one must draw in a deep breath and recite:

### Bacterial meningitis

In an authorative review Stanley (1947) described 41 cases of primary meningitis (22 deaths) due to Ps. pyocyanea 32 of which were iatrogenic; he also reported 28 cases of secondary meningitis caused by Ps. pyocyanea of which only four survived. Cases of iatrogenic infection still occur and those following lumbar puncture performed for diagnostic or therapeutic purposes as well as spinal anaesthesia are all the more tragic since they are entirely preventable if present day knowledge of sterilisation and aseptic techniques were properly practised.

### Infection of the eye.

Infection of the eye usually takes the form of corneal

ulceration (Shih and Hsu, 1960) which can spread with alarming rapidity to a panophthalmitis (Forkner, 1960). Necrosis of the eyelid (Fraenkel, 1922) and conjunctivitis (Kwantes, 1960) have also been encountered. Ps. pyocyanea is said to be the most virulent organism which attacks the eye and loss of the organ is a common outcome (Ayliffe et al., 1966). Bignell (1951) noted that almost all severe corneal ulcers leading to the loss of an eye were the result of infection with Ps. pyocyanea.

Infection of the ear.

Acute otitis media due to Ps. pyocyanea has been recorded by several workers including Carithers (1950) and Kwantes (1960). Infants and young children are particularly susceptible to this form of infection which is apparently more prevalent in tropical climates (Morley, 1938). The infection is especially important because of the risk of an extension to the meninges.

Surface infections.

Ecthyma gangrenosum, the characteristic cutaneous lesions occurring in septicaemia due to Ps. pyocyanea has been well documented (Forkner et al., 1958). Disease of the nails have also been reported by Bauer and Cohen (1957) and Chernovsky and Dukes (1963). Erysipelas (Kopetzsky and Almour, 1927) and various other skin lesions, including ulceration of the lips, mouth and tonsils (Stanley, 1947) mastitis (Kwantes, 1960) and other body

surfaces (Kohlenbrener et al., 1958; Post and Hopper, 1951) have been encountered.

The essentially saprophytic nature of the organism can double the risk of infection since invasion may occur at the time of an injury or afterwards during treatment.

The burn wound is extremely susceptible to invasion by Ps. pyocyanea since there is impairment of the normal defence mechanisms combined with a rich supply of easily accessible nutrients in burns; in addition, the almost inevitable use of broad-spectrum antibiotic therapy may lead to the elimination of competitive organisms and thus give an opportunist species such as Ps. pyocyanea an even greater prospect of establishing itself. Whilst the danger of septicaemia lurks in the background (Jackson et al., 1951; Markley et al., 1957) the proteolytic enzymes of the microbe begin to digest the surrounding tissues making later skin grafting extremely difficult (Lowbury, 1960). Feller and Hendrix (1964) reported that infection was the main cause of death in burned patients and Ps. pyocyanea the most common pathogen.

Gastro-intestinal infection.

Severe gastro-intestinal involvement is rare in this country but subacute infection resulting in dysenteric and typhoid-like illness has been reported in tropical climates (Ghosh, 1938; Gohar, 1939).

Septicaemia.

The onset of septicaemia following infection with *Ps. pyocyanea* is almost invariably fatal. Forkner *et al*. (1958) described 23 cases between 1954 and 1956 and all but one were fatal. Tze-Ying (1964) reported a study of 20 cases between 1958 and 1961 and in this series there were 12 deaths, despite intensive therapy.

Genito-urinary tract infections.

Urine is an excellent culture medium for *Ps. pyocyanea* (Aurelius, 1962). The introduction of the organism into the urinary tract frequently follows instrumental or operative procedures and reports have indicated that it can account for up to 17 per cent. of such infections (Dube and Shriniwas, 1965). Although there is no legal requirement for the notification of infections with *Ps. pyocyanea* it seems likely that these are more commonly acquired in the hospital environment than in the open community (Gould, 1963); for example Pyrah *et al.* (1955) found that over a five month period the organism could be isolated from 100 per cent. of patients with indwelling catheters.

Infection in the young.

The types of infection occurring in children closely parallel those encountered in adults (Geppert *et al.*, 1952) with the

additional hazards of respiratory and gastro-intestinal involvment. Such infections, though not normally serious in adults, are much more severe in the case of young children.

Premature babies and infants are particularly susceptible to attack. Baginski (cited by Paul and Margret, 1964) as early as 1908 considered Ps. pyocyanea to be the most dangerous enemy of infancy. Intestinal infection with concomitant diarrhoea and bowel ulceration has been reported by many authors. Hunter and Ensign (1947) reported nine deaths in an epidemic of 24 cases, and in the following year Schaffer and Oppenheimer (1948) reported that seven of eight cases of such infection died and emphasised the ability of the organism to cause severe gastro-intestinal lesions.

Respiratory infections resulted in the death of 19 of 55 cases in children investigated by Jacobs (1964) over a four year period.

In Germany, Eggers and Wőckel (1958) reported 16 fatalities in 34 cases of infection due to Ps. pyocyanea between 1953 and 1956 whilst Asay and Koch (1960) encountered 21 such fatalities in 1957 alone.

## THE INCIDENCE OF INFECTION WITH PS. PYOCYANEA

Reference has already been made to the lack of accurate information on the incidence of disease and mortality associated with Ps. pyocyanea, however, the dramatic increase both in

incidence and severity of infection encountered since the introduction and widespread use of antimicrobial agents is no less frightening even if the increased morbidity were only apparent rather than absolute (Finland et al., 1959; Rogers, 1959; Curtin et al., 1961; Barber, 1961). The latter author attributes the increasing frequency of infections due to Ps. pyocyanea to several factors e.g. the use of antibiotics has led to a relaxation in the vigilance necessary to avoid sepsis and the infected patient is less commonly isolated in an attempt to control the spread of infection. Modern medical procedures, lengthy and extensive surgery, prophylactic antibiotic treatment together with the administration of corticosteroids and antimitotic drugs, though prolonging life, render the patient extremely susceptible to infection, especially with such an infamously resistant organism as Ps. pyocyanea. Indeed, this very resistance leads to an increased communicability of infection with this organism.

Rogers (1959) comparing the nature and incidence of microbial pathogens during the periods 1937-38 and 1957-58 in America i.e. prior to and during the antibiotic era noted a dramatic alteration of the microbial agents concerned. The common pathogens of the first period namely pneumococci, streptococci and tubercle bacilli had virtually been eliminated but

by 1957 the Gram-negative bacilli, little heard of in 1938, represented "... the most common microbial cause of death in the medical service during this period". Within this particular group Ps. pyocyanea had replaced Escherichia coli as the most common pathogen and in the 1957-58 survey such infections were primarily acquired in hospital and many were of endogenous origin. By this time infection had come to be regarded as the terminal stage in progressive disease, the nature of the disease determining the risk of infection and the nature of the antibiotic determining the particular organism.

The notorious natural resistance of Ps. pyocyanea to antibiotics, chemotherapeutic agents and disinfectants is well known and has resulted in the unique role of the organism amongst the Gram-negative bacilli (Yow, 1952; Garrod et al., 1954). Dube and Shriniwas (1965) examined the sensitivity of 190 strains of Ps. pyocyanea against nine antibiotics i. e. Penicillin G, Streptomycin, Chloromycetin, Oxytetracycline, Chlortetracycline, Sulphadiazine, Erythromycin, Synermycin and Nitrofurantoin. The greatest sensitivity was shown towards Chloromycetin (8 per cent. of strains sensitive).

The introduction of the sulphonamides followed by penicillin led to an increase in the incidence of infection due to the Gram-

negative bacilli at the expense of other organisms; the arrival of the broad-spectrum antibiotics has led to the emergence of Ps. pyocyanea because of it's particular resistance.

Barber (1961) commented "Among the Gram-negative bacilli, Ps. pyocyanea is the most serious menace and is beginning to rival Staph. pyogenes as the hospital scourge. Not only is it found with increasing frequency in all kinds of infections in hospital, but the infections tend to be severe and, since there is no really effective chemotherapy, have a high mortality, reaching almost 100% in cases of established septicaemia".

Finland et al. (1959) in an extensive survey in Boston covering 22 years prior to and including the appearance of antibiotics found little evidence for pyocyaneus bacteraemia in 1935 but by 1957 there was an average annual incidence of 20 such cases. Infections due to the Gram-negative bacteria in general rose from 24 cases in 1941 to 200 in 1957 in the City Hospital, Boston, U. S. A.

Yow (1952) traced 56 examples of infection due to Proteus species of Ps. pyocyanea following antibiotic therapy and over a two year period he noted that the incidence of the latter had doubled.

In this country, Williams et al. (1960) observed an

increased incidence in the isolation of the organism from the sputa of patients. In the first quarter of 1959 more than 60 patients were found to be harbouring Ps. pyocyanea compared to nine in the same period of 1958; at the same time the consumption of broad-spectrum antibiotics had nearly doubled in the course of the year.

Asay and Koch (1960, 1962) reported on the isolation of Ps. pyocyanea in a children's hospital and found a rise from eight cases in 1952 to 152 cases in 1957 and in 1962, 366 cases of infection were detected. During this 11 year period there was no increase in the number of patients at risk nor in the number of specimens submitted to the laboratory. Fatalities due to the organism had numbered 21 in 1957 compared with 2 in 1952. Rogers (1960), investigating bacterial infections in a children's hospital in this country, reported that Ps. pyocyanea had become the most dangerous pathogen in outbreaks of cross-infection.

Many other workers have noted the increasing importance of this species since the introduction of antimicrobial agents and also the role of other drugs used in therapy.

Forkner et al. (1958) reported on 23 cases of septicaemia caused by Ps. pyocyanea; 16 patients were leukaemic and of these 10 had received antibiotic therapy, eight had been treated with steroids and nine with cytotoxic drugs. In an analysis of the bacteria responsible for 63 other cases of septicaemia in patients suffering

from leukaemia the same authors found that Ps. pyocyanea was second only to staphylococci as the aetiological agent. It's role as a secondary or superinfecting organism was also confirmed with the finding that 77 per cent. of pseudomonas septicaemias occurred in patients receiving broad-spectrum antibiotics, whereas only 33 per cent. of staphylococcal septicaemias occurred in similar circumstances. Williams et al. (1960) described five cases, three of septicaemia, one chest infection and one intestinal infection, Ps. pyocyanea being implicated in the death of all five patients. Of the three patients with septicaemia, two had had leukaemia and the third bone-marrow aplasia. All five had received broad-spectrum antibiotics previously and all had been given corticosteroids. The effect of the latter in fatal infections with Ps. pyocyanea has also been stressed by Jacobs (1964).

That an essentially saprophytic species such as this can account for the various syndromes mentioned in this brief review demonstrates the Jekyll and Hyde characteristics which Ps. pyocyanea can assume. The organism can grow harmlessly in a variety of reservoirs within the hospital area and yet, given the opportunity, can assume a pathogenic role transforming it into an obstinate menace which is extremely difficult to eradicate. There seems little doubt today that Ps. pyocyanea can be both a disabling and fatal agent of disease. Lacking the invasive properties of it's

more constantly virulent colleagues, it nevertheless proclaims itself by choosing it's victims carefully or gaining entry via contaminated apparatus. At the extremes of life the premature infant and the geriatric patient are very susceptible to infection; in addition, congenital and urinary tract abnormalities, as well as pre-existing disease are conditions readily taken advantage of regardless of age. As in the case of the cowardly, scavenging hyena this microorganism is ever ready to take advantage of any breakdown or weakness in the defence mechanisms of the body and no part of the human anatomy is sacrosanct.

The human reservoir.

The incidence of carriage of Ps. pyocyanea in man, a possible precursor of endogenous infection, has been investigated; Lowbury and Fox (1954) reported a 3 per cent. faecal carriage amongst students but a higher incidence, 20 per cent,, when burned patients were examined in hospital. Shooter et al. (1966) found the faecal carriage rate to be 28 per cent. in patients within two days of their admission to hospital and found 38 per cent. of patients to harbour the organism at one time or another during their stay in hospital. In the same year Grogan (1966) reported a 20-30 per cent. isolation rate in specimens of urine and faeces and 19 per cent. in various other specimens. Other workers have examined skin carriage and carrier rates, saliva (Shkleir et al., 1963) and

sputum (Takigami et al., 1965). In contrast to staphylococcal carriage, nasal carriers of Ps. pyocyanea are rare.

Transmission of infection.

The natural adaptibility of the organism enables it to survive under a wide range of environmental conditions and it's ability to use simple nutrients and it's innate resistance to antimicrobial agents has resulted in innumerable vehicles being implicated in it's spread; from an extensive list of such vehicles the following may be noted:

Spinal anaesthetics (Evans, 1945)

Nurses' hands (Lowbury and Fox, 1954)

Catheters (Shickman et al., 1959; Turck et al., 1962)

Lumbar puncture needles (Potel et al., 1955)

Steroid creams (Noble and Savin, 1966)

Oxygenators sterilised in benzalkonium chloride (Keown et al., 1957)

Corks and antiseptic solutions (Anderson and Keynes, 1958)

Eye medicaments (Ridley, 1958; Williams et al., 1966)

Urine bottles and bed pans (McLeod, 1958)

Surface-acting cationic agents (Plotkin and Austrian, 1958)

Polythene tubing and electric suction machines (Rogers, 1960)

Hexachlorophene soap (Anderson and Keynes, 1962)

Plaster of paris and cellulose wadding (Sussman and Stevens, 1960)

Lignocaine jelly (Phillips, 1966)

Saline for operative irrigation (Ayliffe et al., 1966)

Anaesthetic equipment (Tinne et al., 1967).

All of these and other vehicles form convenient portals of entry for such an essentially poorly invasive species.

Walter (1958) noted that the organism grew quite freely on the cooling coils of ventilation equipment whilst Anderson (1959) observed it's dissemination from such apparatus into the environment. The role of humidifiers, through contaminated water and mists, as a means of spreading infection in the newborn has been stressed by Hoffman and Finberg (1955) and Lancet (1966).

Selwyn (1963, 1965) studying bacterial cross-infection in dermatological wards found evidence of Ps. pyocyanea in air samples, dust and communal baths; contaminated bath water was also implicated by Victorin (1967) in the spread of infection.

Hurst and Sutter (1966) found large numbers of the organism in mop water and also on the floor of a Burns Unit. These strains were shown to be identical in phage type to those infecting burn wounds within the Unit. The organisms were capable of surviving on the hospital floor in large numbers for a considerable length of time, e.g. 8 weeks.

Ps. pyocyanea can survive and multiply in drains (Fierer et al., 1967), sinks and faucet aerators (Wilson et al., 1961; Cross et al., 1966). Hunter and Ensign (1947) described an epidemic in the U. S. A. in which nine out of 24 children died after drinking pasteurised milk which had been contaminated from a dripping rag used to repair a leaking water pipe.

An especially disturbing feature in recent years has been the involvment of modern assisted respiration apparatus in the spread of pyocyaneus infections resulting in numerous fatalities (Basset et al., 1965; Rubbo et al., 1966; Fierer et al., 1967: Tinne et al., 1967).

Pathogenicity for animals, plants etc.

The pathogenicity of Ps. pyocyanea for species other than man might, at first sight, have little pertinence to the investigations reported in this thesis. The part played by such sources in promoting human disease is unknown but a review of the literature leaves little doubt of such a possibility; a very great number of species of animals, reptiles, fish, birds and insects can suffer from infection or at least parasitisation by Ps. pyocyanea.

It would seem in fact that Ps. pyocyanea has entered the jet age at least at the same time as man since Crum et al. (1967) report it to be a contaminant of aircraft fuels leading to the corrosion of fuel tank linings.

# CHAPTER III

## THE NEED FOR SUBDIVISION OF PS. PYOCYANEA.

From the brief résumé already presented it will be obvious that Ps. pyocyanea has an increasing significance in modern medical practice. Indeed, the comment of Graber et al. (1962) that the organism is bidding for the role of "Ghengis Khan of the Microbial World" is not perhaps without foundation. It is pertinent therefore that studies of the sources and modes of spread of infection, particularly in the hospital environment, are vital and prerequisite to it's eradication; such studies would be impracticable unless there was available a method of marking individual strains of this ubiquitous organism for epidemiological purposes.

Furthermore the organism is primarily an inhabitant of soil, sewage etc. and not a human parasite thus a variation in virulence amongst different strains would seem a reasonable prospect; therefore attempts to eradicate all strains from the environment would seem unnecessary. Experience gained in the work of this thesis has shown that in many outbreaks of infection due to a particular strain of Ps. pyocyanea the actual reservoir of infection is usually limited, although many other strains can be isolated from the environment.

### Early attempts at classification.

Because of variation in colonial morphology attempts to

subdivide the species on this basis are of little value. Similarly, the restricted biochemical activity of the organism does not allow it's subdivision on the basis of activity towards various chemical substrates and it's innate resistance to most antibiotics defeats any attempts to characterise strains on the basis of their different patterns of sensitivity towards such agents.

Ps. pyocyanea is capable, however, of producing a variety of extracellular organic pigments e.g. pyocyanine-green; fluorescein-yellow and pyorubrin-red; this property has been used to differentiate strains (Gessard, 1920; Simon, 1956). Most workers would agree, however, that in addition to the limited number of types which such a schema affords the variable nature of pigment production does not lend itself as a basis for a typing technique. Evidence will be given later to show that apart from the variability of pigment production, strains producing uncommon pigments e.g. violet or red, are not homogeneous when investigated by other techniques.

Attempts to subdivide the species by means of serological and phage typing methods have been described by numerous authors and would appear to offer more satisfactory results. A summary of the development of these techniques and an assessment of their value and validity is offered in the following pages.

## The Serology of *Ps. pyocyanea*

The agglutination reaction, destined to become one of the most important and constantly used techniques in serological studies, was first observed in tests with *Ps. pyocyanea*. As early as 1889, Charrin and Roger found that when the bacillus was grown in serum obtained from an animal previously infected with the same strain, it did not grow diffusely through the medium as it does in normal fluid cultures, but in this case, the bacilli clumped together and fell to the bottom of the tube in small aggregates.

The serological heterogeneity of *Ps. pyocyanea* has been recognised for over half a century; Trommsdorf (1916) allocated 25 of 27 strains of the organism to one or other of three serotypes. Aoki (1926) could not differentiate among *Ps. pyocyanea*, *Ps. fluorescens* or *Ps. putida* and thus accepting such organisms as one species he found 22 serological types among 50 strains. Conversely Meader (1924) had found the species to be serologically homogeneous but later Kanzaki (1934) and Elrod and Braun (1942) agreed with Aoki's finding in regard to *Ps. pyocyanea*.

Many of the early studies were hampered by a failure to differentiate heat-labile and heat-stable antigens. The general acceptance, however, of heat-stable somatic (O) and heat-labile flagellar (H) antigens in the genus followed the work of Gaby (1946)

and Munoz et al. (1949). Gaby, investigating bacterial dissociation in strains of Ps. pyocyanea, found that in 10 of 12 strains each of three dissociative types differed serologically but analogous variants of such strains were serologically identical. By means of cross-absorption experiments he was able to divide the strains into three groups on the basis of their H antigens but also found them to be homogeneous in regard to their O antigen. This latter observation may have been due to his investigation of a single serological group since the number of strains examined was so small.

Interest in the serotyping of Ps. pyocyanea was stimulated by the increasing importance of the organism in hospital-acquired infections. Mayr-Harting (1948) working with 54 strains of Ps. pyocyanea found evidence of three main antigens occurring singly or in combination. She could not differentiate between O and H reactions; antisera prepared against formalin-treated flagellate strains could be completely absorped by ethanol-treated organisms. Agglutination was always of the granular O variety. She observed, in addition, that preparations of the O agglutinogens by heating in a water bath at $60^{0}$C for half an hour destroyed the agglutinability of a strain but not it's antigenicity. Brutsaert (1924) had reported a similar loss of agglutinability when antisera, prepared against a suspension of Ps. pyocyanea heated at 56°C., was used to test

suspensions of the same strain which had been heated for two hours at 100°C.; paradoxically such suspensions had retained their agglutinability for serum prepared with organisms heated for two hours at 100°C. Mayr-Harting also found that treatment with formalin did not prevent agglutination of flagellate strains with somatic antisera and suggested that that antigenic alteration of monotrichously flagellate organisms, in this case Ps. pyocyanea, by formalin might not be analogous with that of peritrichously flagellate organisms e.g. Salmonellae or Proteus.

Similarly from Australia Christie (1948) described the serological typing of 138 strains of Ps. pyocyanea by tests with 38 antisera. He found evidence of 13 O and two H antigens. In addition to 18 strains which failed to agglutinate with any of the antisera Christie noted that strains lost their agglutinability on subculture.

Fox and Lowbury (1953) used strains typed by Christie's method to investigate the serum antibody titres of burned patients. In every instance the patients' sera possessed antibodies to all 14 strains used in the investigation, although to varying titres. These authors found that saline suspensions of agglutinogens gave higher titres than alcoholised suspensions and that agglutination was of the granular, O variety no matter which type of suspension was used. This agrees with the earlier findings of Mayr-Harting.

Since the serological techniques used had not altered greatly, one is tempted to believe that the organisms themselves capitulated when in 1957 Habs, in Germany, reported a satisfactory typing schema based on heat-stable O agglutinogens. She used boiled bacterial suspensions to immunise rabbits and with the resultant sera was able to identify 12 types in 70 strains in tube agglutination tests although she encountered cross-reactions among the different groups. Kleinmaier (1957) confirmed the existence of Habs' O groups by slide agglutination tests and in 1958, together with Müllar, once again confirmed Habs' findings on this occasion using a gel diffusion technique. Habs' schema has since been used by other workers (Sandvik, 1960; Véron, 1961; Wokatsch, 1964; Wahba, 1965) in the identification of serotypes from human and animal sources in Europe and South-East Asia. Strains of Habs' types 1 and 6 were most commonly encountered and her schema extended by the discovery of additional serotypes.

In this country, Gould and McLeod (1960) used both serological and phage typing methods simultaneously to distinguish hospital strains of Ps. pyocyanea and found evidence of three main antigens using antisera which they had developed. Difficulties were encountered due to cross-reactions and reduction or loss of agglutinability after a strain had been subcultured.

Verder and Evans (1961) satisfactorily typed 326 isolates

of _Ps. pyocyanea_ from three areas in North America; they identified 13 O and 10 H antigens and thus recognised 29 serotypes and claimed that no cross-agglutination reactions occurred even when low dilutions of antisera were used. Contrary to the findings of Aoki (1926) and Sandiford (1937) the antisera employed did not agglutinate any of 56 strains belonging to other _Pseudomonas_ species. Furthermore, it is intriguing to note that the O agglutinogens, used by Verder and Evans, were heated at 100°C. for one hour since Mayr-Harting had reported a loss of agglutinability after heating suspensions for only half an hour at 60°C.

Van den Ende (1952) employed precipitation reactions in an endeavour to resolve the difficulties of agglutination techniques; similarly other authors (Kleinmaier and Müller, 1958; Van Eeden, 1967) used gel diffusion techniques and employed a variety of procedures for the preparation of the precipitinogens. Though type differentiation was possible the latter author summarises several difficulties encountered namely, variation in results after the test strains had been subcultured and also in the number and nature of antigens produced by one strain under different cultural conditions. After 24-48 hr individual lines of precipitation would sometimes part to show a double band and whether or not this is due to the presence of two antigens is a matter for conjecture; precipitation

reactions with extracts, though precise, fail to reveal the number of antigens involved. In addition, since interpretation of the gel diffusion test is not always straightforward Van Eeden also used immuno-electrophoretic methods to confirm his precipitation results; he considered, however, that the technique was useful when carried out in conjunction with agglutination tests.

The epidemiological validation of serological typing techniques for Ps. pyocyanea, is as scantily reported in the literature as the information on the various techniques is copious.

Gould and McLeod (1960) described type variation in replicate isolates from only two out of 28 patients involving 95 strains. Verder and Evans (1961), examined 574 replicate strains from 166 patients and found only three instances when strains from the patient were of different serotypes. Christie (1948) showed that the strains responsible for an epidemic involving four people were serologically identical. Finally Chia-ying (1963) serotyped 151 strains of Ps. pyocyanea and found from one patient that the strain from a blood culture and that from an infected burn area were of the same serotype and suggested, on these grounds, that such a typing technique might be useful in epidemiological investigations!

Characterisation of Ps. pyocyanea by Bacteriophage activity.

The primary interest of early workers in studies of phage activity on Ps. pyocyanea was concerned with the phenomenon now

known as iridescence in which plaques or areas of metallic-like pitting, closely resembling phage activity, appeared spontaneously on the surface of strains growing on solid media. Since this property may be linked with phage activity, and is, at the present time, still of uncertain origin, it will be considered along with a résumé of phage methods of typing the organism.

Though Hadley (1924) is generally considered to be the first to have investigated this autoplaque phenomenon others had previously noted it's occurrence and considered it to be due to the action of phages (Quiroga, 1923; Hauduroy and Peyre, 1923; Lisch, 1924). Quiroga (1923) and Hauduroy and Peyre (1923) had obtained lysis of sensitive strains using filtrates prepared from areas of metallic pitting, whilst Lisch (1924) obtained the same result with non-autoplaque-producing strains. Hadley tested filtrates from iridescent and non-iridescent strains and found that both could cause lysis of the same sensitive strain. Asheshova (1926) described the isolation of phage from water in the Port of Dubrovnik which reacted with pyocyanea strains giving rise to lytic plaques, non-metallic in appearance, which occurred in direct proportion to the quantity of phage suspension added. When she investigated the filtrates from autoplaque-producing strains, however, the plaques which appeared bore no relation to the quantity of additive and were extremely variable in number. Even after many

successive passages such filtrates showed no activity towards non-autoplaque-producing strains even though the latter were phage-sensitive. In contrast to Hadley, Asheshova observed no lysis of broth cultures when filtrates of autoplaque-producing strains were added.

Such results might be explained by the fact that the authors were on occasion investigating strains capable of both autoplaque production and lysogeny. The apparent transmission of the former phenomenon, observed by Asheshova, can be explained if her "sensitive strain" was itself capable of autoplaque production. This is all the more likely an explanation when one considers her failure to produce iridescence in a normally non-autoplaque producing strain. The predominant use of broth cultures by these early workers adds to the likelihood of such possibilities.

Sonnenschein (1927) confirmed Asheshova's findings and considered that the agents responsible for iridescence were distinct from phage and he called them "pseudobacteriophagen". Close examination of the pitted areas revealed the presence of needle-like crystals which were absent from the lytic areas of true phages. He went on to show that, unlike the pseudophages, true phages could be increased quantitatively by passage through a sensitive host strain and this was confirmed by Jadin (1932).

Contradictory reports had in the meanwhile arisen regarding the specificity of the filtrates. Hadley (1924) and Sonnenschein (1927) had found their preparations inactive against *Escherichia coli*, *Salmonella typhi*, Shiga's bacillus, *Vibrio cholerae* and *Bacillus anthracis*; but Jadin (1932), noted activity on *Esch. coli* and *Shigella dysenteriae*. The use of broth cultures and a lack of purified preparations together with the ability of *Ps. pyocyanea* to produce a variety of antimicrobial agents may account for such discrepancies in these early investigations.

Congé (1948) was unable to produce lytic plaques on *Esch. coli* even with phage suspensions of *Ps. pyocyanea* exhibiting high titre. He showed that iridescence was dependent on the medium used but he was unable to isolate the active agent from the metallic plaques either by filtration, heat or centrifugation. Warner (1950) also failed to determine the cause of iridescence but by employing certain media could prevent it's occurrence and concluded that the phenomenon was not due to phage activity.

At the present time this strange phenomenon, in which metallic-like areas of lysis, sometimes in the form of single phage-like plaques, can appear spontaneously without the need for a sensitive host strain, is as yet little understood. In recent years Berk (1963) found that certain amino acids were involved in the

production of iridescence but still could find no direct evidence for it's cause.

It's frequent occurrence amongst strains of Ps. pyocyanea and at times it's close resemblance to the lytic areas due to phage has rendered it troublesome in obscuring lytic reactions when phage typing of the species is attempted (Warner, 1950; Terry, 1952).

The common occurrence of lysogeny in Ps. pyocyanea has been reported by several authors (Congé, 1948; Warner, 1950; Don and Van den Ende, 1950; Holloway et al., 1960) and has resulted in numerous attempts to characterise members of the species by noting the sensitivity reactions obtained in response to a set of phages (Don and Van den Ende, 1950; Terry, 1952; Gould and McLeod, 1960; Grogan and Artz, 1961; Postic and Finland, 1961; Pavlatou and Hassikou-Kaklamani, 1961; Graber et al., 1962; Sutter et al., 1965).

From the investigations of these workers in typing strains from various countries several deductions can be made; lytic activity appears in many forms; the onset of resistant growth is more frequent and profuse than in the case of phage typing of coliform organisms making reactions less clear-cut; a relatively large proportion of strains are resistant to all phages of a typing set; such typing sets of phage preparations though suitable for one geographical area are in some cases of little use in another part of the world.

Undaunted, however, Warner (1950) had considered that the stability of his phage preparations (two - six months at 4°C.), their wide range of activity and the large percentage of organisms which were sensitive justified the use of the technique.

Terry (1952) in his attempts at phage typing noted that iridescence could be eliminated by adjustment of the cultural conditions of the test strains i.e. 6-8 hr at 37°C followed by overnight incubation at room temperature (approx. 21°C.). Other difficulties, however, had been encountered in phage typing studies. Spontaneous loss of sensitivity to phage action (Hadley, 1924; Linz, 1938; Postic and Finland, 1961) could result in the loss of useful phages due to the lack of a propagating host. According to Feary et al. (1963) since the majority of his strains were found to be lysogenic for more than one phage a considerable amount of misinterpretation of phage sensitivity could arise if such strains were used for the propagation of stock phage lysates. Drawbacks such as these do not ease the task of developing and maintaining typing sets of phages.

In general, the development of phage typing techniques has not presented as many practical difficulties as serological typing and more emphasis has been placed on investigations into the nature of the phages used and to a lesser extent in epidemiological applications of the technique.

The number of phage groupings obtained by the various investigators varies from four (Grogan and Artz, 1961) to 19 (Postic and Finland, 1961). Alteration in lytic pattern on storage or subculture has been reported by many of the authors (Hadley, 1924; Warner, 1950; Don and Van den Ende, 1950; Pavlatou and Hassikou-Kaklamani, 1961; Sutter et al. 1965); Gould and McLeod (1960) reported that a strain frequently varied in two successive tests on the same day. Such in-vitro instability detracts from the value of this method of typing strains of Ps. pyocyanea.

Sutter et al. (1965) found an even greater variation on examining the in-vivo stability of phage sensitivity by typing replicate isolates from the same site in any one patient. In only 26 out of 116 isolates did the pattern of sensitivity remain unchanged. These authors considered that such findings were more likely the results of infection with multiple types of Ps. pyocyanea than due to variation in sensitivity. Pavlatou and Hassikou-Kaklamani (1961), however, had observed similar variations and in a later investigation, using micromanipulation techniques to examine individual cells, they proved the in-vitro occurrence of such instability (Pavlatou and Hassikou-Kaklamani 1962); they noted that Polymyxin, the drug of choice in infections due to Ps. pyocyanea, increased the tendency towards variation and recommended that for practical purposes, isolation of the organism must be carried out before administration of antibiotics and typing performed immediately after isolation. In

contrast, Grogan and Artz (1961) found that 73 per cent. of multiple isolates from 34 patients retained their type patterns; they recognised only four types, however, in strains from 117 patients and since 60 per cent, of these strains fell into two types their method has restricted application.

Phage typing then must be regarded as unsatisfactory since the retention of a sensitivity pattern in repeated isolations from a patient is essential if a typing method is to be of any value in clinical or epidemiological investigations.

A somewhat different approach to phage typing was described by Holloway (1960). This was based on the ability of strains to produce phages rather than their sensitivity to such agents; production of the phages being detected by their activity against a set of bacterial indicator strains. He detected six types amongst 214 hospital strains of _Ps. pyocyanea_. Although Feary _et al._ (1963) confirmed the value of this method it has not been generally adopted and since these workers did not investigate either the in-vitro or in-vivo stability of phage production little can be said regarding the validity of the technique. It would seem, nevertheless, that the method has an obvious drawback relying as it does on the sensitivity of strains to detect phages when it is already known that such reactions are variable.

A feature of the considerable number of investigations into the use of phage typing has been the lack of standardisation of the typing phages, media and techniques; similarly few authors have attempted to prove the reliability of phage typing as an epidemiological tool and on the few occasions where validation has been attempted the evidence presented is very flimsy.

From these summary comments it will be obvious that there are difficulties in both serological and phage typing methods as applied to _Ps. pyocyanea_. In addition, the preparation of antisera and the isolation and maintenance of phage sets is time-consuming as are indeed the actual typing procedures themselves. In their practical applications both methods show evidence of faults which are undesirable in a schema of strain differentiation namely a failure to produce replicable results when a series of strains are examined from the same site in a given patient or when retested after being subcultured, or held in storage. Finally, the relatively small number of types of _Ps. pyocyanea_ which are recognised by either method has led some authors (Gould and McLeod, 1960) to employ both methods simultaneously in order to enlarge the number of epidemiological types.

## Bacteriocinogeny.

Attention has been directed in recent years to the use of

bacteriocine typing as a possible method of characterising several bacterial species.

The term bacteriocine was first introduced by Jacob et al. (1953) to describe an unusual class of antibiotic substances, produced by various species of bacteria, which differed from the classical antibiotics in acting only on strains of the same or closely related species.

Though bacterial antagonism had been described by previous authors including McLeod and Govenlock (1921) whose 'bactericidins' seem to possess certain bacteriocine-like properties the real development of the study of these substances stems from the intensive investigations of Gratia which he reported in a series of papers commencing in 1925. His initial experiments into the study of the antagonism of Esch. coli V against Esch. coli ϕ, were continued by his associate Fredericq (see for example Fredericq, 1948) who extended the investigations to include strains other than Escherichia and the reports of these investigators have formed a compendium of classical experiments for the elucidation of many of the properties of bacteriocines.

Gratia and Fredericq (1946) introduced the term colicine to describe the lethal and specific substances produced by Esch. coli, Shigella sonnei and other shigella and salmonella species. These

colicines appeared to be of a protein nature but differed in various other properties such as the extent and specificity of their spectrum of activity, the specificity of resistant mutants, diffusibility, the morphology of the inhibition zones formed on a sensitive strain grown on agar, susceptibility to proteolytic enzymes, thermoresistance, electrophoretic mobility and antigenic properties.

Colicines of different strains gave rise to resistant mutants which retained their sensitivity to the colicines of other strains. Using such mutants in conjunction with the other variable properties already mentioned Fredericq (1948) was able to classify the various colicines into 17 groups, in each case a mutant resistant to one group generally retained it's sensitivity to the colicines of all the other groups.

Gratia and Fredericq (1946) had observed that Esch. coli also inhibited shigella and salmonella strains and since then these and many other genera have been shown to produce bacteriocines e.g. megacines from Bacillus megaterium (Ivánovics and Alföldi, 1957), pesticines from Pasteurella pestis (Ben-Gurion and Hertman, 1958), marcescines from Serratia marcescens (Hamon and Péron, 1961) and enterococcines from enterococci (Brock et al., 1963).

Much of our present knowledge regarding the production, mode of action, chemical composition and other properties of these

antibacterial substances has resulted from intensive studies carried out on a limited number of bacteriocines. Bacteriocines produced by different genera, though having some characteristics in common, can exhibit differences in size, antigenicity etc.; the same holds true amongst the bacteriocines produced by a single species.

In general, bacteriocinogeny i. e. the ability of a strain to produce bacteriocine, is a hereditary property which can, in the case of some colicines, be genetically transferred by a colicinogenic to a non-colicinogenic strain. Though, normally, only a small number of cells within a growing clone are responsible for production at any one time, production can be enhanced and sometimes induced in a normally non-bacteriocine-producing strain, by exposure to ultraviolet light or other mutagenic agents.

The factors responsible for the genetic control of production are still not completely understood but it is thought that they are autonomous units which can exist either independently of the chromosome or attached to it, a property shared by other genetic elements namely fertility factors and the temperate phages and for which Jacob and Wollman (1958) proposed the name episomes.

The genetic basis of bacteriocine production by species other than Esch. coli has as yet been scantily investigated; Ben-Gurion and Hertman (1958) failed to transfer pesticinogeny and

Holland and Roberts (1963) were also unsuccessful in their attempts to transmit megacinogeny. Though Hamon (1956) could not demonstrate pyocinogenic transfer, Holloway and Fargie (1960) have shown that recombination mechanisms exist in Ps. pyocyanea, albeit not so widespread as in Esch. coli, and perhaps the genetic background of pyocinogeny will be revealed in time.

Since bacteriocinogenic factors must be capable of autonomous replication within a cell and in phase with the bacterial chromosome without bringing about lysis of the cell, they resemble the fertility factors rather than temperate phages. Their release, however, into the environment with concomitant cell death is more akin to the release of mature phage particles by lysogenic strains.

The lethal activity of bacteriocines follows adsorption to specific receptors on the cell walls of sensitive strains. The actual mechanisms responsible for lethality, studied in the case of a few bacteriocines only, have been shown to depend on the particular bacteriocine under investigation though all would seem to exert specific biochemical effects on the sensitive strain.

Bacteriocine production as a bacterial marker.

The use of bacteriocinogeny for the typing of bacterial species has been reported by several authors. Shannon (1957) studied bacteriocine production in enteropathogenic Esch. coli and

described a simple technique which he used to divide 240 strains into six types. A not dissimilar technique has been employed by McGeachie (1965) to type strains of Esch. coli isolated from cases of urinary tract infection.

Sh. sonnei.

Abbott and Shannon (1958) detected bacteriocine production in Sh. sonnei and by using 15 indicator strains were able to divide their strains into 15 types on the basis of the sensitivity patterns obtained. Gillies (1964) simplified the technique and by typing over 25,000 strains of Sh. sonnei was able to demonstrate beyond doubt the validity of bacteriocine production as an epidemiological marker of this species; the indices which this author used to judge the reliability of the technique were firstly the constancy of type in replicate isolates from any one individual and the uniformity of type in strains isolated from different individuals in an epidemic outbreak. In addition, the stability of bacteriocine production by strains after many subcultures and/or prolonged storage in vitro was noted. This form of typing has been used successfully by other workers in the study of Sonne dysentery e.g. Cooke and Daines (1964) and Naito et al. (1966).

Attempts at bacteriocine typing of strains of Sh. sonnei by noting their sensitivity to stock bacteriocine-producing strains were

undertaken by Abbott and Shannon (1958) but this method was not pursued because replicable results could not be obtained. Since, however, no quantitative measurement of activity was made their variable results might have been due to variation in bacteriocine production by the stock strain; such variation has been observed in Esch. coli (McGeachie and McCormick, 1967).

Klebsiella species.

Durlakowa et al. (1964) using 18 strains of Klebsiella species as indicators obtained 16 sensitivity patterns when they examined 32 strains of Klebsiella for bacteriocinogeny and these potential producer strains were representative of 21 serotypes.

Proteus mirabilis.

The successful typing of Proteus mirabilis strains by bacteriocine production was described by Cradock-Watson (1965) who found three major types amongst 139 strains.

Ps. pyocyanea.

In 1954, Jacob described a bacteriocine elaborated by a strain of Ps. pyocyanea and named this agent pyocine. He investigated in detail the biosynthesis and the mode of action of this substance and showed that it's production could be induced by ultraviolet light and other mutagenic agents. A sensitive strain was destroyed if only one unit of pyocine became adsorped on to it. The

specifity of action of pyocines, primarily, for other strains of Ps. pyocyanea (Hamon, 1956; Hamon et al, 1961) differentiated it from the various other antibiotic substances for which the species is well known (Hays et al., 1945). Holloway (1960) reported pyocinogeny to be of common occurrence in the species and suggested that this characteristic might be used in epidemiological studies of the organism.

An investigation into the possibility of characterising strains of Ps. pyocyanea on the basis of their ability to produce pyocines was undertaken as the primary object of this thesis.

Shortly after the commencement of these studies Darrell and Wahba (1964) described a method of pyocine typing; their technique allowed the recognition of 12 pyocine types using 12 indicator strains to detect pyocine production. The in-vivo stability of pyocine production was suggested by the constancy of types obtained in replicate isolations from the same patient; in retrospect, it is surprising that of the 1090 strains examined all could be allocated to one type or another.

Osman (1965), on the other hand, recognised 10 types among 101 strains of Ps. pyocyanea by noting their sensitivity to the pyocines of four stock producing strains. He found that sensitivity to his preparations was unaltered by subculture of the test strains and that replicate isolations from the same patient and strains

recovered after passage through mice retained their sensitivity towards the four preparations.

Such "sensitivity typing" demands the preparation of bacterial lawns of the test strain and the examination of serial dilutions of these active preparations to ensure the presence of pyocine and not phage activity. A strain used by Osman to prepare one of his stock pyocine solutions was both pyocinogenic and lysogenic. Because of this Osman states that it was necessary to study closely the nature of inhibition produced on a sensitive strain under test to determine whether the response was caused by phage or pyocine activity. This might be quite a feasible procedure if the test strain was sensitive only towards the pyocine fraction but if phage plaques were to appear it would seem impossible to judge whether this was due to phage action alone or to phage plus pyocine activity, the pyocine acting only at a lower dilution.

Preliminary reports had shown the possibility of purifying pyocine preparations (Hamamura, 1962; Kageyama and Egami, 1962). It was therefore considered that pyocinogeny in Ps. pyocyanea was a subject worthy of study to learn new facts, gain fresh insights and to develop further techniques which might be of use in the much needed exploration of this species.

# MATERIALS AND METHODS

# CHAPTER IV

## MATERIALS AND METHODS

### Strains

Five thousand six hundred and ninety strains of Ps. pyocyanea were obtained from specimens involving almost every part of the human body and from a variety of geographical sources (see Table 1).

Table 1. Sources of 5690 strains of Ps. pyocyanea examined.

| Origin | No. of strains |
|---|---|
| The Royal Infirmary of Edinburgh (R.I.E.) | 3730 |
| Stobhill General Hospital, Glasgow | 847 |
| Bacteriological Service, Edinburgh University | 479 |
| P.H.L.S., Colindale, London | 198 |
| The Glasgow Royal Maternity Hospital | 135 |
| The Glasgow Royal Infirmary | 28 |
| St. Mary's Hospital, London | 24 |
| The College of Agriculture, Edinburgh | 20 |
| The Montreal Children's Hospital, Canada | 16 |
| Others | 213 |
| | 5690 |

The majority of the strains were examined shortly after

isolation; those obtained from Colindale and the first 400 strains received from the R. I. E. had been maintained on storage media for many months.

In the early phase of the investigations strains were received as subcultures of a single colony picked from a diagnostic plate; however, so that several colonies from each specimen could be examined it was later arranged that the diagnostic plates themselves were submitted.

A modified set of Wahba's indicator strains was obtained from Dr. M. T. Parker together with 186 strains of Ps. pyocyanea which he had previously serotyped.

Freeze-dried cultures of the strains used by C. S. Terry (1952) in his phage typing investigations were acquired but only 25 strains of Ps. pyocyanea were recovered out of the 158 received.

Identification of strains of Ps. pyocyanea.

Pure cultures were examined for a positive oxidase reaction and production of the blue-green pigment, pyocyanine. However, since the production of this pigment depends upon growth conditions, age of the culture etc. and as several authors have commented upon the apparent increase in the number of non-pigmented strains in recent years, strains were accepted as Ps. pyocyanea if they exhibited a positive oxidase reaction and in addition produced a characteristic odour of trimethylamine, showed active motility,

gave rapid liquefaction of gelatin and abundant growth at 42°C., even if they did not produce pyocyanine on nutrient agar.

Culture media

Nutrient Broth ('Oxoid' No. 2). This comprised 1.0 g. meat extract ('Oxoid' Lab-Lemco), 1.0 g. peptone ('Oxoid' L37), 0.5 g. NaCl and 100 ml. distilled water; the pH was 7.3.

Nutrient Agar. This consisted of nutrient broth with the addition of 1.5% agar ('Oxoid' No. 3) and dispensed as 7 ml. and 12 ml. amounts in 4" Petri dishes or as 5 ml. amounts sloped in 6" x 5/8" test tubes. This medium, 2 ml. volume in 1/4 oz. screw-capped bottles, was also used to store strains.

Semi-solid Agar. This was prepared as nutrient agar but with only 0.25% agar ('Davis' Type P). Five ml. amounts were dispensed in 6" x 5/8" test tubes.

Sterilisation of the above media was carried out by autoclaving at 121°C. for 15 min.

Blood Agar. This consisted of nutrient agar with the addition of 7% sterile, defibrinated horse blood ('Evans' or 'Burroughs-Wellcome').

Peptone Water

| | |
|---|---|
| Peptone ('Oxoid' L37) . . | 10 g. |
| Sodium chloride . . . | 5 g. |
| Water . . . . . | 1 l. |

Ingredients dissolved in warm water, pH adjusted to 7.3.

Autoclaved at 121°C. for 15 min.

Selective media

The following selective media were used in the isolation of *Ps. pyocyanea* from infected mice; 'Cetrimide' agar, 'Dettol' agar, sodium nitrofurantoin agar, MacConkey's agar ('Oxoid') and desoxycholate citrate agar (DCA). The first three media were prepared by the addition to nutrient agar of the appropriate chemicals to give 1%, 0.2% and 20 mg. per cent. respectively. MacConkey's agar was prepared according to the maker's instructions and DCA as in Cruickshank *et al*. (1965, p. 760).

Tryptone Soya Broth

This comprised the broth base used in the preparation of Tryptone Soya Blood Agar, described overleaf.

Prepared according to the maker's instructions pH 7.3.

Autoclaved at 121°C. for 15 min.

Tryptone Soya Blood Agar (TSBA).

| | |
|---|---|
| Tryptone ('Oxoid' L42) . | 15 g. |
| Soya Peptone ('Oxoid' L44) . | 5 g. |
| Sodium chloride . . | 5 g. |
| Agar . . . | 15 g. |
| Horse blood (defibrinated) . | 70 ml. |
| Water . . . | 930 ml. |

Prepared according to the maker's instructions but with the addition of blood after autoclaving at 121°C. for 15 min. pH 7.3.

Medium of Darrell and Wahba (1964).

This consisted of the Tryptone Soya Agar (TSA) described above without the addition of blood but incorporating the following triad of chemicals:

| | |
|---|---|
| Dipotassium hydrogen phosphate | 1 g. |
| Sodium citrate . . | 1 g. |
| Iodo-acetic acid . . | $10^{-5}$ M. |

Phage Typing Medium (Sutter et al., 1963).

| | |
|---|---|
| Sodium lactate (60% syrup) . | 2 ml. |
| Sodium chloride . . | 5 g. |
| Magnesium sulphate, $MgSO_4 . 7H_2O$ | 0.2 g. |
| Ammonium dihydrogen phosphate | 1 g. |
| Dipotassium hydrogen phosphate | 10 g. |

| | |
|---|---|
| Agar . . . | 15 g. |
| Water . . . | 1 l. |

Autoclaved at 121°C. for 15 min.

Medium of Kageyama and Egami (1962).

| | |
|---|---|
| Sodium glutamate (60% syrup) . | 20 g. |
| Glucose . . . | 5 g. |
| Magnesium sulphate, $MgSO_4 . 7H_2O$ | 0.1 g. |
| Disodium hydrogen phosphate, $Na_2HPO_4 . 12H_2O$ . | 5.63 g. |
| Dihydrogen potassium phosphate | 0.25 g. |
| Calcium nitrate . . | 0.01 g. |
| Yeast extract . . | 1 g. |
| Water . . . | 1 l. |

pH adjusted to 7.3: Sterilisation by autoclaving at 121°C. for 15 min.; sterile solution of glucose added separately.

Media for the Enhancement of Pigment Production (King et al., 1954).

Medium A.

| | |
|---|---|
| Bactopeptone . . | 20 g. |
| Glycerol C. P. . . | 10 ml. |
| Magnesium chloride (anhydrous), | 1.4 g. |
| Potassium sulphate (anhydrous), | 10 g. |
| Agar . . . | 15 g. |
| Distilled water . . | 1 l. |

Medium B.

| | | |
|---|---|---|
| Proteose peptone No. 3 | . | 20 g. |
| Glycerol C. P. | . . | 10 ml. |
| Dibasic potassium phosphate | . | 1.5 g. |
| Magnesium sulphate, $MgSO_4 . 7H_2O$ | | 1.5 g. |
| Agar | . . . | 15 g. |
| Distilled water | . . | 1 l. |

These media were prepared according to Edwards and Ewing (1962, p. 254).

After inoculation Medium A, for the enhancement of pyocyanine production, was incubated at 37°C. for 48 hr or longer and in the case of Medium B, for the enhancement of fluorescein production, incubation was at 37°C. for 24 hr and then left for 2-3 days at room temperature before being examined.

Tris buffer solution.

0.1 M NaCl and 0.01 M Tris buffer solution, pH 7.5, was made up in distilled water as follows: 39.9 ml. of 0.2 M HCl was added to 50 ml. of 0.2 M solution of tris-(hydroxy-methyl)-amino-methane (2.42 g. in 100 ml. water) and then diluted to 1 l. before adding 5.85 g. NaCl and shaking thoroughly.

Saline.

Normal physiological saline (0.85% NaCl) was used routinely for dilution of pyocine and phage preparations and for the preparation

of test substrates e.g. red blood cell suspensions.

## Methods

### Test for pyocyanine production.

Strains were inoculated on to nutrient agar slopes and incubated for 24 hr at 37°C.; if pigmentation had not developed the slopes were then left at room temperature for a further 6-7 days. When there was doubt as to the presence of pyocyanine 2-3 ml. of $CHCl_3$ was added and after several hours made acidic with N/2 HCl. The development of a blue colouration in the $CHCl_3$ which became red on acidification was taken as proof of pyocyanine production. In further investigations strains failing to produce pyocyanine on nutrient agar were grown on the media of King et al. (1954), under the appropriate conditions, and examined as above.

### Test for bacterial motility.

A straight wire was used to stab the surface of semi-solid nutrient agar contained in a 6" x 5/8" test tube to a depth of about 1 cm. After 2-3 hr incubation at 37°C. motility was observed as a diffuse spreading of growth from the original inoculum line.

### Test for the presence of cytochrome oxidase.

The presence of cytochrome oxidase was investigated using a modification of Kovac's technique (1956).

Several drops of a 1 per cent. solution of tetramethyl-p-phenylene-diamine-dihydrochloride were allowed to fall on to the surface of the bacterial growth present on the semi-solid medium

used in the motility test. Development of a purple colouration within 30 sec. was taken as a positive result.

The freeze-thaw extraction of pyocines.

The strain of Ps. pyocyanea being investigated for pyocine production was inoculated over the entire surface of several TSA plates ( 12 ml. medium in 4" Petri dishes) and the plates incubated for 14-16 hr at 32°C. The resultant macroscopic growth was then killed by placing 3-5 ml. $CHCl_3$ in the lid of the Petri dish and the dish with the medium was replaced on the lid for 15 min. after which the lid was removed and $CHCl_3$ vapour eliminated by exposure to the air for a few minutes. The plates were then placed in a deep-freeze cabinet at -70°C. for an hour after which they were removed and the contents allowed to thaw at room temperature. The expressed fluid was removed with a sterile pipette and centrifuged at 3,000 r.p.m. for 15 min. to remove the dead bacterial cells. Chloroform was then added to the supernatant to give a 5 per cent. v/v mixture and the contents thoroughly shaken. This last procedure killed off any bacteria which may have escaped the earlier procedures and at the same time, any pyocyanine, was absorbed into the $CHCl_3$ layer. After allowing the two layers to separate the top layer, containing pyocine, was decanted or removed by means of a syringe or pipette and transferred into a sterile screw-capped container.

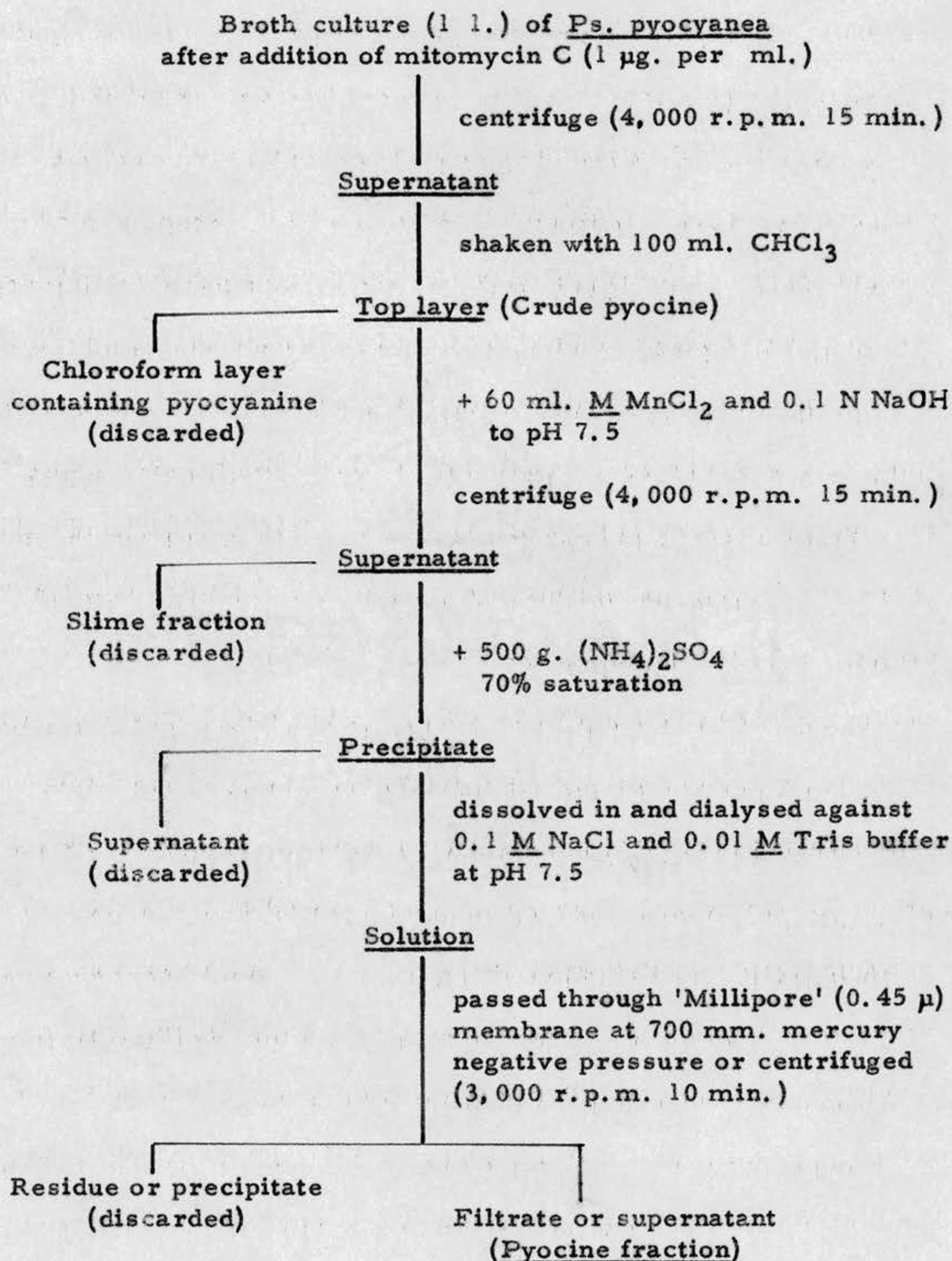
Preparation and purification of pyocines from liquid media (Fig. 1).

The preparation and partial-purification of high titre pyocine extracts was based on the technique described by Kageyama (1964).

Mitomycin C was added after varying intervals of time to an actively growing agitated culture of *Ps. pyocyanea* incubated at 32°C. in Tryptone Soya or Sodium glutamate broth to give a concentration of approximately 1 μg. per ml. The culture increased steadily in turbidity for 3-4 hr and then rapidly lysed; after about 5-6 hr a lysate exhibiting a pyocine titre of 1 part in 32, 000 or more was usually obtained.

After removal of the disintegrated bacterial cells by centrifugation at 4, 000 r.p.m. the lysate was sterilised and pigment removed by thorough agitation in 10 per cent. $CHCl_3$. After allowing the $CHCl_3$ layer to settle the top layer was decanted into a sterile container. To one litre of this pyocine-containing fraction was added 60 ml. of M $MnCl_2$ and the pH adjusted to 7.5 with NaOH under vigourous agitation. The precipitate, containing the slime fraction, was removed by centrifugation at 4, 000 r.p.m. for 15 min. and 500 g. $(NH4)_2SO4$ was added to the supernatant to give 70 per cent. saturation. After standing for at least 2 hr the precipitate, containing almost all of the pyocine activity, was dissolved in 50 ml.

Fig. 1. Purification of pyocine



of 0.01 M Tris buffer containing 0.1 M NaCl and then dialysed against 2 litres of the same buffer overnight. If necessary the resulting solution could be cleared by passage through a membrane filter (Millipore, 0.45 μ under 700 mm. mercury, negative pressure) or by centrifugation (3,000 r.p.m. for 15 mins.).

## Assay of pyocine activity.

### Preparation of indicator plates.

A 4 hr nutrient broth culture of a pyocine-sensitive strain (containing approximately $10^7$ organisms per ml.) was used to flood the surface of a nutrient agar plate (7 ml. contained in 4" Petri dish); excess of the broth culture was removed with a sterile Pasteur pipette and the bacterial lawn allowed to dry.

### Titration of material under assay.

Doubling dilutions of the pyocine-containing preparation were made in sterile saline and were then individually applied to the bacterial lawn with sterile calibrated, platinum-tipped, dropping pipettes delivering 0.02 ml. per drop; when these had dried the plates were incubated overnight at 37°C. The titre of pyocine activity was read as the reciprocal of the highest dilution showing complete inhibition of the sensitive strain.

## Phage Typing of *Ps. pyocyanea*.

### Isolation of phages and preparation of typing suspensions.

18 hr sodium lactate broth cultures of strains of *Ps.*

pyocyanea were centrifuged for 15 min. at 3,000 r.p.m. in order to remove the majority of the bacterial cells and the supernatants were then sterilised by shaking in 5 per cent. $CHCl_3$. Ten-fold dilutions of the supernatants were then examined for phage activity using the method as previously described for the assay of pyocines. In this instance discrete phage plaques were removed by means of a Pasteur pipette and mixed with a 6 hr broth culture of a non-lysogenic and sensitive strain of Ps. pyocyanea. After overnight incubation at 37°C. the resulting lysate was centrifuged and examined for activity as before. In order to obtain high concentrations of phage material the highest dilution showing confluent lysis of the host strain was then used to flood the surface of sodium lactate agar lawns of the same strain, the plates allowed to dry then reincubated overnight at 37°C.; a small area of the lawn was left uninoculated to test for spontaneous lysogenicity of the propagating strain. The plates were then freeze-thawed and the contents centrifuged and sterilised with $CHCl_3$ before being assayed. For the purpose of phage typing the routine test dilution (RTD) was taken as the highest dilution showing confluent lysis of the propagating strain. Only suspensions exhibiting an RTD of $10^{-3}$ or more were considered for use in the phage typing set. After a series of preliminary trials 12 preparations were chosen for the phage typing trial.

Phage typing procedure.

The surface of a sodium lactate agar plate was flooded with a 4 hr nutrient broth culture of the test strain and then allowed to dry. The phage preparations (at RTD concentrations) were then individually applied each in 0.02 ml. amounts and when these had dried the plate was incubated overnight at 37°C. and the lytic patterns then observed.

Preparation of a pyocine gradient plate.

10 ml. of molten nutrient agar was poured into a Petri dish set at an angle (Fig. 2). When this layer had set the dish was

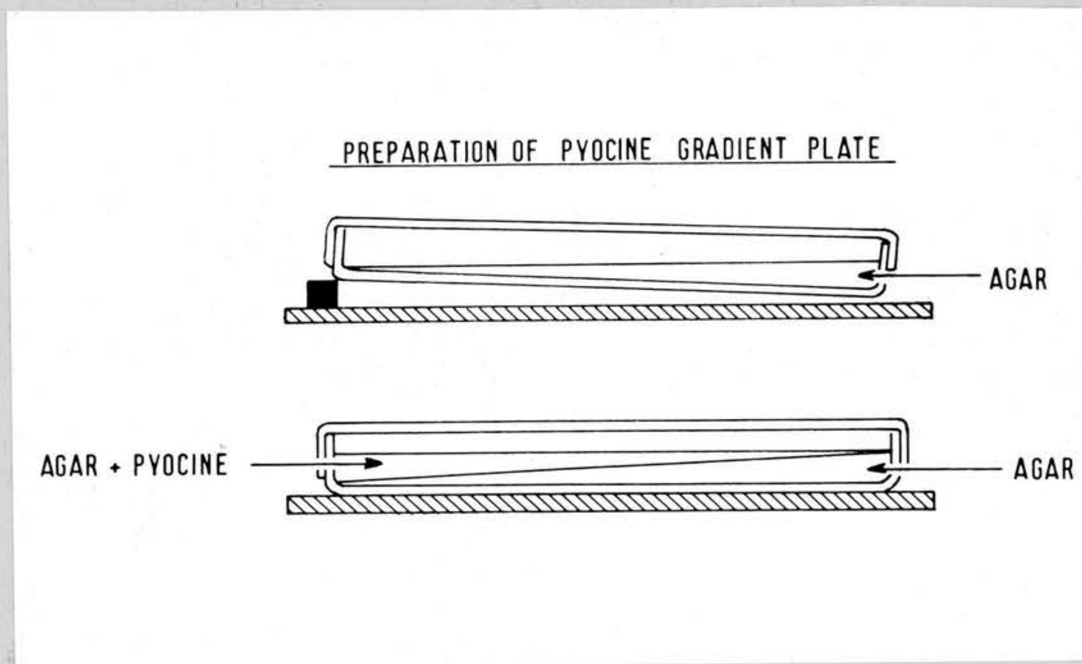



Fig. 2.

placed horizontally and 10 ml. of pyocine-agar (5 ml. of a 1 in 8,000 concentration of pyocine extract + 5 ml. of molten nutrient agar, mixed at 50°C.) was added and allowed to set

Diffusion of the pyocine downwards results in a concentration gradient across the plate with the highest concentration towards the left hand side. The surface of the gradient plate was flooded with a broth suspension of the sensitive strain and allowed to dry before it was incubated overnight at 37°C. Confluent growth of the indicator strain was observed over the right hand portion of the plate together with several discrete colonies to the left of the growth area i. e. in the region of the higher pyocine concentrations. These variants were streaked towards the left (an area of even higher pyocine activity) to yield on further incubation second step variants. The process was continued until colonies were obtained at the extreme left hand edge of the plate.

Test for gelatinase activity.

In solid culture

Charcoal-impregnated denatured gelatin (Kohn, 1953) was prepared. Dehydrated nutrient gelatin medium was mixed with water to give a 15 per cent. w/v mixture and heated. 7 per cent. w/v of finely powdered charcoal was then added to the medium. After cooling slightly the mixture was poured into a flat tray to give a layer of approximately 3 mm. thickness. After setting the entire

sheet was removed and placed in 10 per cent. formalin for 24 hr. The formalin-gelatin sheet was then cut into pieces approximately 1 cm. x 0.5 cm. which were wrapped in muslin and washed in running tap water for 24 hr. The pieces of charcoal-impregnated gelatin were then placed under distilled water in a wide necked screw-capped bottle and sterilised by Tyndallisation.

Small pieces of the impregnated gelatin were placed mid-way on the surface of an 18 hr nutrient agar slope culture of an organism grown at 37°C. After several hours further incubation at the same temperature gelatinase activity could be observed by the release of charcoal particles from the liquefying gelatin.

In fluid preparations

Doubling dilutions of the pyocine preparations were made in sterile saline and 0.02 ml. amounts dropped on to the gelatin emulsion face of a $3\frac{1}{4}$" x $3\frac{1}{4}$" photographic lantern plate (Kodak, Contrasty) by means of a calibrated dropping pipette. The plates were then placed in a moist chamber at 37°C. for 30 min. and then washed gently in running tap water before being developed. Gelatinase activity could be observed by the appearance of clear zones in the emulsion surrounded by a black background and thus the titre of gelatinase activity could be measured.

Tests for lecithinase, haemolysin and lipase activity.

0.3 per cent. sheep or human red blood cells, 1.0 per cent.

egg yolk and 1.0 per cent. Tween 80 suspensions were prepared separately in sterile saline and distributed in 0.5 ml. amounts in small (3" x$\frac{1}{2}$") test tubes. Doubling dilutions of the pyocine-containing preparation, also in sterile saline, were added in 0.5 ml. amounts to each tube. The presence of haemolytic activity was measured by lysis of the red cells after 1 hr at room temperature (approximately 21°C). After incubation in a water bath at 37°C. for 24 hr lecithinase and lipolytic activity was measured by the appearance of a yellow curdled surface layer and clouding of the clear solution respectively.

Measurements of pH.

pH measurements were made using a mains operated multi-range instrument (Pye, Universal).

Turbidimetric measurements.

Turbidimetric measurements were made using a photo-electric colorimeter (Unicam, S. P. 1300) and employing a bright spectrum yellow filter (Ilford, No. 626) with a transmission range of 545 - 635 mμ.

Cytopathogenicity of pyocine preparations.

The possible cytopathogenic effects of pyocine preparations were observed on the following tissue cell lines; chick fibroblasts, Hep 2 epithelial cells and monkey kidney cells.

The cell cultures were distributed as monolayers on the sides of 5" x ½" glass test tubes stoppered with grey, virgin rubber caps and containing 0.8 ml. of Hank's solution buffered at pH 7.3. To each tube was added 0.2 ml. of doubling dilutions of the test preparation in sterile saline to give final dilutions of 1 in 5, 1 in 10, up to 1 in 80. The tubes were then incubated on a drum revolving at 18 rev. per. hr. at 37°C. After 1 hr, 3 hr, 24 hr and 48 hr intervals the monolayers were examined microscopically to observe any cytopathogenic effect. Several tubes of each cell line were used in the examination of each dilution; control tubes were also examined using the saline or Tris buffer preparations.

Animal pathogenicity of pyocine preparations.

Adult white mice were injected with pyocine preparations, subcutaneously, intramuscularly and intraperitoneally using 0.1 ml., 0.5 ml. and 1.0 ml. amounts respectively.

For certain purposes the drinking water was replaced with pyocine-containing preparations.

Isolation of Ps. pyocyanea from mouse faeces.

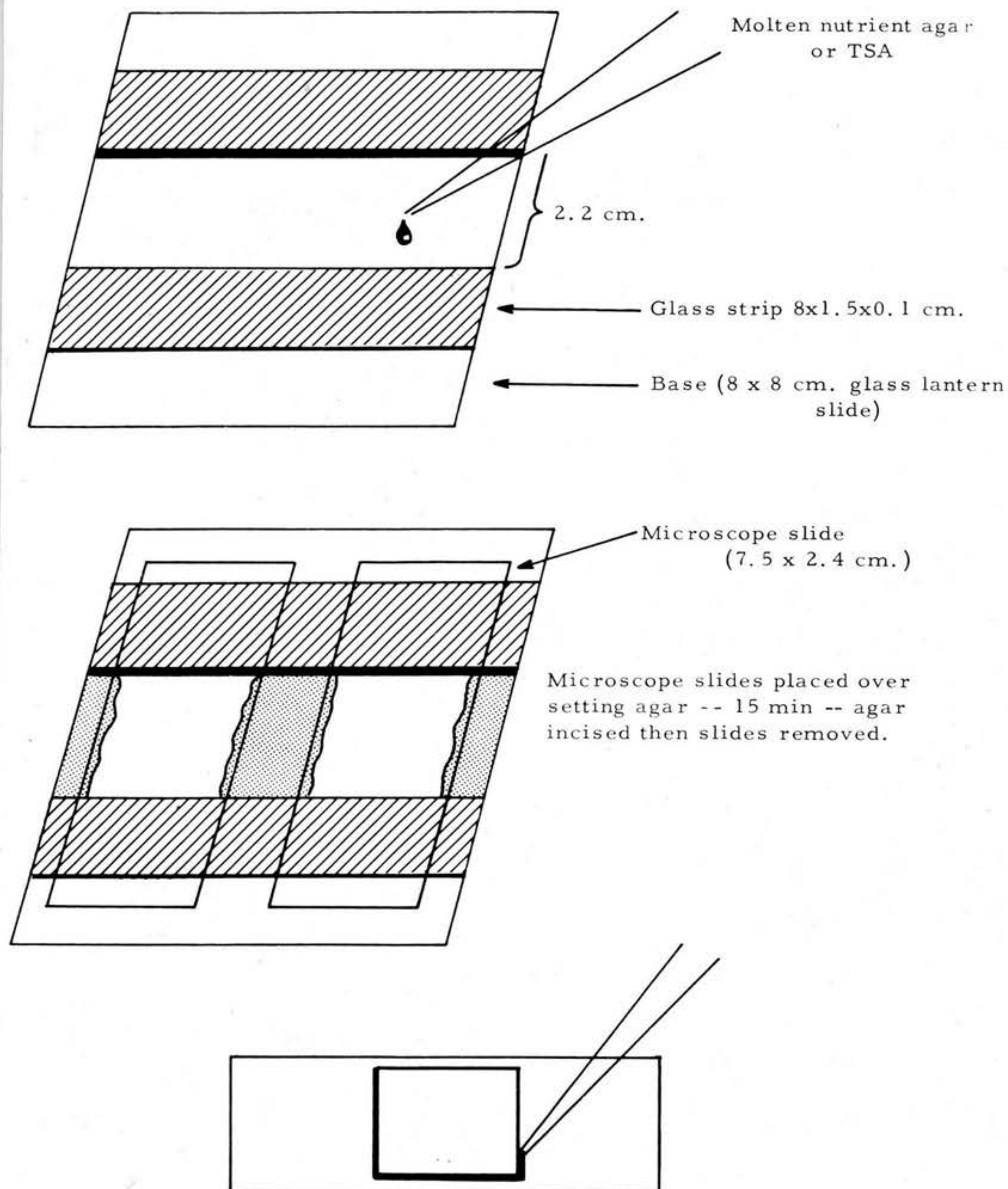
Ten to twelve pellets of faeces were emulsified in 10 ml. of sterile saline and using a nickel loop (3 mm. diam.) six loopfuls were plated out on the following media; DCA, 1% 'Cetrimide', agar 0.2% 'Dettol' agar and 20 mg. per cent. sodium nitrofurantoin agar.

The plates were incubated for 18-24 hr at 37$^{o}$C. and then left at room temperature for several days to allow characteristic colonies to develop.

Agar slide technique (Fig. 3).

Sterile nutrient agar was melted in a water bath and poured into a channel measuring 8.0 x 2.2 x 0.1 cm. prepared on a mould which had previously been sterilised by passage through a bunsen flame. Before the agar set, clean sterile microscope slides were placed at right angles across the agar channel ensuring that no air bubbles formed. After 15 min. excess agar was cut from around the edge of a slide which was then inverted bearing a block of agar.

A 4 hr broth culture of Ps. pyocyanea together with an aliquot of pyocine preparation was then placed on the surface of the agar block; the preparation was allowed to dry at room temperature in a closed container and a sterile cover glass was applied to the surface of the agar. Excess agar was then removed with a sterile scalpel, the exposed edges of the block was sealed with melted paraffin wax and the preparation was then incubated at 37$^{o}$C. in a moist chamber. The culture was examined at various intervals using phase-contrast microscopy in a Leitz Ortholux microscope fitted with an Orthomat camera.




After addition of the bacterial culture, with or without pyocine, a cover slip is placed on the preparation, excess agar trimmed off and the edges sealed with melted paraffin wax.

**Fig. 3. PREPARATION OF AGAR SLIDE-CULTURE**

An alternative method was by seeding an agar (TSA) block with a known pyocine-producing strain and after incubation for 14 hr at 32°C. the macroscopic growth was removed and the surface of the agar block sterilised by exposure to $CHCl_3$ vapour; the agar surface was then seeded with a strain of Ps. pyocyanea and after incubation for various periods of time at 37°C. microscopic observations were made as above.

Electron microscope techniques.

Pyocine and Phage preparations

Preparations exhibiting pyocine or phage activity were centrifuged for 2 hr at 30,000 r.p.m. (100, 000 G.) in a Spinco preparative ultracentrifuge (S. W. 39 Head) under refrigeration. The sediment was resuspended in 1 per cent. ammonium acetate buffer at pH 7.2; fixation when undertaken was with a final concentration of 0.25 per cent. formaldehyde.

The preparations were examined using a negative staining technique (Brenner and Horne, 1959) carried out as follows:

Equal volumes of specimen and 2 per cent. phosphotungstic acid at pH 7.2 were mixed on the surface of a clean glass slide and a drop of the suspension transferred by means of a fine platinum loop on to the surface of a collodion membrane supported on a copper electron microscope grid (NEW 200). After 30 sec. excess fluid was removed by allowing a piece of filter paper to

come into contact with the edge of the specimen drop and the preparation dried in a dessicator over anhydrous calcium carbonate. On occasions the specimens did not spread smoothly over the collodion membrane and in such instances a trace of serum albumin was added to the acid prior to staining with improved results.

Bacterial suspensions

Peptone water cultures of bacteria were fixed with 0.25 per cent. formaldehyde, centrifuged at 6,000 r.p.m. for 45 min. and the cells resuspended in 1 per cent. ammonium acetate buffer before staining the preparations as above.

In order to observe the effects of pyocine preparations on macroscopically sensitive and resistant strains of Ps. pyocyanea, 1.0 ml. of a pyocine preparation of titre 1 in 8,000 was added to 7 ml. of a 4 hr peptone water culture of the organisms. After incubation at 37°C., the suspension was fixed in 0.25 per cent. formaldehyde, centrifuged at 6,000 r.p.m. for 45 min. and resuspended in ammonium acetate buffer. The preparations were then stained by the method described.

The electron dense staining materials permeate the crevices in the bacterial cell walls or in the structures of the smaller particles and allow them to show up as light areas against a dark background. The specimens were screened at maximum

brightness in an electron microscope (Associated Electrical Industries, E. M. 6) at 50 kV. and observations recorded on photographic plates (Kodak, Contrasty).

# RESULTS

# CHAPTER V

## TYPING OF PS. PYOCYANEA BY PYOCINE PRODUCTION

### Preliminary experiments.

Previous experiments by Abbott and Shannon (1958) had shown the importance of conditions of incubation in the production of bacteriocines; in the case of pyocines incubation of the producer strains had been made at 37°C. (Jacob, 1954; Hamon, 1956; Holloway, 1960). Thus it was decided to investigate various combinations of temperature and duration of incubation in the production and detection of pyocine activity.

Initially 32 strains of *Ps. pyocyanea*, isolated from various sites were tested for the production of and sensitivity to pyocines (Fig. 4) by cross-testing experiments on plates of TSBA; several such plates were inoculated with each strain and then incubated under various combinations of time and temperature varying from 6 to 72 hr and from 21°C. to 42°C.

The inhibitory reactions observed on any one sensitive (indicator) strain took a variety of forms viz. complete inhibition without any resistant growth; clear-cut inhibition with a few resistant colonies; inhibition zones largely occupied by coalescent resistant colonies forming areas of confluent growth and on occasion inhibition of an indicator strain was represented by a narrow slit in the growth. This latter phenomenon, demonstrated to varying

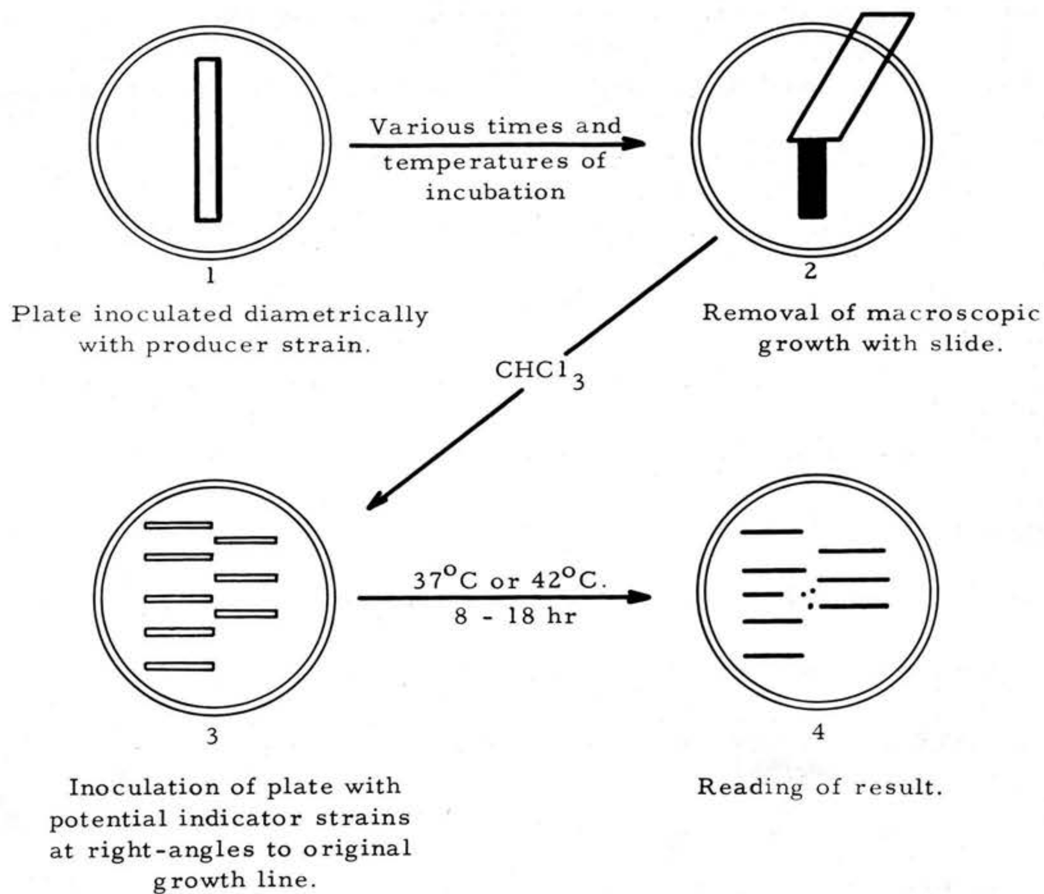


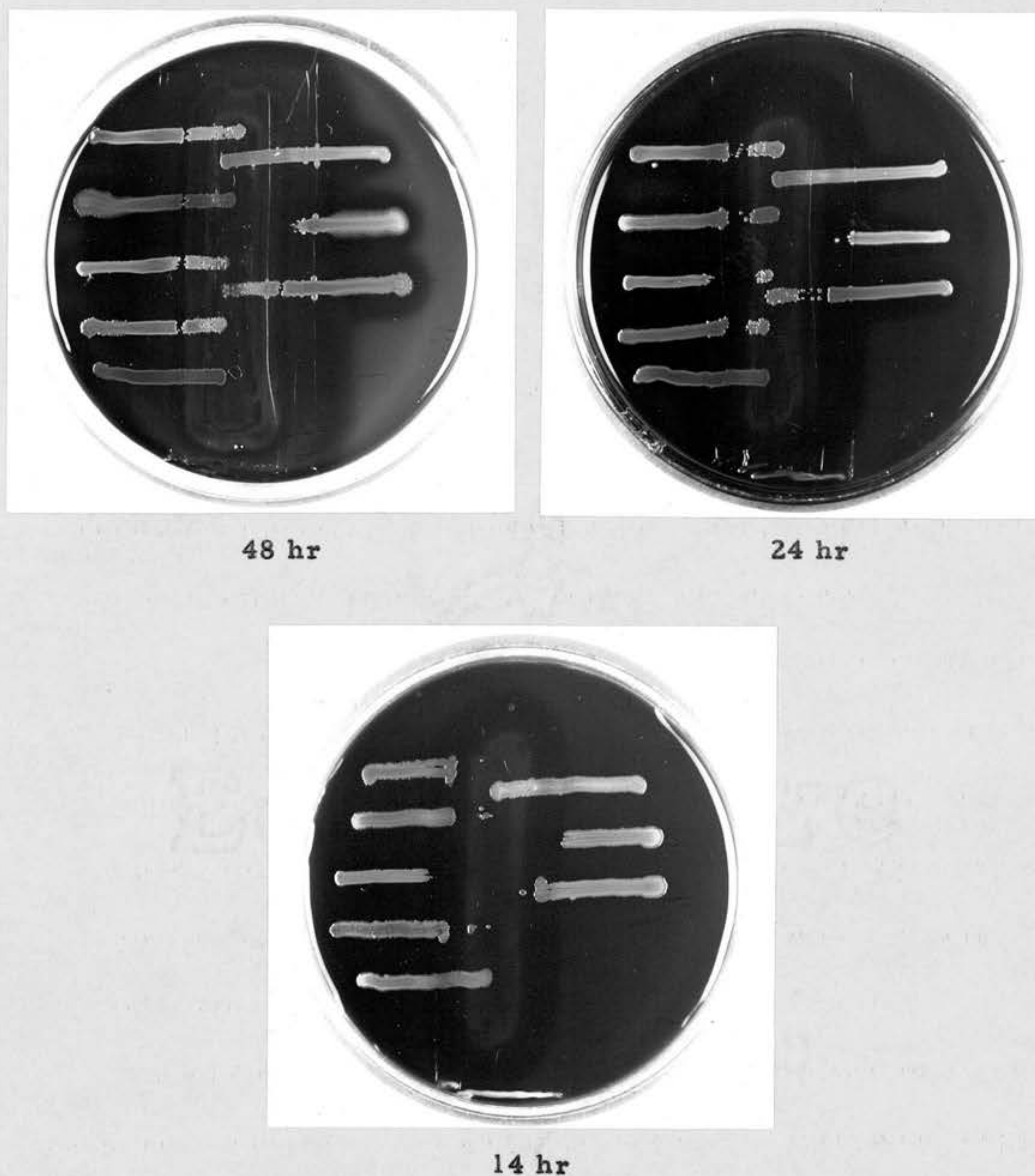

**Fig. 4.**

degrees in Fig. 5, was also encountered by Abbott and Shannon (1958) in the colicine typing of Sh. sonnei and referred to as 'D zones'. The extent and clarity of the zones of inhibition depended also on the producer and indicator strains, there being an apparent variation in the clarity of inhibition produced by different strains; in addition some strains, when used as indicators of pyocine activity, gave rise to more easily interpretable results than did others.

Though negative reactions were in general easily read, incubation of a producer strain for prolonged periods e.g. more than 24 hr often gave rise to a considerably reduced growth of the indicator strain over the area of the original inoculum. This is probably due to a reduction in the amount of nutrient materials available and is referred to as 'thinning'.

The production of pyocines appeared to be influenced by both the duration and the temperature of incubation of the pyocinogenic strain. Fig. 5 demonstrates the gradual disappearance of 'D zones' and the considerable improvement in the clarity of inhibition when primary incubation, at 35.5°C, is reduced from 48 to 24 and finally to 14 hr respectively before the plates are processed and the indicator strains applied.

Even more important than the duration of primary incubation of the potential producer strain was the temperature employed during this phase; regardless of the duration of incubation



48 hr　　　　24 hr

14 hr

Fig. 5. Effect of the duration of the primary incubation period upon pyocine production by a strain of Ps. pyocyanea.

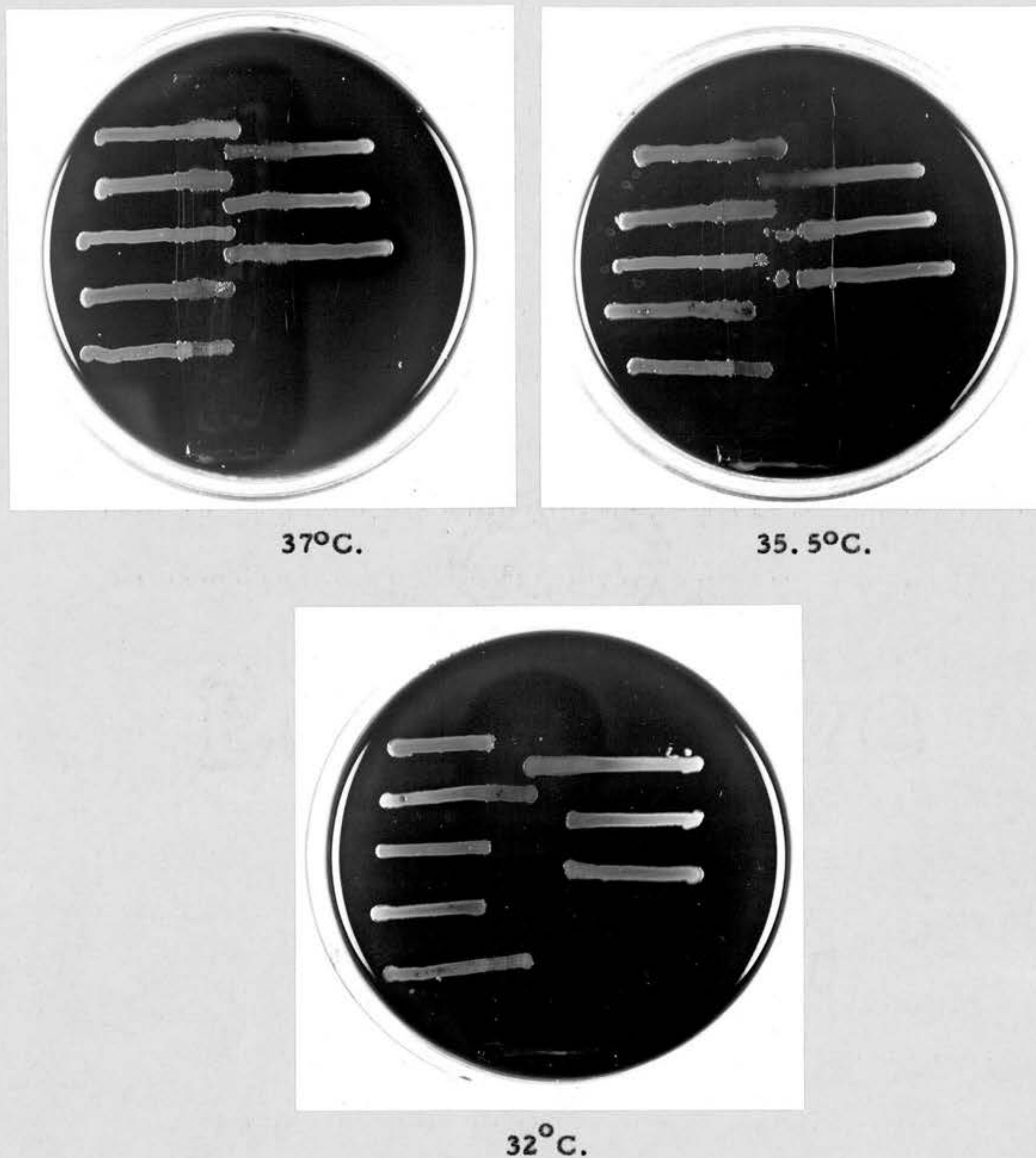
Strain incubated at 35.5°C. during primary incubation period and for 48, 24 or 14 hr. Thereafter macroscopic growth was removed, the medium sterilised and eight potential indicator strains (4 hr nutrient broth cultures, 37°C,) applied before the plate was reincubated at 37°C. for 18 hr.

employed, many strains which produced clear-cut inhibition patterns when incubated at 32°C., gave inferior results at 35.5°C. and failed to show any pyocine activity whatsoever after primary incubation at 37°C. Some strains, however, produced identical patterns of inhibition at all three temperatures, though once again the inhibition patterns were more clear-cut at the lower temperature. Examples demonstrating these temperature effects are shown in Figs.6 and 7 with strains later designated as pyocine types 16 and 3 respectively.

A further disadvantage of incubating the potential producer strain at 37°C. was that the resultant growth was often viscid and adherent to the medium.

The uniformity of response of certain potential indicator strains against the producer strains being tested allowed many of them to be rejected and similarly potential indicator strains which were uniformally resistant were deleted from the trial. In addition to these 32 strains, 200 strains of Ps. pyocyanea, isolated from a variety of pathogenic lesions, were then examined for pyocine production by cross-testing, for potential use as indicator strains.

As a result of these pilot studies it was decided that in the development of the typing technique the potential pyocine-producing strains should be incubated for 14-18 hr at 30° - 32°C.; the use of



37°C. 35.5°C.

32°C.

Fig. 6. Effect of temperature during primary incubation period upon pyocine production.

Producer strain of pyocine type-16 incubated for 14 hr at 37°, 35.5° and 32°C. before removal of macroscopic growth, sterilisation of medium, application of the eight standard indicator strains and reincubation at 37°C. for 18 hr.

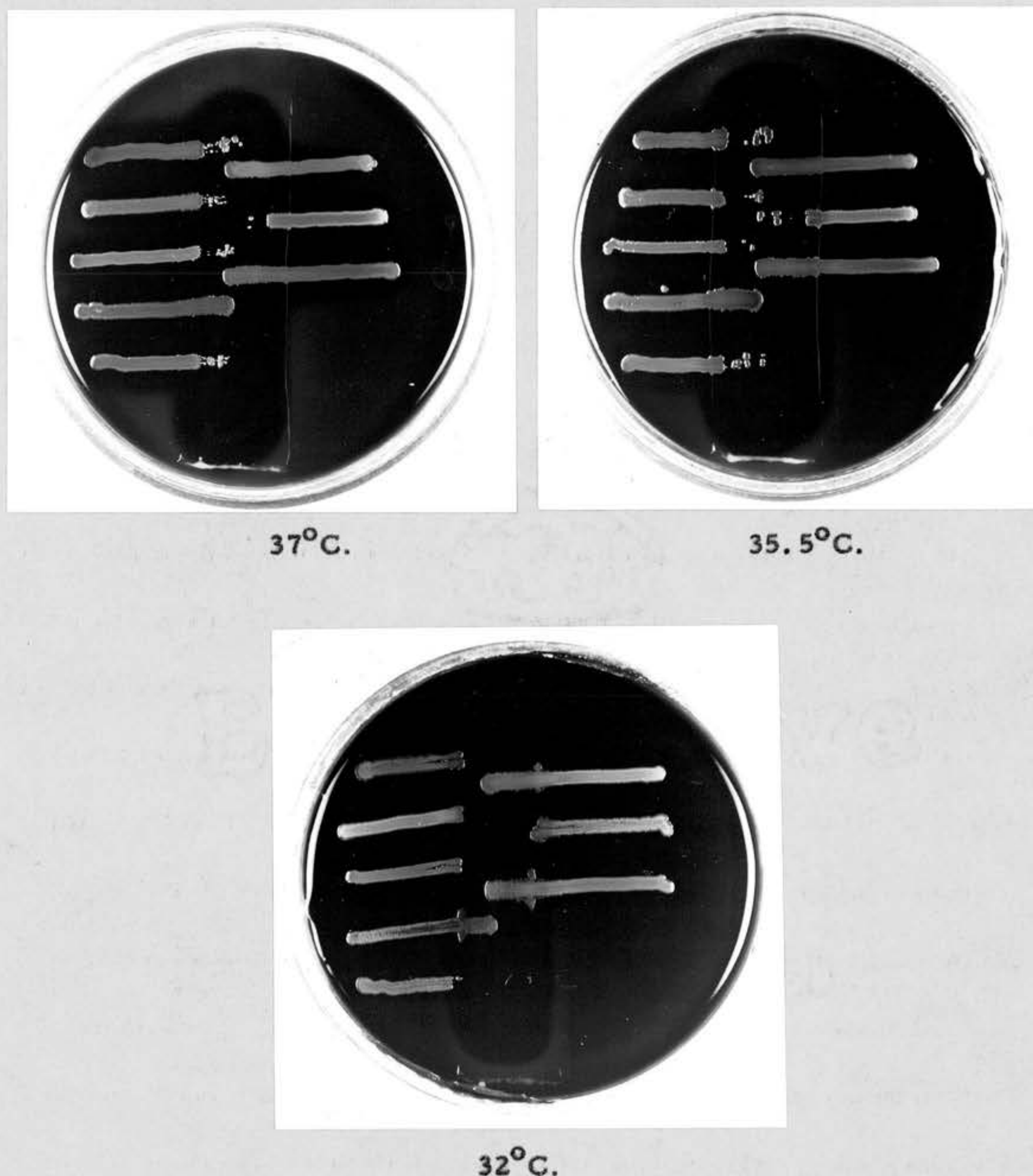



Fig. 7. Effect of temperature during primary incubation period upon pyocine production by a strain of Ps. pyocyanea.

Producer strain of pyocine type-3 incubated for 14 hr at 37°, 35.5° or 32°C. before removal of macroscopic growth, sterilisation of medium, application of the eight standard indicator strains and reincubation at 37°C. for 18 hr.

a 12 hr primary incubation period, though equally as satisfactory in regard to the results obtained, is not convenient for service laboratories.

Selection of indicator strains.

Coliform strains

The set of 15 indicator strains (which includes 14 strains belonging to the genus Shigella and also Fredericq's Esch. coli strain Row) for the typing of strains of Sh. sonnei by means of their colicine production were all completely inhibited when used as indicators against pyocine-producing strains when the latter were primarily incubated under the chosen conditions; similar results were also obtained when the set was examined against strains of Ps. pyocyanea which had shown no pyocine activity when tested against the 232 strains of Ps. pyocyanea used as indicator strains.

In addition 190 strains of Esch. coli were also completely inhibited when assayed as potential indicators of pyocine activity. The total inhibition of these non-pseudomonas strains, especially by apparently apyocinogenic strains of Ps. pyocyanea would seem to indicate that the phenomenon is due not to pyocine activity but instead to other, less specific antibacterial agents e.g. the pigment pyocyanine; later investigations with purified pyocine preparations did in fact indicate that pyocine activity was not involved in such widespread inhibiting phenomena.

The failure of such coliform strains to be of any value as indicator strains in pyocine typing studies is perhaps fortunate since the necessity of using indicator strains of the self-same species, in this case already well known for it's antibacterial properties, ensures that the inhibition patterns dealt with are in fact due to bacteriocine activity. From the several hundred strains of Ps. pyocyanea which had been examined it became increasingly evident that not only was pyocinogeny of common occurrence but that a considerable variety of inhibition patterns could be obtained using relatively few indicator strains. Eight strains of Ps. pyocyanea were chosen, therefore, to form an indicator set for a full scale investigation into the possible value of pyocine production as a means of characterising strains of Ps. pyocyanea. Before doing so, however, further preliminary experiments were carried out.

Influence of incubating indicator strains at 42°C.

Since many strains of Ps. pyocyanea are capable of slime production and since this function can be reduced by incubation at relatively high temperatures the effects of growing the eight indicator strains at 42°C. were investigated. For the purpose of this test seven pyocine-producing strains were tested for production after primary incubation for 14 hr at 32°C. Two sets of indicator strains were employed one being grown in nutrient broth at 37°C.

for 4 hr before being applied to the test plates and the other being incubated in a similar medium at 42°C. for 4 hr; thereafter the test plates were incubated at the respective temperatures for 18 hr. In all instances the set which had been incubated at 37°C. gave the most clear-cut results.

The effect of indicator strain preparation on typing results.

To determine whether the satisfactory results obtained, when the indicator strains were grown for only 3-4 hr at 37°C. before use, were due to the relative youth of the cells or to the less dense nature of the inoculum in comparison with the use of indicator strains grown for 24 hr, the following experiments were undertaken.

Four sets of indicator strains were prepared in nutrient broth: A set incubated for 24 hr at 37°C.; two other sets derived from 24 hr cultures but diluted respectively in nutrient broth to give 1 in 10 and 1 in 100 dilutions and finally a set grown for 4 hr was also employed. The 1 in 100 dilutions resembled the 4 hr cultures when measured in a photoelectric colorimeter. Using a bacteriological loop of internal diameter 2 mm., the four sets were employed in parallel tests as indicator strains against seven pyocine-producing strains. From the results, (Table 2) it can be seen that the most satisfactory inhibitions were obtained using indicator strains which had been grown up for only 4 hr.

Table 2. The effect of indicator strain preparation on pyocine typing results.

| Producer strain of type | Indicator strains grown at | | | |
|---|---|---|---|---|
| | 24 hr/37°C | | | |
| | Neat | 1 in 10 | 1 in 100 | 4 hr/37°C. |
| 1 | Ex | Ex | Ex | Ex |
| 3 | rc | rc | G | G |
| 5 | rc | rc | G | G |
| 9 | C | G | Ex | Ex |
| 10 | rc | rc | G | Ex |
| 11 | C | C | G | Ex |
| 16 | C | C | G | Ex |

Key to table:-

Ex (Excellent) = clear typing pattern with no resistant growth.

G. (Good) = clear typing pattern with 2-3 resistant colonies.

rc (resistant colonies) = readable pattern but with up to ten resistant colonies.

C (Confluent) = readable pattern but with areas of confluent resistant growth.

Typing strains of Ps. pyocyanea by their sensitivity to pyocines.

Twenty four strains of Ps. pyocyanea representing 10 pyocine types together with one apyocinogenic and one unclassifiable strain i. e. one which produced against the indicator strains under normal typing conditions inhibitions which were unacceptable in clarity for typing purposes were used in the form of 4 hr nutrient broth cultures as indicators of the pyocine activity of strains of types 1, 3, 9 and 10 which had previously been inoculated into TSBA and incubated and prepared for typing in the chosen manner. The reactions of these 24 strains to the pyocines produced by the four

test strains are shown in Table 3.

Table 3. Sensitivity patterns of 24 strains of Ps. pyocyanea to the pyocines of four test strains.

| Pyocine type of indicator strain | Ps. pyocyanea type no:- | | | |
|---|---|---|---|---|
| | 1 | 3 | 9 | 10 |
| 1 | + | - | - | + |
| 1 | + | - | - | + |
| 1 | + | - | - | + |
| 1 | - | - | - | - |
| 1 | - | - | - | - |
| 3 | + | - | - | + |
| 3 | + | - | - | + |
| 3 | + | - | - | + |
| 3 | + | - | - | + |
| 3 | + | - | - | + |
| 5 | + | + | - | + |
| 5 | + | + | - | + |
| 5 | + | + | - | + |
| 5 | + | + | - | + |
| 9 | - | + | - | - |
| 8 | + | + | - | + |
| 10 | - | - | - | - |
| 10 | - | - | - | - |
| 11 | + | + | - | + |
| 14 | + | + | + | + |
| 16 | + | + | - | + |
| 16 | + | + | - | + |
| uc | - | - | - | - |
| ut | + | + | + | + |

Key to table:-

+ = inhibition, - = no inhibition
uc = unclassifiable i. e. uninterpretable typing pattern.
ut = untypable i. e. no pyocine production noted.

It may be noted that two distinct patterns of sensitivity were obtained with the five strains of pyocine type-1; these results were

later explained by the successful subdivision of this common pyocine type. No such variation in sensitivity was noted in the case of the other pyocine types represented and the number of patterns obtained was very much less than the number of types examined e.g. no distinction can be made between strains of types 1 and 3, or between types 5, 8, 11 and 16. With the exception of the three strains of type-1, which later proved to be of a different subtype from the pyocine-producing test strain, no strain proved sensitive to the pyocines of strains of it's own type. Under the present conditions, then, this system of typing did not recognise as many types as did the method dependent upon pyocine production.

Effect of medium on pyocine production.

The effect of duration and temperature of incubation on the production of pyocines has already been demonstrated; the influence of various culture media on the pyocine typing of 28 strains of Ps. pyocyanea is now summarised: Typing was carried out using the eight standard indicator strains and the producing strain incubated under the normal conditions (Fig. 8). Using TSBA as a control the following media were examined; Tryptone Soya agar, nutrient agar, nutrient agar incorporating 7 per cent. horse blood, MacConkey's medium, TSBA incorporating 1 per cent. glucose and finally TSBA incorporating 2.5 per cent. calcium carbonate (CaCO3). The latter medium was investigated as a result of Morihari's (1964)

findings that the presence of $Ca^{++}$ ions greatly enhanced the production of proteinase by strains of Ps. pyocyanea.

Results.

The inhibition patterns obtained with the nutrient agar media (with or without added blood) and with MacConkey's medium in particular were by far inferior to those obtained with the other media, primarily because of large amounts of resistant growth in the zones of inhibition. Clear-cut patterns of inhibition were obtained using both TSBA and TSA plates though the incorporation of blood gave greater contrast between indicator strain growth and the medium and thus led to a quicker appraisal of the results. The incorporation of glucose into TSA did not lead to clear patterns of inhibitions but instead to greater amounts of resistant growth than with the glucose-free medium. Finally, though the presence of $Ca^{++}$ ions gave equally as good results as did the use of TSA alone they led to enhanced pyocyanine production both by the test strain and by each of the indicator strains and the diffusions of this pigment into the medium, to vary degrees, provided a difficult background on which to interpret typing.

# CHAPTER VI

## STANDARDISED TECHNIQUE OF TYPING STRAINS OF PS. PYOCYANEA BY PYOCINE PRODUCTION

Since it appeared that pyocines, in general, did not diffuse rapidly into the medium the original inoculum was fairly wide and in order to facilitate a quick and adequate inoculum the indicator strains were applied across half the plate only. The technique for the pyocine typing of strains of Ps. pyocyanea finally adopted for epidemiological studies is as follows (Fig. 8):

The strain to be examined for it's pyocine-producing properties is streaked diametrically across the surface of a plate containing TSBA to give a primary inoculum width of c. 1 cm. The plate is then incubated for 14-18 hr at $30^{o}$-$32^{o}$C. The macroscopic growth is removed with a glass slide, 3-4 ml. $CHCl_3$ is placed in the lid of the Petri dish and the medium containing portion is replaced on the lid for 15 min. so that microscopic remnants of the culture are killed.

The plate is then opened and traces of $CHCl_3$ vapour eliminated from the culture plate by exposing it to the air for a few minutes.

Cultures of the eight indicator strains, grown in nutrient broth for 3-4 hr at $37^{o}$C. are streaked on to the medium at right-angles to the line of the original inoculum; five strains are applied

on the left side of the plate and the other three on the right side. The plate is then reincubated at 37°C. for 8-18 hr.

Any pyocines produced by the original inoculum diffuse into the medium during the first period of incubation and then exert their inhibitory action on the indicator strains during subsequent incubation. The pyocine types of strains under examination are recognised from the patterns of inhibiton which they produce on the indicator strains (Figs. 9-12).

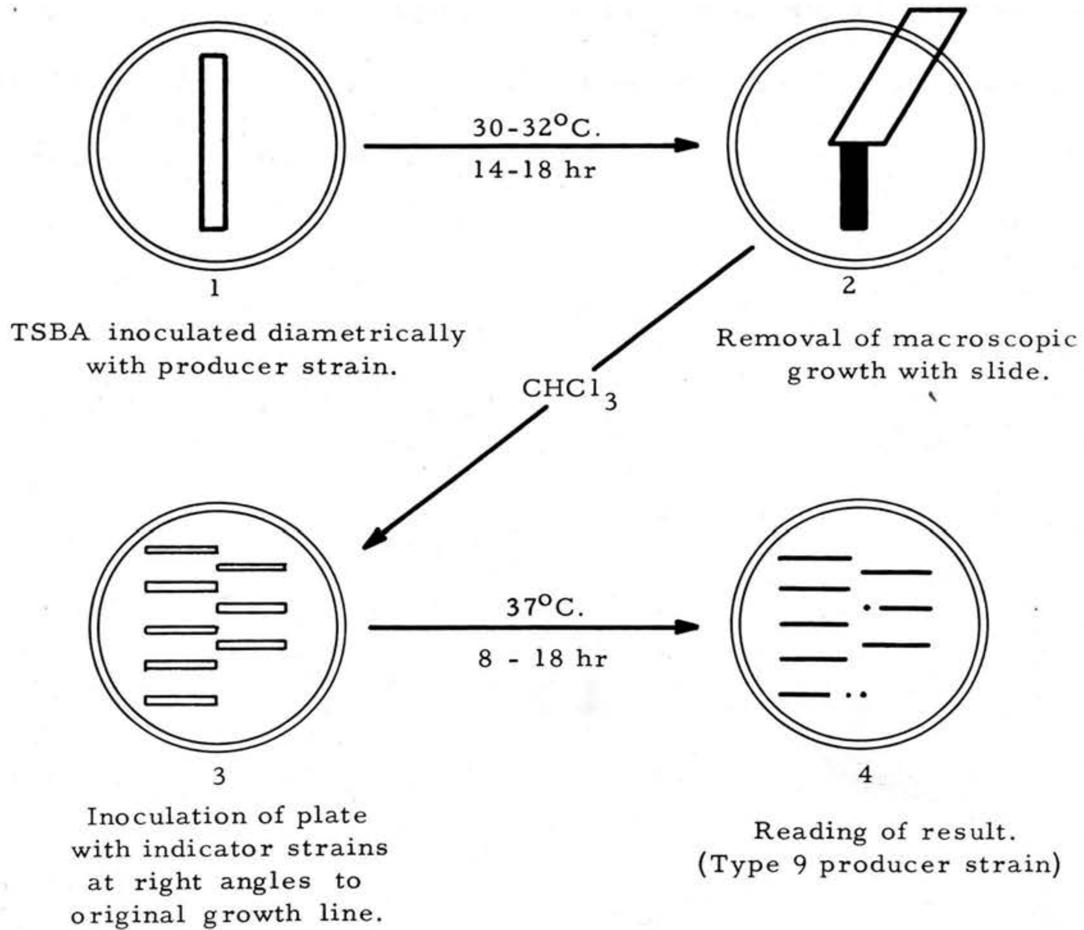



**Fig. 8.**

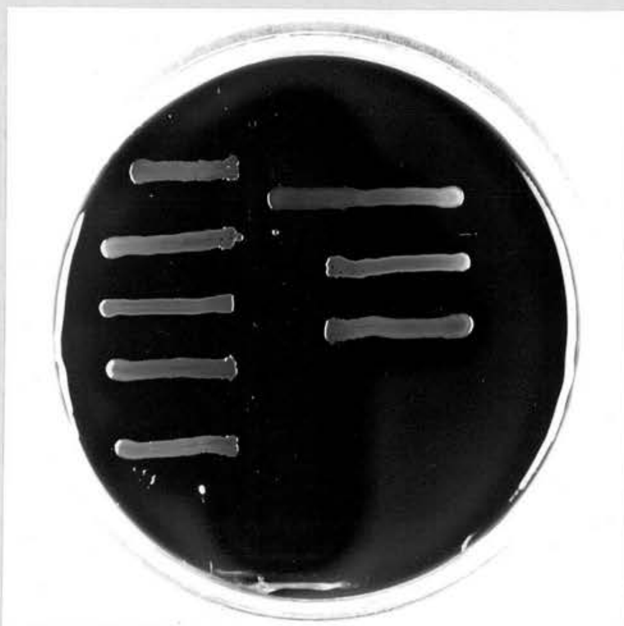


Fig. 9. Inhibition pattern given by pyocine type-1 strain of *Ps. pyocyanea*; all indicator strains except no. 6 (top right), are inhibited.

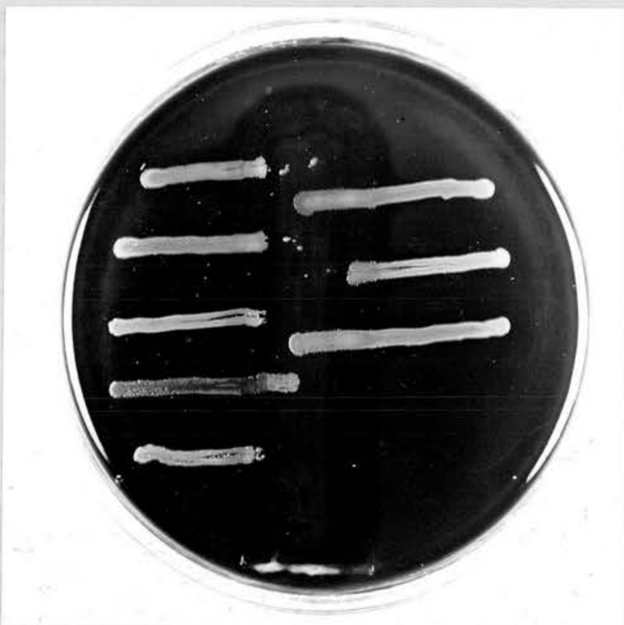


Fig. 10. Inhibition pattern given by pyocine type-3 strain of *Ps. pyocyanea*; indicator strains nos. 4, 6, and 8 remain uninhibited.

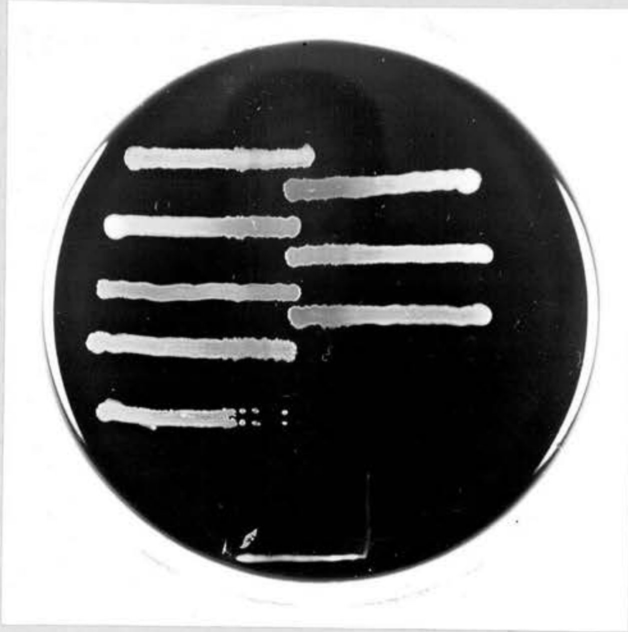


Fig. 11. Type-5 strains have restricted activity and inhibit only the indicator strain no. 5 (bottom left).

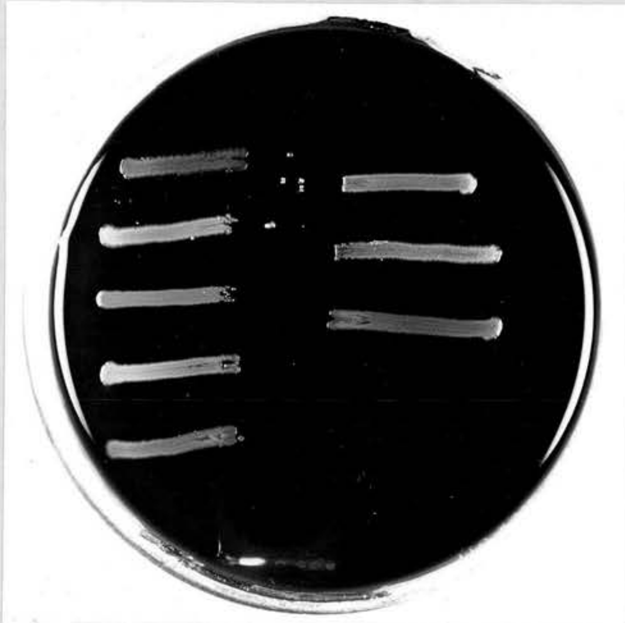


Fig. 12. Type-10 strains have wide inhibitory activity and inhibit all eight indicator strains.

# CHAPTER VII

## PYOCINE TYPING OF 5690 STRAINS OF PS. PYOCYANEA

The standardised technique of pyocine typing (Fig. 8) was then used to type 5690 strains of Ps. pyocyanea isolated from a wide variety of human and animal sources including pathological material, as well as from the inanimate environment. 88.5 per cent. of the strains fell into one or other of the 37 pyocine types of Ps. pyocyanea which have so far been established. The patterns of inhibition of these 37 pyocine types on the eight indicator strains are shown in Table 4. Twelve of the types have been regularly isolated in the Edinburgh area.

### Validity of the technique.

The indices used in determining the validity of this method of characterising strains of Ps. pyocyanea were as follows:

(1) In-vitro - strains were examined for constancy of pyocine production after storage and/or subculture.

(2) In-vivo - a. the constancy of pyocine type from replicate isolates made from the same site in a given patient and b. the uniformity of pyocine type in strains from an epidemic outbreak.

### In-vitro stability of pyocine production.

#### Subculture

In order to investigate the in-vitro stability of pyocine

Table 4. Patterns of inhibition of pyocine types of Ps. pyocyanea.

| Pyocine type of producer strain | Inhibition of indicator strain no. | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| 1 | + | + | + | + | + | - | + | + |
| 2 | - | + | - | - | - | - | - | - |
| 3 | + | + | + | - | + | - | + | - |
| 4 | + | + | + | + | + | - | - | + |
| 5 | - | - | - | - | + | - | - | - |
| 6 | + | + | + | + | + | - | + | - |
| 7 | + | + | + | - | - | - | + | + |
| 8 | - | + | + | + | - | - | + | - |
| 9 | - | - | - | - | + | - | + | - |
| 10 | + | + | + | + | + | + | + | + |
| 11 | + | + | + | - | - | - | + | - |
| 12 | + | + | - | + | + | - | - | + |
| 13 | - | - | - | + | - | - | - | + |
| 14 | - | - | + | - | + | - | + | - |
| 15 | - | + | - | - | + | - | + | - |
| 16 | + | - | + | + | - | - | + | + |
| 17 | - | - | + | - | - | - | + | - |
| 18 | + | - | + | + | + | - | + | + |
| 19 | - | - | + | + | - | - | + | - |
| 20 | - | - | - | - | + | + | - | - |
| 21 | - | + | - | + | + | - | - | - |
| 22 | + | + | + | - | + | + | + | - |
| 23 | + | - | - | - | + | - | + | - |
| 24 | - | - | + | + | + | - | + | + |
| 25 | + | - | + | - | - | - | + | - |
| 26 | + | - | - | - | - | - | + | - |
| 27 | + | - | + | - | + | - | + | - |
| 28 | - | - | - | + | - | - | + | - |
| 29 | - | + | - | - | + | - | - | - |
| 30 | - | + | + | - | - | - | - | - |
| 31 | - | - | - | - | - | - | + | - |
| 32 | - | - | - | + | + | - | - | + |
| 33 | + | + | + | + | + | + | + | - |
| 34 | - | - | - | - | - | - | - | + |
| 35 | + | + | - | - | + | - | + | - |
| 36 | - | + | - | + | - | - | - | + |
| 37 | - | + | + | + | + | - | + | - |

+ = inhibition, - = no inhibition

production after numerous subcultures eight strains of Ps. pyocyanea, one each of pyocine types 1, 3, 5, 9, 10, 11, 16 and 31 were subcultured on nutrient agar once a fortnight and typed at least weekly for over two years. These same types were employed as control producer strains to check the reliability of the indicator strains whenever a large number of tests was being carried out i.e. on average once every three days. In no instance was there any alteration in the patterns of inhibition nor in the clarity of such inhibition even when the inoculum of the producer strains was made directly from cultures stored on nutrient agar for up to a year at room temperature (c. 21°C.).

Storage

In a much larger series of 260 strains, however, representing 22 pyocine types and stored for periods ranging from three months to three years at room temperature certain variations were noted. On retyping, 15 strains (5.7 per cent.) were found to produce patterns of inhibition different from the original. All but two of these strains, however, had been stored for more than six months and in the two exceptions loss of activity towards only one indicator strain was observed.

In-vivo stability of pyocine production.

Constancy of pyocine type of replicate isolates

In order to assess the in-vivo stability of pyocine

production an investigation was carried out to determine whether isolates obtained on different occasions from the same site in a patient were of the same or of a different type from the strain originally isolated from that patient. The interval between isolations varied from one day to six months. The results of this investigation in patients treated in hospital or at home are summarised in Table 5 and 6.

It can be noted that not all isolates from the same site in a patient were of identical pyocine type. 530 replicate isolates from patients treated in hospital were of the same type as the original isolate but in 68 instances strains of different types were encountered on different days of testing. In some instances this variation in type involved only one isolate differing from the majority, e.g. the patient with 17 replicate isolates yielded 17 strains of pyocine type-1 and one strain of pyocine type-3.

In the case of patients treated at home, however, (Table 6) though they are fewer than those in hospital, the variation in type of replicate isolates was very much less: 27 patients showed no variation in the pyocine type of their replicate isolates and in only one instance was there variation in the pyocine type noted from that of the original isolate.

Some series of replicate isolates of Ps. pyocyanea from the same site in a patient were of considerable duration and

Table 5. Constancy of pyocine type in replicate isolates of *Ps. pyocyanea* obtained from different occasions from the same site in the same patient; results from 483 hospitalised patients.

| No. of replicate isolates obtained from patient* | No. of patients whose isolates were | |
|---|---|---|
| | of one type | of more than one type |
| 1 | 274 | 33 |
| 2 | 91 | 19 |
| 3 | 53 | 2 |
| 4 | 23 | 4 |
| 5 | 22 | 1 |
| 6 | 13 | 1 |
| 7 | 11 | 2 |
| 8 | 9 | 0 |
| 9 | 2 | 1 |
| 10 | 6 | 0 |
| 11 | 4 | 1 |
| more than 11 + | 22 | 4 |

*Excluding the first strain isolated from the patient.

+See table 5A.

Table 5A. (extension of Table 5).

Constancy of pyocine type in replicate isolates of <u>Ps. pyocyanea</u> obtained on different occasions from the same site in the same patient; results from 26 hospitalised patients.
(i. e. those patients with more than 11 replicate isolates)

| No. of replicate isolates obtained from patient* | No. of patients whose isolates were | |
|---|---|---|
| | of one type | of more than one type |
| 13 | 1 | 1 |
| 14 | 0 | 1 |
| 15 | 2 | 0 |
| 16 | 3 | 0 |
| 17 | 2 | 1 |
| 18 | 1 | 1 |
| 19 | 1 | 0 |
| 20 | 1 | 0 |
| 21 | 1 | 0 |
| 25 | 2 | 0 |
| 27 | 1 | 0 |
| 29 | 2 | 0 |
| 30 | 1 | 0 |
| 37 | 1 | 0 |
| 43 | 1 | 0 |
| 44 | 1 | 0 |
| 54 | 1 | 0 |

*Excluding the first strain isolated from the patient.

Table 6. Constancy of pyocine type in replicate isolates of Ps. pyocyanea obtained on different occasions from the same site in the same patient; results from 28 patients treated at home.

| No. of replicate isolates obtained from patient* | No. of patients whose isolates were | |
|---|---|---|
| | of one type | of more than one type |
| 1 | 18 | 0 |
| 2 | 7 | 0 |
| 3 | 0 | 0 |
| 4 | 1 | 1 |
| 5 | 0 | 0 |
| 8 | 1 | 0 |

*Excluding the first strain isolated from the patient.

involved many isolations (up to 55 in one instance). Strangely, the incidence of type variation in these cases was no more frequent than when only one or two replicate isolates were examined.

Though not so pertinent to the validation of pyocine typing as an aid in epidemiological investigations the examination of replicate isolations made on different days from different sites in the same patient reveal some interesting findings and these are summarised in Table 7.

Table 7. Constancy of pyocine type in replicate isolates of Ps. pyocyanea obtained on different occasions from different sites in the same patient; results from 168 hospitalised patients.

| No. of sites from which isolates were obtained | No. of patients whose isolates were | |
|---|---|---|
| | of one type | of more than one type |
| 2 | 84 | 21 |
| 3 | 24 | 7 |
| 4 | 11 | 5 |
| 5 | 8 | 1 |
| 6 | 3 | 1 |
| 10 | 1 | 0 |
| 12 | 2 | 0 |

The period of examination varied from several days to 5 months. Ample evidence for both the stability of pyocine production and the ubiquity of Ps. pyocyanea in regard to it's sites of isolation are revealed by a more detailed examination of the last three cases noted in this Table. These patients retained constancy of pyocine type in replicate isolates throughout 10 and 12 different specimens involving up to 138 replicate isolates and the relevant data is summarised in Table 8.

Table 8. Replicate isolates of Ps. pyocyanea obtained on different occasions from different sites in the same patient; isolates from 3 hospitalised patients.

| Patient | I. D. | | W. A. | | L. S. |
|---|---|---|---|---|---|
| Period | Sept. '66- Jan. '67 | | Apr. - Aug. '66 | | Nov. '64-Jan. '65 |
| No. of isolates | 227 | | 88 | | 101 |
| Specimens | No. | Specimens | No. | Specimens | No. |
| Urine | 44 | Urine | 61 | Urine | 38 |
| Ileostomy swabs | 30 | Nasal | 7 | Sputum | 9 |
| Throat | 30 | Wound | 6 | Throat | 8 |
| Nasal | 17 | Throat | 4 | Perineal | 8 |
| Urethral | 6 | Scrotum erosion | 1 | Drains | 7 |
| Teflon swabs | 4 | Stool | 2 | Blood | 6 |
| Catheter swabs | 2 | Groin ulcer | 1 | Arterial cannulae | 2 |
| Blood | 2 | Tongue | 1 | Stool | 1 |
| Perineal | 2 | Catheter | 1 | Groin | 1 |
| Stool | 1 | Blood | 2 | Catheter | 1 |
| Exposed plates | 89 | Exposed plates | 21 | Nasal | 2 |
| Necropsy | no specimen | Spleen | 1 | Exposed plates | 16 |
| | | Renal abscess | 1 | Necrotic area of kidney | 1 |
| Pyocine type | 35 | | 1 | | 16 |

Plates of culture medium placed in the patient's environment yielded strains of Ps. pyocyanea which in most instances were of the same pyocine type as those isolated from the patient and this is perhaps significant in determining the modes of spread of this organism. Patient I. D. (Table 8) showed no variation in pyocine type in the 227 strains examined even though an average of six colonies were examined in each of the exposed plates. Of the strains examined from patient W. A. two exposed plates showed the presence of both types 1 and 3; these being further differenciated since with the exception of the two type-3 strains which did not produce pyocyanine on nutrient agar all the type-1 strains produced pyocyanine and the unusual red pigment, pyorubrin on this medium.

Type stability in infected mice.

Over a three-month period 150 isolates of Ps. pyocyanea obtained from the faeces of two infected mice, showed no alteration in pyocine type from that of the original isolate.

In addition, when with four adult mice, the drinking water was replaced, for six days with a broth culture of a type-16 strain of Ps. pyocyanea they were found to excrete strains of this pyocine type for up to a month after withdrawal of the broth culture.

Uniformity of pyocine type in epidemic outbreaks.

The uniformity of pyocine type in any one epidemic of infection due to Ps. pyocyanea was investigated by typing the isolates from 20 outbreaks each involving from 5 to 90 patients; in each instance the strains from one epidemic focus were found to belong to the same pyocine type; further details of these outbreaks will be given later.

Type distribution in two Scottish hospitals and in an open community.

The type distribution of strains of Ps. pyocyanea in two large teaching hospitals and of isolates obtained from patients treated at home is shown in Table 9. Hospital 1 was situated in the South East of Scotland and Hospital 2 in the West; the University Diagnostic Laboratory serving patients treated at home was also in the South East region. The strains were isolated between August 1961 and June 1967. In order to obtain a more realistic estimate of the incidence of the various types only one strain of a particular type from any one patient is included in the Table e.g. a patient who provided 237 strains, all of pyocine type-35, from ten varieties of specimen and from plates exposed at his bedside, accounts for only one instance of type-35 in the Table. Likewise in epidemic outbreaks due to a single pyocine type only one strain is represented in this material.

Table 9. Distribution of pyocine types in 2396* strains of <u>Ps. pyocyanea</u> isolated in two Scottish hospitals and from a University Diagnostic Service Laboratory.

| Pyocine type | Percentage of strains belonging to stated pyocine type in | | | |
|---|---|---|---|---|
| | Hospital 1 (1395) | Hospital 2 (550) | Diagnostic Lab. (451) | Total (2396) |
| 1 | 36.3 | 28.7 | 34.4 | 34.3 |
| 3 | 25.0 | 31.1 | 18.8 | 25.3 |
| 5 | 5.4 | 5.1 | 7.1 | 5.7 |
| 10 | 3.4 | 1.6 | 3.1 | 2.9 |
| 35 | 4.0 | 0.3 | 2.7 | 2.9 |
| 29 | 2.7 | 0.5 | 2.0 | 2.1 |
| 27 | 0.9 | 5.8 | 0.9 | 2.0 |
| 16 | 1.5 | 0.3 | 3.8 | 1.7 |
| 11 | 1.1 | 3.3 | 1.3 | 1.6 |
| 9 | 0 | 5.5 | 0.4 | 1.3 |
| ut | 7.3 | 6.5 | 10.2 | 7.7 |
| uc | 5.7 | 6.7 | 3.1 | 5.5 |
| Other types | 6.7 | 4.7 | 12.2 | 7.3 |
| | (20 types) | (13 types) | (14 types) | (24 types) |

*Only one example of a type from each patient is represented and only one type from each epidemic situation.

ut = untypable strains i. e. not producing pyocines detected by the standardised method.

uc = unclassifiable strains i. e. individual strains giving patterns other than those shown in Table 8 or alternatively uninterpretable typing results.

The predominance of pyocine types 1 and 3 is evident in each instance although the relative incidence of these types varies from one centre to another. It is interesting to note the frequency of type-9 (5. 5 per cent.) in hospital 2 in comparison with it's complete absence from the much larger series of strains obtained from hospital 1. Similarly type-27 accounts for only 0. 9 per cent. of strains isolated from the hospital and the neighbouring community in the South East yet represents almost six per cent. of the strains isolated in hospital 2 situated only 40 miles distant.

# CHAPTER VIII

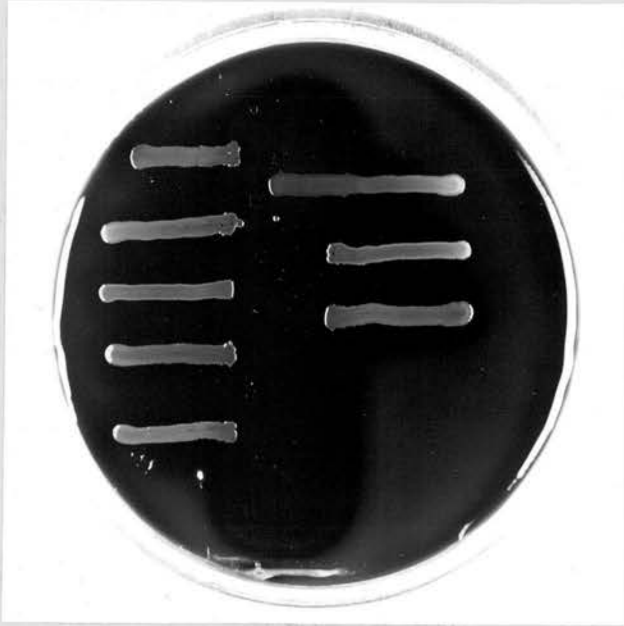
## SUBDIVISION OF PYOCINE TYPE-1 STRAINS OF PS. PYOCYANEA

### Predominance of pyocine type-1 strains.

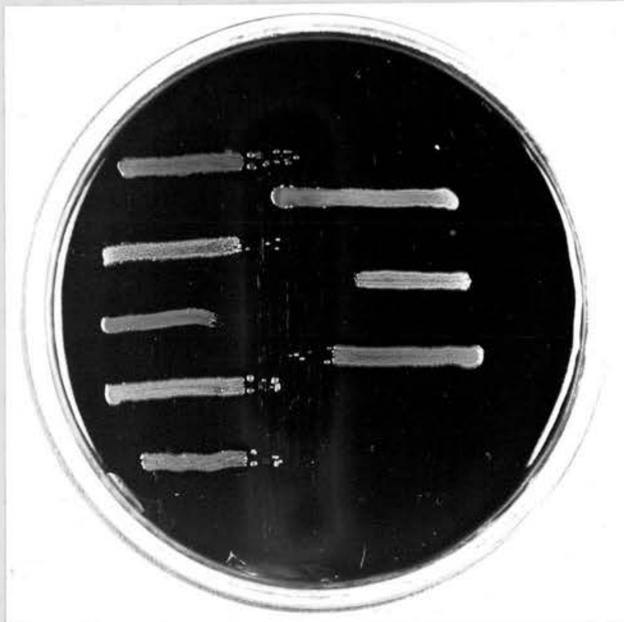
From Table 9 it will be noted that pyocine type-1 strains are those most commonly encountered and account for some 30 per cent. of isolates. Obviously if it were possible to recognise subtypes within such a common pyocine type the epidemiological value of the technique would be enhanced; a number of previous observations had suggested such a prospect.

In general, strains producing a type-1 pattern of inhibition on the eight indicator strains i.e. inhibition of all save indicator strain no. 6, displayed inhibition zones of uniform extent towards each of the individual indicators affected; in some instances, however, strains were encountered which though producing a type-1 pattern gave variation in the width of the inhibition zones with certain indicator strains. Such differences are demonstrated in Fig. 13. The top plate shows the pattern of a type-1 strain producing uniform inhibition of each of the sensitive indicator strains; the lower plate, though revealing a similar pattern of inhibition shows indicator strains nos.3 and 7 attacked to a greater extent than the others. In addition, when strains of pyocine type-1 were examined for their sensitivity towards the pyocines of standard producing strains (p. 85) different patterns of sensitivity were obtained. Since pyocine-

Fig. 13.



Ps. pyocyanea strain of pyocine type-1 showing uniform inhibition of each of the sensitive indicator strains.



Strain of Ps. pyocyanea exhibiting a pyocine type-1 pattern with enhanced inhibition of indicator strains nos.3 and 7.

sensitivity is a stable characteristic it would seem likely then that several pyocines acting singly or in combination are capable of producing a type-1 pattern.

Subdivision of type-1 strains.

Using a fresh group of 12 strains of Ps. pyocyanea as potential indicator strains various patterns of inhibition were noted when several hundred strains of Ps. pyocyanea of pyocine type-1 were tested for pyocine-producing activity under the standard conditions. From this group of 12 potential indicator strains a set of five strains was selected which allowed the recognition of eight clear-cut subtypes within the pyocine type-1 series. These subtypes are referred to as 1/1, 1/2, 1/3 and etc. (Table 10)

Table 10. Patterns of inhibition of subtypes of pyocine type-1.

| Subtype of pyocine type-1 | Inhibition of indicator strain no. | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| 1/1 | + | + | + | + | + |
| 1/2 | - | + | + | + | + |
| 1/3 | - | - | + | + | + |
| 1/4 | + | - | + | + | + |
| 1/5 | - | + | + | + | + |
| 1/6 | - | - | - | - | - |
| 1/7 | - | - | + | - | + |
| 1/8 | - | + | - | + | - |

+ = inhibition, - = no inhibition.

In the subtyping of pyocine type-1 strains of Ps. pyocyanea the duration and temperature of incubation of the producer strain was as important as in the primary typing method. The typical appearance of the plates when subtyping was performed with primary incubation at 32°, 35.5° and 37°C. for 14 hr in each case is shown in Fig. 14.

Plate 1 shows the characteristic pattern produced by strains of pyocine subtype 1/2 namely the inhibition of all the indicator strains save strain no. 1. This plate was incubated at 32°C. for 14 hr after inoculation with the producer strain.

The results obtained when the primary incubation temperature was raised to 35.5°C. and for 14 hr is shown in Plate 2 in which, although the same inhibition pattern is discernable, the amount of resistant growth in the inhibition zones has increased. Furthermore indicator strain no. 3 shows evidence of 'D zone' inhibition referred to earlier (p. 76) where an area of confluent resistant growth appears over the central region of the original inoculum area; this differs from the clear-cut inhibition obtained when incubation is at 32°C.

When incubation of the producer strain is carried out at 37°C. for 14 hr (Plate 3) the lack of inhibition of any of the indicator strains can be noted.

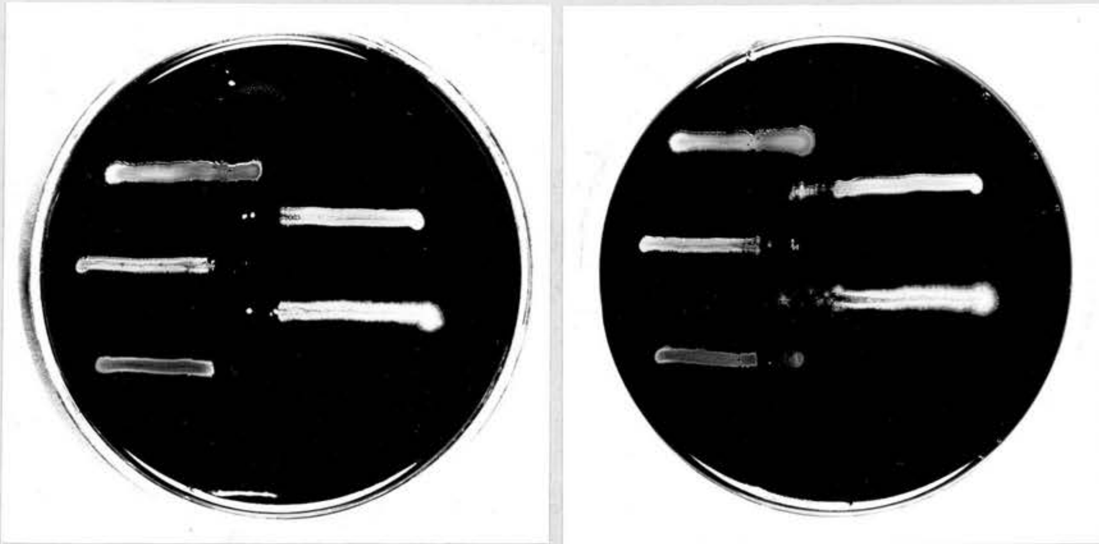


Plate 1. 32°C. Plate 2. 35.5°C.

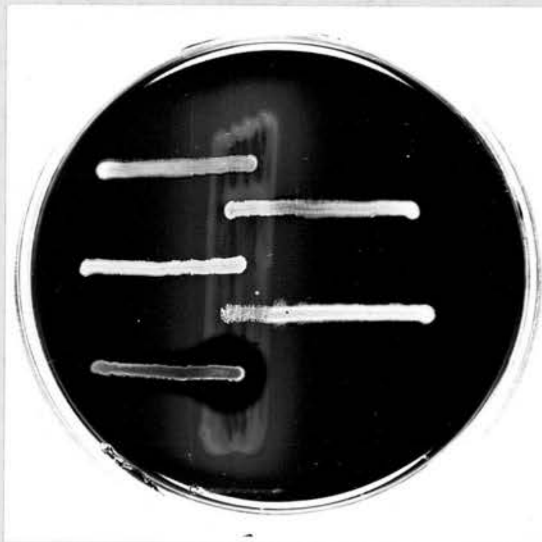


Plate 3. 37°C.

Fig. 14. Influence of temperature during primary incubation period on the patterns yielded in the subtyping of a pyocine type-1 producer strain.

Strain of <u>Ps. pyocyanea</u> R21, pyocine subtype 1/2, incubated for 14 hr at 32°, 35.5° or 37°C. before removal of macroscopic growth, sterilisation of the medium, application of five standard indicator strains and reincubation at 37°C. for 18 hr.

Validation of the subtyping technique.

Validation of the method of subtyping of pyocine type-1 strains was carried out using the same in-vitro and in-vivo indices as for the primary method of pyocine typing.

No variation was noted when strains representing each of the subtypes were examined after numerous subcultures and/or storage for periods of up to one year. The results obtained when replicate isolates were examined i. e. strains from the same site in the same patient, are summarised in Table 11. It can be noted that in most instances the production of pyocine is a stable characteristic of strains isolated on different occasions from a given site in a patient. Nevertheless, as with similar investigations into the validity of the primary typing technique (Table 5) in a number of instances replicate isolates from a patient do not show the same pyocine type as the original strain.

Table 11. Constancy of subtype of pyocine type-1 strains of Ps. pyocyanea in replicate isolates obtained on different occasions from the same site in the same patient; results from 162 patients.

| No. of replicate isolates | No. of patients whose isolates were | |
|---|---|---|
| | of one subtype | of more than one subtype |
| 1 | 95 | 4 |
| 2 | 23 | 0 |
| 3 | 12 | 5 |
| 4 | 10 | 0 |
| 5 | 8 | 3 |
| 6 | 4 | 0 |
| 7 | 3 | 0 |
| 8 | 3 | 1 |
| more than 8 | 16+ | 3† |

+No. of replicate isolates from patients in whom all were of the same subtype; one instance of 13, 14, 15, 16, 19, 22, 44 and 55, two instances of 17 and three instances of 10 and 11 replicates.

†No. of replicate isolates from patients yielding more than one subtype; one instance of 10 and two instances of 25 replicates.

The distribution of subtypes of pyocine type-1 strains, collected from the same laboratories as in the case of the primary typing technique is given in Table 12.

**Table 12. Distribution of subtypes of pyocine type-1 in 795 strains of *Ps. pyocyanea* isolated in three laboratories.**

| Pyocine subtype | Percentage of strains belonging to stated subtype of pyocine type-1 in | | | |
|---|---|---|---|---|
| | Hospital 1 (504) | Hospital 2 (158) | Diagnostic Laboratory (133) | Total (795) |
| 1/1 | 6.9 | 4.4 | 2.3 | 5.7 |
| 1/2 | 28.8 | 30.4 | 36.1 | 30.3 |
| 1/3 | 25.6 | 34.8 | 17.3 | 26.0 |
| 1/4 | 17.7 | 18.4 | 15.0 | 17.4 |
| 1/5 | 3.0 | 2.5 | 1.5 | 2.6 |
| 1/6 | 4.4 | 4.4 | 12.0 | 5.7 |
| 1/7 | 3.6 | 1.3 | 2.3 | 2.9 |
| 1/8 | 6.5 | 3.2 | 6.7 | 5.9 |
| 1/uc | 3.6 | 0.6 | 6.7 | 3.5 |

1/uc = Pyocine type-1 strains producing uninterpretable inhibition against the subtyping indicator strains or patterns other than those shown in Table 10.

# CHAPTER IX

## EPIDEMIOLOGICAL ASPECTS OF PYOCINE TYPING

### Occurrence of more than one pyocine type of Ps. pyocyanea in a single specimen.

It has already been noted both in the primary typing technique and in subtyping of pyocine type-1 strains of Ps. pyocyanea that there were occasional discrepancies in that in a series of replicate isolates from the same site in any one patient a minority of strains differed in type or subtype from the remainder of the series. Originally strains of Ps. pyocyanea had been received as subcultures of a single colony picked from diagnostic plates and so the possible presence of more than one pyocine type from any one site could not be determined; on occasion, however, the opportunity had arisen to examine several colonies of Ps. pyocyanea from the same diagnostic plate and in a few instances more than one pyocine type had been obtained. It was decided therefore to determine the frequency with which more than one pyocine type could be detected from the same specimen by obtaining primary diagnostic culture plates and examining an average of six colonies of Ps. pyocyanea for pyocine production. The findings are summarised in Table 13.

It can be noted that in general the prospect of encountering more than one pyocine type in any one specimen is greater in the case of hospital patients (1 in 11.1) as compared with those treated

at home (1 in 18.2). Indeed, in specimens other than faeces and urines the isolation of two or more pyocine types is five times more common in hospital patients (1 in 10.9) than in those treated at home (1 in 53). Such findings confirm the results already obtained in the examination of replicate isolates where the isolation of more than one pyocine type from any one specimen was more common in hospital patients than in those treated at home. The significance of such findings will be discussed later.

Table 13. Frequency with which more than one pyocine type of Ps. pyocyanea is encountered in specimens from patients at home or in hospital.

| Nature of specimens | No. of specimens: | | | | Ratio of column 2 to column 1 | |
|---|---|---|---|---|---|---|
| | (1) Total | | (2) Those with more than one type | | | |
| | Home | Hospital | Home | Hospital | Home | Hospital |
| Faeces | 158 | 18 | 10 | 1 | 1/15.8 | 1/18 |
| Urine | 80 | 73 | 5 | 7 | 1/16 | 1/10.4 |
| Others | 53 | 131 | 1 | 12 | 1/53 | 1/10.9 |
| Total | 291 | 222 | 16 | 20 | 1/18.2 | 1/11.1 |

Pyocine production: An epidemiological marker of Ps. pyocyanea.

Many strains were examined retrospectively and little detail was available regarding the background of epidemic outbreaks of infection due to Ps. pyocyanea e.g. in a small rural hospital 11 patients suffered post-prostatectomy urinary tract infections caused

by the relatively rare strains belonging to pyocine type-8; the infections were sporadic and involved two or more patients at a time over an eighteen-month period. Although attempts to investigate the situation thoroughly were thwarted, strains of Ps. pyocyanea of the same pyocine type were isolated from various environmental situations and especially alarmingly from the disinfectant solution employed in the unit.

As the technique of pyocine typing became more widely recognised requests for typing of strains were often received from hospitals encountering outbreaks of infection due to Ps. pyocyanea. When cooperation was forthcoming and strains from both patients and fomites were examined systematically many interesting details were obtained regarding the vehicles of spread of this organism. There follow summaries of a few of the epidemics which were investigated.

Epidemic 1. (Genito-urinary Unit).

A particular feature of the results of pyocine typing in the hospital in the West of Scotland was that all strains isolated from patients in a particular ward, over a period of time, belonged to pyocine type-9. Most of the patients had had a prostatectomy and were on bladder drainage; enquiry showed that such infections were of quite frequent occurrence but no specific investigation of potential sources and methods of spread had been made. A survey of the

ward environment revealed the existence of the causal type (i.e. pyocine type-9) together with three other pyocine types of Ps. pyocyanea. (Table 14).

Table 14. Pyocine type of strains of Ps. pyocyanea isolated from various environmental sites.

| Site | Pyocine type | Site | Pyocine type |
|---|---|---|---|
| Slunge outlet | 3 | Slunge | 3 |
| Bottle mop | 5 | Lavatory brush | 9 |
| Drainage jars (3) | 9 | Lavatory brush holder | ut |
| Urine bottles (2) | 9 | Gibbons bottle | 3 |
| Slunge water hose | 9 | Vacuum dust bag | 3 |

ut = untypable i.e. no pyocine production noted

Discussion with the ward staff revealed that the facilities to wash drainage jars etc. were quite inadequate and it had become the practice to rinse out such receptacles by hand and 'sterilise' them afterwards by allowing them to stand overnight in a bath of water which contained a few drops of a well known proprietary disinfectant. The drainage jars were then reconnected to the drainage tubes of the patients and little imagination is required to see how this highly motile organism could gain access to the urinary tract. The introduction of a proper method of cleaning

and sterilising drainage jars and other potential vehicles of infection coincided with an abrupt cessation of this epidemic.

Epidemic 2. (Maternity Unit).

From March 1964 until July 1965, 56 cases of infection due to Ps. pyocyanea were observed in neonates in the Maternity Unit of a large hospital and 40 of these were caused by strains of pyocine type-3. No further strains were obtained from this hospital until October 1966 when an explosive outbreak of gastro-intestinal infection occurred in the neonatal population. During October and November of 1966, 71 babies suffered such infection and they were aged from 1 day to 21 days; primarily the epidemic was noted by the babies showing " a failure to thrive" and though the majority of infections were of the gastro-intestinal tract two babies suffered infection of the eye and two cases of urinary tract infection were also noted. This sudden involvement of such a large number of bottle-fed babies and the failure to isolate Ps. pyocyanea from the few breast-fed babies in the Unit broughtthe artificial milk supply under suspicion.

Artificial milk-feeds were prepared in a large ultramodern kitchen using heat sterilisation of the bottles and milk and chemical sterilisation of the rubber stoppers by immersion in a basin of water containing a well-known proprietary antiseptic; the stoppers were used to seal the milk-feed until such time as it was required,

the stoppers were then replaced with rubber teats.

A large scale swabbing of the milk kitchen and the environment of three infected babies (Table 15) pointed to the rubber stoppers as likely vehicles of spread. Both the bulk milk source and the glass bottles themselves appeared to be uncontaminated but Ps. pyocyanea of pyocine type-3 was isolated from the rubber stoppers which were fitted to the prepared feeds prior to their leaving the kitchen; indeed, a strain of this type was isolated from a milk-feed just before it's intended use.

Table 15. Strains of Ps. pyocyanea of pyocine type-3, isolated from the following vehicles.

| | |
|---|---|
| 1. | Surface of stoppers removed from antiseptic solution. |
| 2. | 'Milk surface' of stoppers removed from feeding bottle. |
| 3. | Prepared milk-feed prior to administration. |
| 4. | Bed cupboard of infected child. |
| 5. | Wash basin sump. |

The introduction of terminal heat sterilisation of the milk-feeds brought the epidemic to a close.

Epidemic 3. (Cardiac Surgery Unit).

During December 1966 - February 1967 five of eight patients undergoing open-heart surgery, with the establishment of an extracorporeal circulation, developed respiratory tract infection due to Ps. pyocyanea of pyocine type-10 and three patients subse-

quently died. The five cases which developed such infection had required tracheostomy and thus a respiratory source of infection was suspected; as will be seen (Table 16) the causal pyocine type of Ps. pyocyanea was isolated from the respirator equipment.

Table 16. Strains of Ps. pyocyanea isolated in Epidemic 3.

| Site of isolation | Pyocine type |
|---|---|
| Tracheal aspirate (patient C) | 10 |
| Sputum (patient H) | 10 |
| Blood (patient H) | 10 |
| 'Bennet' ventilator tube | 10 |
| Nebuliser water | 10 |
| 'Ambu' rebreathing bag | 10 |

Random swabbing of the equipment of the theatre suite and adjacent rooms showed Ps. pyocyanea to be limited to the anaesthetic equipment and such vehicles were seriously implicated when it was shown that these strains also belonged to pyocine type-10. The last three cases occurred after the anaesthetic and resuscitation equipment had been 'sterilised' by washing with chlorhexidene digluconate and also exposure to ethylene oxide. Large scale swabbing of the entire Unit revealed the presence of several pyocine types of Ps. pyocyanea including type 10 located in a variety

of sites even after the use of various disinfectants.

The Unit was closed and intricate equipment was dismantled and thoroughly cleaned before being exposed to ethylene oxide.

Epidemic 4 (Maternity Hospital).

In May 1967 two cases of meningitis with septicaemia due to Ps. pyocyanea occurred in new-born babies in a large maternity hospital; no previous infections of this sort had been encountered since 1964. All strains isolated at necropsy from lung tissue and brain belonged to pyocine type 1 (subtype 1/4); this prompted a search for this subtype in the environment and also it's carriage by healthy infants; the results were disturbing.

During the period June-November 1967, 65 babies were shown to be excreting Ps. pyocyanea in their faeces; of these 57 carried strains of pyocine type-11 and eight were carriers of pyocine type-5. In addition, 16 cases of infection due to Ps. pyocyanea occurred during this period and with five deaths (Table 17). The infections involved a variety of sites and pyocine typing of the isolates revealed 13 cases due to strains of pyocine type-11, one each of types 1/4 and 5 and an untypable strain.

Strains isolated at necropsy, on the seven babies who died, showed that the first three babies suffered infection with pyocine type 1/4 and it appeared at this stage that the pyocine type-11 strains though more prevalent were less pathogenic. Such optimism was

Table 17. Pyocine types of strains of Ps. pyocyanea from pathogenic sources in a Maternity Hospital.

| Patient | Date of isolation | Nature of specimen | Pyocine type |
|---|---|---|---|
| Baby D | 22nd May | Lung (p.m.*) | 1/4 |
| " | | Brain (p.m.) | 1/4 |
| Baby C | 1st June | Lung (p.m.) | 1/4 |
| " N | 6th June | Urine | 11 |
| " G | 9th June | Eye | 11 |
| " K | 12th | Faeces | 11 |
| " B | 13th | Vagina | 11 |
| Mrs. O | 13th | Sputum | 11 |
| Baby Q | 31st July | Lung (p.m.) | 1/4 |
| " T | 10th August | Skin | 11 |
| " McG | 12th | Eyes and mouth | 11 |
| " Be | 24th | Nose | 11 |
| " Ca | 4th September | Lung (p.m.) | 5 |
| " Co | 4th | Ear | ut† |
| " F | 5th | Faeces | 11 |
| " H | 13th | Lung and brain (p.m.) | 11 |
| " S | 14th | Lung and brain (p.m.) | 11 |
| " Ke | 16th | Nose | 11 |
| " McN | 6th November | Lung (p.m.) | 11 |

* = post-mortem, † = untypable, i.e. no pyocine production noted.

premature, however, for later investigations showed that isolates from three of the last four necropsies involved strains of pyocine type-11, the remaining fatality being due to a strain of pyocine type-5.

The pyocine typing of strains of Ps. pyocyanea obtained from various environmental sites (Table 18) revealed the existence of strains of the three pyocine types incriminated in the fatal cases together with strains of four other pyocine types. The environmental reservoirs of the causal organisms, however, seemed primarily to be restricted to the sinks (nos. 1 and 2) of the main

neonatal wards where aspirator (2) was also in use. On occasion isolates of more than one type were isolated from the same sink and at the same time.

Table 18. Pyocine types of strains of Ps. pyocyanea isolated from environmental sites in a Maternity Hospital.

| Environmental sites | Date of isolation | | | | |
|---|---|---|---|---|---|
| | 26th June | 29th June | 10th July | 25th July | 23rd August |
| Sink 1 | 5 | 5 & 11 | 1/2 | 1/2 & 1/4 | 1/2 & 11 |
| " 2 | 11 | 5 & 11 | 1/4 & 11 | 1/4 & 11 | - |
| " 3 | - | 11 | 11 | 3 | 3 |
| " 4 | - | - | - | 3 | - |
| " 5 | - | 3 | 3 | - | 3 |
| " 6 | - | - | 11 | - | - |
| " 7 | 3 | 3 | 3 | - | - |
| " 8 | - | - | 3 | - | - |
| " 9 | 11 | 3 | 3 | - | - |
| " 10 | 1/2 | - | 1/2 | 3 | 3 |
| " 11 | - | - | - | - | 1/2 |
| " 12 | 33 | - | - | - | - |
| Labour room | 1/2 | - | - | - | 11 |
| Aspirator (1) | 1/2 | - | - | - | - |
| Aspirator (2) | 1/4 | - | - | - | - |
| Labour cubicle | - | 1/2 | 1/2 | 1/2 | - |

- = No strain of Ps. pyocyanea isolated.

## Hospital infection due to _Ps. pyocyanea_

As pyocine typing studies continued it was noted, on occasion, that certain pyocine types of _Ps. pyocyanea_ were occurring regularly in particular wards; therefore, an attempt was made to analyse retrospectively the possibility of an endemic state of cross-infection in such situations. The results provided confirmatory evidence that cross-infection existed and on a substantial scale.

From 1962 - 1967 of 82 patients, male and female, treated in the same surgical unit from whom strains of _Ps. pyocyanea_ were isolated no less than 52 (63.4 per cent.) harboured strains of pyocine type-3; other strains encountered belonged to types, 1, 10 and 35. Sampling of the ward environment revealed the existence of types 3, 10 and untypable strains. The significance of these findings was reduced somewhat by the frequency with which strains of pyocine type-3 are routinely encountered (Table 9). More conclusive evidence, however, was the finding of the relatively uncommon pyocine type-35 in each of 8 patients infected in an intensive care unit over a three-month period; indeed, it was in this unit and during this period that the patient I. D. who features in Table 8 (p. 101) first received treatment. These investigations also brought to light two further long-term episodes of apparent cross-infection in surgical units involving in one instance strains

of pyocine type-35 and in the other, strains of the equally uncommon pyocine type-29. The relevant details regarding these two episodes are listed in Tables 19 and 20.

It can be noted from Table 19 that during the period June 1965 until October 1966 strains of pyocine type-35 accounted for 26 (72. 2 per cent.) of the 36 cases of infection with Ps. pyocyanea encountered in unit A. Concurrently, in unit B, strains of pyocine type-29 were isolated from 23 (69. 7 per cent.) of the 33 similar cases of infection No instance of infection due to a type-29 strain of Ps. pyocyanea was found in unit A and the only instance of infection with a type-35 strain in unit B was the patient F. F. who, prior to his confinement in this unit, had been treated in unit A where presumably infection had occurred. In contrast to the first episode described in this section strains of pyocine type-3 were never isolated from patients in these units though they were certainly present in the environment of unit B (ward survey, Table 20). Similarly strains of the most common pyocine type, namely type-1, accounted for only 13. 9 per cent. and 18. 2 per cent. of the isolates from units A and B respectively.

A final interesting observation involved the examination of strains, during the same period of time, from patients in the convalescent hospital which served the two units (Table 21). The isolates from 14 such patients were, with only two exceptions, found to belong to either pyocine type-29 or 35.

Table 19. Pyocine typing of strains* of Ps. pyocyanea isolated in surgical unit A from June 1965 until November 1966.

| Patient | Date of isolation | Specimen | Pyocine type |
|---|---|---|---|
| J. H. | 2. 6.65 | Sputum | 35 |
| J. C. | 27. 6.65 | Wound | 35 |
| H. McC. | 27. 6.65 | Wound | 35 |
| P. F. | 29. 6.65 | Ear | 35 |
| A. D. | 5. 7.65 | M. S. U. | 35 |
| L. G. | 17. 7.65 | Wound | 35 |
| W. McL. | 25. 7.65 | Sputum | 1/4 |
| J. B. | 3. 8.65 | Gangrenous foot | 1/2 |
| J. R. | 4. 8.65 | C. S. U. | 5 |
| G. B. | 17. 8.65 | Wound | 35 |
| P. S. | 18. 8.65 | Perineum | 35 |
| J. S. | 23. 8.65 | C. S. U. | 35 |
| J. G. | 11. 9.65 | Wound | 35 |
| T. S. | 9.10.65 | Stool | 35 |
| G. B. | 11.10.65 | M. S. U. | 35 |
| J. B. | 9.11.65 | Wound | 1/3 |
| W. L. | 26.11.65 | Sputum | 35 |
| A. N. | 30.11.65 | Drain | 5 |
| S. F. | 22. 1.66 | Wound | 5 |
| E. L. | 15. 5.66 | Rectal swab | 35 |
| G. B. | 6. 6.66 | Wound | 35 |
| A. S. | 12. 6.66 | C. S. U. | 35 |
| J. P. | 25. 6.66 | Wound | 35 |
| J. W. | 1. 7.66 | C. S. U. | 35 |
| W. W. | 1. 7.66 | C. S. U. | 35 |
| D. C. | 3. 7.66 | Sputum | 1/4 |
| J. H. | 6. 7.66 | Bile | 35 |
| G. F. | 11. 7.66 | Perineum | 1/4 |
| A. Ni. | 12. 7.66 | Wound | ut |
| J. R. | 12. 7.66 | Wound | ut & 5 |
| J. D. | 15. 7.66 | Bladder | ut & 35 |
| T. C. | 2. 8.66 | C. S. U. | 35 |
| A. C. | 3. 8.66 | C. S. U. | 35 |
| H. B. | 6. 9.66 | C. S. U. | 35 |
| J. S. | 23. 9.66 | Sore | 35 |
| A. S. | 10.10.66 | Wound | 35 |

*Includes first isolate only from each patient

ut = untypable, i.e. no pyocine production noted.

Table 20. Pyocine typing of strains* of Ps. pyocyanea isolated in surgical unit B from May 1965 until June 1966.

| Name | Date of isolation | Specimen | Pyocine type |
|---|---|---|---|
| J.F. | 27. 5.65. | Wound | 29 |
| M.T | 28. 5.65. | C.S.U. | 29 |
| R.G. | 28. 5.65. | M.S.U. | 1/3 |
| J.F. | 23. 6.65. | M.S.U. | 29 |
| J.D. | 19. 7.65. | Ulcer | 3 |
| E.K. | 13. 8.65. | Wound | 29 |
| W.P. | 25. 8.65. | M.S.U. | 29 |
| R.T. | 20.10.65. | M.S.U. | 29 |
| F.F. | 20.10.65. | C.S.U. | 35 |
| W.L. | 23.10.65. | C.S.U. | 29 |
| W.A. | 24.10.65. | Ulcer | 11 |
| F.D. | 27.10.65. | Ulcer | 29 |
| A.J. | 18.11.65. | M.S.U. | 29 |
| P.F. | 5.12.65. | Urine | 1/3 |
| J.Fo. | 5. 1.66. | Peritoneum | uc |
| W.McK. | 6. 1.66. | M.S.U. | 29 |
| G.S. | 7. 1.66. | Wound | 29 |
| W.McL. | 5. 3.66. | Sputum | 29 |
| S.C. | 7. 3.66. | C.S.U. | 29 |
| H.C. | 29. 3.66. | Wound | 29 |
| A.W. | 18. 4.66. | Mastoid | 29 |
| R.J. | 25. 4.66. | Drain | 29 |
| A.D. | 28. 4.66. | C.S.U. | 29 |
| A.W. | 5. 5.66. | C.S.U. | 1/2 |
| G.McL. | 20. 5.66. | Trachea | 1/3 |
| L.M. | 23. 5.66. | Wound | 29 |
| A.B. | 26. 5.66. | Burn | 29 |
| A.M. | 28. 5.66. | Sputum | 29 |
| Ward survey | 28. 5.66. | Drain | 1/3 |
| | | Pump | 3 |
| | | Suction bottle | 1/3 |
| | | Sink | 3 |
| | | Basin | 1/8 |
| | | 4 Bedpan washers | 29 |
| H.S. | 1. 6.66. | Sputum | 29 |
| J.S. | 4. 6.66. | Sputum | 1/3 |
| D.C. | 6. 6.66. | C.S.U. | 29 |
| J.W. | 9. 6.66. | Sputum | 1/2 |
| J.B. | 21. 6.66. | C.S.U. | 29 |

*Includes first isolate only from each patient

uc = unclassifiable i.e. uninterpretable typing result.

Table 21. Pyocine types of strains* of <u>Ps. pyocyanea</u> isolated in a convalescent hospital serving surgical units A and B, from June 1965 until August 1966.

| Patient | Date of isolation | Specimen | Pyocine type |
|---|---|---|---|
| J. C. | 3. 6.65. | Colostomy fluid | 29 |
| J. R. † | 16. 8.65. | M. S. U. | 35 |
| J. S. † | 13. 9.65. | M. S. U. | 35 |
| L. F. | 24. 9.65. | M. S. U. | 35 |
| R. T. x | 29. 9.65. | M. S. U. | 29 |
| T. G. | 7. 10.65. | M. S. U. | 35 |
| A. G. | 11. 11.65. | Faeces | 5 |
| F. F. x | 13. 11.65. | Wound | 35 |
| M. F. | 10. 1.66. | Stool | 29 |
| R. McT. | 23. 2.66. | M. S. U | 29 |
| J. C. | 21. 3.66. | M. S. U. | 29 |
| T. D. | 10. 4.66. | Stool | 1/2 |
| A. T. | 26. 7.66. | Abscess | 35 |
| W. W. † | 24. 8.66. | M. S. U. | 35 |

*Includes first isolate only from each patient

† Also appears in Table 19
x Also appears in Table 20

# CHAPTER X

# FURTHER ASPECTS OF PYOCINE TYPING

## Another method of pyocine typing.

After initial experiments had provided encouraging evidence of the reliability of pyocine typing as an epidemiological marker of strains of *Ps. pyocyanea* a paper appeared (Darrell and Wahba, 1964) describing a not dissimilar technique in which, however, initial incubation of the potential pyocine-producing strain was carried out for 24 hr at 37°C. The culture medium (TSA) used by these workers also incorporated $10^{-5}$ *M* iodo-acetic acid., 0.1 per cent. sodium citrate and 0.1 per cent. dipotassium hydrogen phosphate. The use of such chemicals, firstly employed by them in a nutrient agar base, then in TSA resulted from the work of Wahba (1963) who considered that the areas of confluent resistant growth ('D zones') sometimes found in the centre of inhibition zones were due to the inactivation of pyocines by proteolytic enzymes produced by the pyocinogenic strain of *Ps. pyocyanea* *pari passu* with pyocine production. The chemicals used in this instance had each previously been shown by other workers to reduce the production of such enzymes. In an earlier part of this thesis (pp. 74 - 81) it has been noted that primary incubation of the potential pyocine-producing

strain at 37°C. for 24 hr was less satisfactory than the conditions finally adopted for the standardised technique. The complete lack of any unclassifiable strains experienced by Darrell and Wahba with their conditions of primary incubation suggested that their technique and medium should be investigated.

Initially experiments involved the pyocine typing, in parallel, of seven standard pyocine-producing strains (pyocine types 1, 3, 5, 9, 10, 11 and 16). In each case pyocine production by these strains was assayed under various conditions using TSA and TSBA; these media were prepared as stated on p. 55 and also with the addition of the chemical additives used by Darrell and Wahba; thus in all, four media were assessed and in each case sufficient plates were inoculated with each producer strain to allow incubation at two temperatures i.e. 32°C. and 37°C. and at each temperature for two different periods of time i.e. 14 hr and 24 hr.

Without exception the ultimate results were more clear-cut after primary incubation of the producer strain at 32°C., regardless of the duration of incubation; where primary incubation of the producer strain was undertaken at 37°C. the patterns of inhibition were very difficult to interpret. Provided that primary incubation was undertaken at 32°C. the results were equally as satisfactory on all four media.

Examples of the influence of temperature and duration of incubation of the producer strain are given in Figs. 15 and 16. Plates a represent the conditions of pyocine typing employed by Darrell and Wahba and plates d the standardised method described in this thesis (p. 90 ).

As can be seen in these figures inhibition was clear-out when incubation was at 32°C. for 14 hr (plates on the right), regardless of the medium used, whereas on plates incubated at 37°C. for 24 hr (plates on the left) inhibition was limited (Fig. 15) or entirely absent (Fig. 16).

Dr. M. T. Parker, The Central Public Health Laboratory, Colindale, London kindly provided 180 strains of Ps. pyocyanea which had been tested by the method of Darrell and Wahba. These additional strains were tested for pyocine production on TSBA and in parallel under the standardised conditions (32°C. for 14 hr) and also under the conditions advocated by Darrell and Wahba (37°C. for 24 hr). The results are summarised in Table 22.

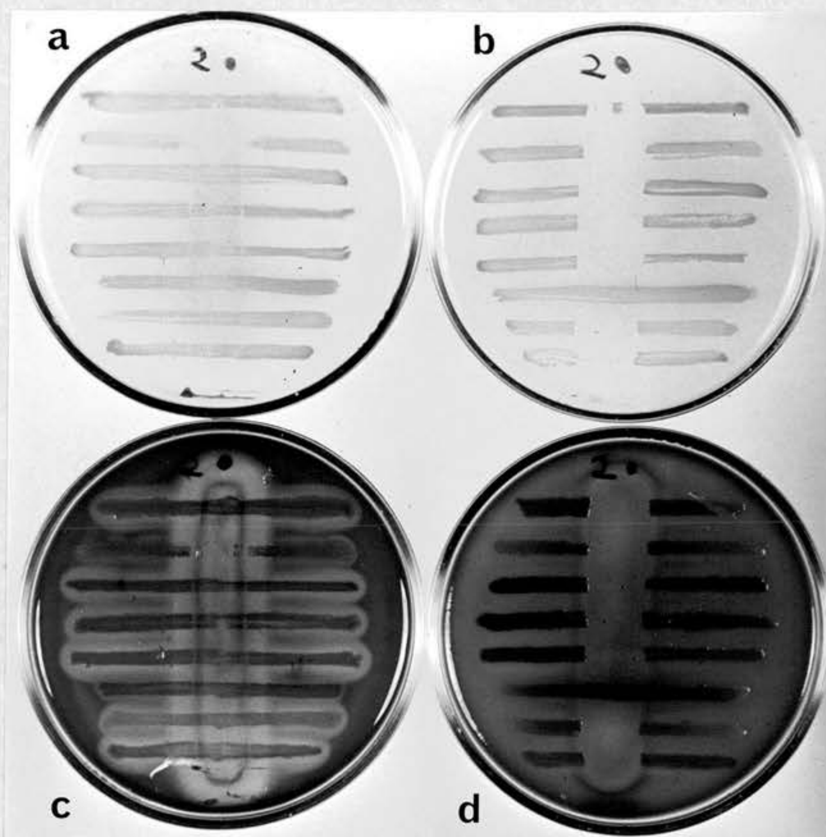



Fig. 15. Patterns of inhibition given by producer strains of pyocine type-1 tested in parallel on two plates of Wahba's medium (above) and two plates of TSBA (bottom) incubated at 37°C. for 24 hr (plates a and c) or at 32°C. for 14 hr (plates b and d) during growth of the producer strain. The producer strain was streaked vertically and the same set of 8 indicator strains was streaked horizontally across the entire width of the plate. Only indicator strain no. 2 has been inhibited in plates a and c (primary incubation at 37°C.) whereas clear-cut inhibition of all save indicator strain no. 6 is shown in plates b and d (primary incubation at 32°C.).

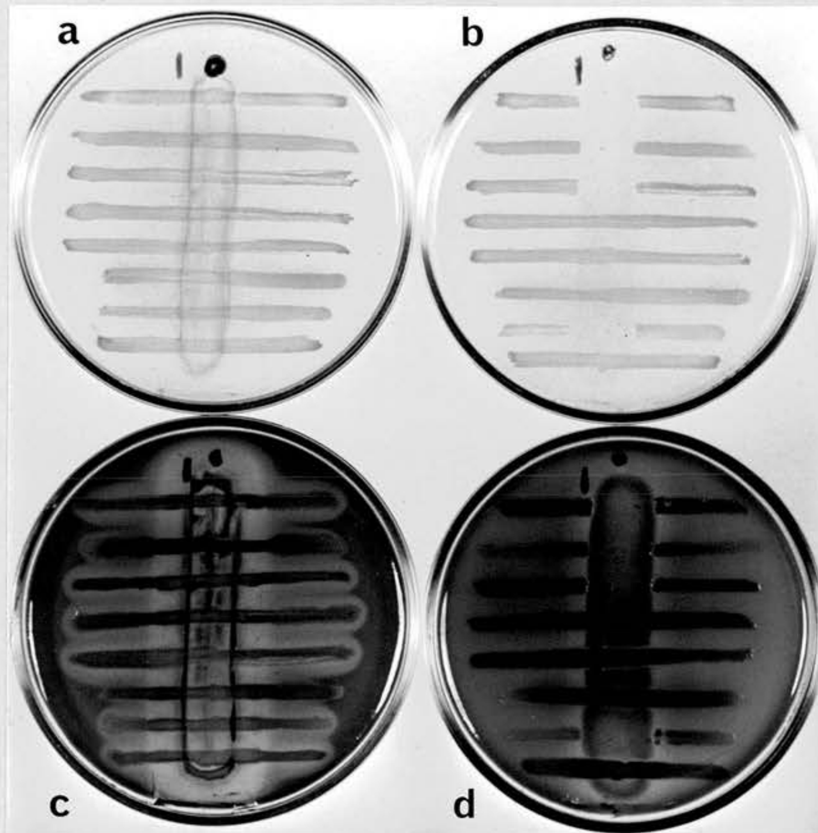



Fig. 16 Patterns of inhibition given by producer strains of pyocine type-11 tested in parallel (Fig. 15). No inhibition has occurred on plates a and c (primary incubation at 37°C.) whereas clear-cut inhibition of the same four indicator strains is demonstrated on plates b and d (primary incubation at 32°C).

Table 22. Comparative results of testing 180 strains of Ps. pyocyanea for pyocine production by the standardised method and by the method of Darrell and Wahba.

| | Standardised method | Method of Darrell and Wahba |
|---|---|---|
| No. typable* | 160 | 95+ |
| No. untypable | 20 | 85 |

* 45 strains (34 of various pyocine types and 11 untypable strains) gave identical results regardless of the method employed.

\+ 31 of the strains gave poorly interpretable patterns of inhibition as compared with the patterns resulting from tests by the standardised method.

Part of the differences in results obtained by the standardised method as compared with that of Darrell and Wahba might have been due to the fact that the indicator strains used in these methods were unrelated. Dr. M. T. Parker generously provided a set of Wahba's 12 indicator strains and 90 potential pyocine-producing strains of Ps. pyocyanea were then tested for activity against this set of 12 indicator strains.

A pair of plates was inoculated with each strain of Ps. pyocyanea one being tested for production of pyocine by primary incubation at 32°C. for 14 hr and it's partner being incubated at 37°C. for 24 hr before processing and the application to all plates

of Wahba's set of indicator strains.

Thirty-one of the 90 strains produced patterns of inhibition when primary incubation was undertaken at 32°C. for 14 hr, but showed no inhibitory activity when primary incubation was at 37°C. for 24 hr. Four strains were untypable and 11 other strains gave identical patterns of inhibition regardless of the conditions of primary incubation. Of the remaining 44 strains, 36 showed more clear-cut inhibition patterns when incubation was under the standardised conditions and the remaining 8 strains showed better inhibition when incubation was at 37°C. for 24 hr.

A further advantage of growing the producer strain at 32°C. is the almost complete absence of adhesion of the primary inoculum to the medium which occurs frequently when strains are incubated at 37°C. and renders removal of growth and eventual reading of the results both prolonged and difficult.

<u>The use of cellulose acetate strips in pyocine typing.</u>

Kohn (1966) considered that the removal of the macroscopic growth of the pyocine-producing strain was time-consuming and that the method could be simplified if inoculation of the producer strain was made on to the surface of a cellulose acetate strip placed on the surface of the medium thus allowing easy removal of the macroscopic growth along with the cellulose acetate membrane.

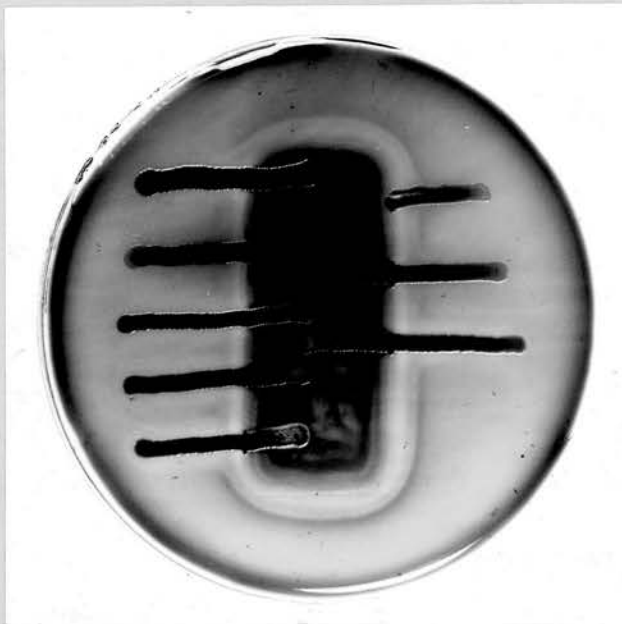
Testing this modification in parallel tests with Wahba's technique over a three-month period Kohn obtained satisfactory results but did not offer detailed evidence of these results.

The use of such strips in conjunction with the standardised pyocine typing technique did not prove so satisfactory. Initial tests with eight standard pyocine-producing strains of Ps. pyocyanea showed that not all pyocines were capable of spontaneous passage through the membrane (porosity approx. 0.5 µ). Strains of pyocine types 5, 9 and 31 produced similar patterns of inhibition with and without the strip; strains of types 1 and 3 produced no inhibition of any of the indicator strains when the strips were used and the strain of pyocine type-16 produced the appropriate pattern but with much less clear-cut inhibition. The strain of pyocine type-10, however, demonstrated a possible use of cellulose acetate namely in the characterisation of individual pyocines for although all the indicator strains were inhibited under the standard typing method only indicator strain no. 6 was inhibited when the 'strip method' was employed (Fig. 17). Similarly in the case of a type-1 strain exhibiting enhanced inhibition of indicator strains nos. 3 and 7 (Fig. 13) the use of a cellulose acetate strip resulted in a type-17 pattern i.e. inhibition of indicator strains nos. 3 and 7 only.

314 strains of Ps. pyocyanea, representing 13 pyocine types were then examined in parallel for pyocine activity i.e. by the

Fig. 17.

Inhibition pattern obtained with a strain of <u>Ps. pyocyanea</u>, of pyocine type-10, assayed under the standard conditions and exhibiting inhibition of all eight indicator strains.



Inhibition pattern obtained with the same strain as above but after inoculating directly on to cellulose acetate before typing in the normal manner.

standardised typing procedure and also with the cellulose acetate modification. Only 116 strains showed identical patterns of inhibition under both systems; the remainder exhibited aberrant patterns of inhibition when the strip method was employed.

Pyocine typing of strains of Ps. pyocyanea from various sources

In order to test the efficacy of the indicator strains against strains of Ps. pyocyanea from different geographic sources cultures were examined from several centres in England and abroad; strains from animals were also examined for pyocine production. The pyocine types of such strains were similar to those previously encountered and no increase was noted in the number of strains in the unclassifiable or untypable categories.

Pigment production and pyocinogeny in Ps. pyocyanea.

Of 940 cultures designated as Ps. pyocyanea, 133 (14.3 per cent.) failed to produce any pigment after growth on nutrient agar slopes for several weeks, at 37°C. or room temperature.

When these 133 non-pigment producing strains were inoculated on to the two pigment-enhancing media of King et al. (1952) and grown under the appropriate conditions the following results were observed:

(1) 64 strains produced pyocyanine on medium A and fluorescein on medium B.

(2) 14 strains produced fluorescein on medium B and pyocyanine together with a deep-violet pigment on medium A.

(3) 50 strains (5 per cent. of the 940 strains) exhibited fluorescein on medium B and no pigment production on medium A.

(4) 5 strains (0.5 per cent. of the 940 strains) produced no detectable pigment on either medium.

All of the latter strains belonged to accepted pyocine types and indeed, of the 70 untypable strains encountered in this series of 940 isolates no less than 64 (91.4 per cent.) produced both pyocyanine and fluorescein, the remaining six strains producing only fluorescein. This lack of pigment production may, however, be the result of instability since all six had been stored for more than six months.

21 of the 940 strains (2.5 per cent.) produced the deep-red pigment, pyorubrin, on nutrient agar. When these strains were grown on the media of King _et al_. all exhibited fluorescein production on medium B and 18 strains showed the production of pyorubrin and pyocyanine on medium A, the remainder exhibiting pyorubrin production only.

There was no correlation with a particular pyocine type in either the pyorubrin-producing strains nor in the 14 strains exhibiting the unusual violet pigment.

# CHAPTER XI

## A COMPARITIVE STUDY OF PYOCINE TYPING WITH SEROLOGICAL AND PHAGE TYPING TECHNIQUES

### Pyocine typing and serological typing.

One hundred and fifty-seven strains of Ps. pyocyanea, previously serotyped, were obtained from Dr. M. T. Parker, The Central Public Health Laboratory, Colindale, London. These strains, representing 11 serotypes (Habs' schema), were then assayed for pyocine production by the standardised typing method. The results are summarised in Table 23.

The predominance of pyocine type-1 strains (69 out of 157 strains examined) was to be expected. The distribution of subtypes of the 69 pyocine type-1 strains in comparison with their serological types is given in Table 24.

Table 23. The pyocine type distribution of 157 strains of <u>Ps. pyocyanea</u> representing 11 serotypes (Habs' schema).

| Pyocine type | Serological type | | | | | | | | | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 9 | 10 | 11 | 13 | nt | |
| 1 | | 12 | 1 | 5 | 9 | 5 | 5 | 6 | 24 | | 1 | 68 |
| 3 | | 1 | | | | 19 | 3 | | 1 | | | 24 |
| 5 | 3 | | 5 | | | | | | | 1 | | 9 |
| 10 | | | | | | 4 | | 1 | | | | 5 |
| 11 | 11 | | | | | 1 | | | | | | 12 |
| 17 | | | | | | 9 | | | | | | 9 |
| 19 | | | | | | 3 | | | | | | 3 |
| 36 | | | 3 | 1 | | | | | | | | 4 |
| uc | 1 | | 1 | | | 7 | | | | | 1 | 10 |
| ut | 5 | | | | | 4 | | | | 1 | | 10 |
| Total | 20 | 13 | 10 | 6 | 9 | 52 | 8 | 7 | 25 | 2 | 2 | 154* |

*Single examples of serotypes 6, 10 and 12 belonged to pyocine types 22, 29 and 1 respectively.

nt = serologically untypable.
ut = untypable i.e. no pyocine production noted.
uc = unclassifiable i.e. uninterpretable typing pattern.

Table 24. The distribution of subtypes of 69 pyocine type-1 strains representing 9 serotypes (Habs' schema).

| Pyocine subtype | Serological type | | | | | | | | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2 | 3 | 4 | 5 | 6 | 9 | 10 | 11 | 12 | nt | |
| 1/1 | | | | | | | | 7 | | | 7 |
| 1/2 | 1 | | 4 | | | | 1 | 14 | | | 20 |
| 1/3 | 7 | | | 6 | 3 | | 1 | | 1 | | 18 |
| 1/4 | 4 | | | 3 | 2 | | 3 | | | 1 | 13 |
| 1/6 | | | | | | 5 | 1 | 1 | | | 7 |
| 1/8 | | 1 | 1 | | | | | 2 | | | 4 |
| Total | 12 | 1 | 5 | 9 | 5 | 5 | 6 | 24 | 1 | 1 | 69 |

nt = serologically untypable

It can be noted from the above Tables that there are varying degrees of relationship between pyocine types and serological types of Ps. pyocyanea. A close linkage is apparent between strains of serological types 2 and 5 and pyocine subtypes 1/3 and 1/4. A more diffuse relationship exists in the case of serological type-6 of Ps. pyocyanea since the 52 strains of this type fell into nine categories of pyocine type. On the other hand the greatest subdivision of pyocine type or subtype obtainable by serological typing was in the case of pyocine subtype 1/4 in which the 13 strains could be allocated to 5 serological categories.

Pyocine typing and phage typing.

Twenty-five strains of Ps. pyocyanea which had been phage typed were obtained from Dr. C. S. Terry, The Bradford Institute of Technology; these were examined for pyocine production and the results are shown in Table 25.

Table 25. Pyocine type distribution of 25 strains of Ps. pyocyanea representing 10 phage types.

| Pyocine type | Phage type | | | | | Total |
|---|---|---|---|---|---|---|
| | I | VIII | IX | X | XIV | |
| 1/1 | 1 | | | | | 1 |
| 1/4 | 2 | | | 1 | | 3 |
| 1/7 | | 2 | | | | 2 |
| 3 | | 1 | | | | 1 |
| 5 | | 1 | | | | 1 |
| 11 | | 1 | 1 | | 2 | 4 |
| 18 | 1 | | | | | 1 |
| 26 | | | 1 | | | 1 |
| 29 | | 1 | 1 | | | 2 |
| uc | | 1 | | 2 | | 3 |
| ut | | 1 | | | | 1 |
| Total | 4 | 8 | 3 | 3 | 2 | 20* |

ut = untypable i.e. no pyocine production noted.
uc = unclassifiable i.e. uninterpretable typing pattern.

*Single examples of phage types V, VII, XI, XIII and CP:H2 belonged to pyocine types uc, 1/3, 1/3 ut and uc respectively.

It can be noted from this brief investigation that the relationship between phage and pyocine typing of strains of Ps. pyocyanea is slight but as in the case of similar studies between pyocine and serological typing pyocine typing gave a finer distinction of strains than did phage typing e.g. the eight strains of phage type VIII could be allocated into seven categories of pyocine type.

In view of the small number of strains obtained from Dr. Terry it was though advisable to extend these studies with locally isolated strains of Ps. pyocyanea and relevant phages.

The 12 phage preparations used locally were prepared as on p. 63, and 139 strains of Ps. pyocyanea, including replicate isolates made on different occasions from the same site in the same patient and involving various pyocine types, were tested for sensitivity to the phage set in an attempt to assess both the validity of the technique and it's relationship to pyocine typing.

Seventy-four strains (53.2 per cent.) showed sensitivity towards one or more of the phage preparations; there was variation in the degree of sensitivity e.g. from several isolated plaques to confluent zones of inhibition. When individual plaques were observed these varied in size with the result that on occasion less than 10 could create a zone of semi-confluent lysis and in other instances 100 or more could be accomodated and still retain their individuality. For this reason the approximate number of plaques

has been noted in the results of this brief investigation.

The patterns of sensitivity obtained with 19 strains, representing 10 pyocine types, when challenged with the typing phages are shown in Table 26. No clear-cut relationships emerge and in addition 33 other strains of Ps. pyocyanea, representing 17 pyocine types, including those already mentioned in this Table with the exception of pyocine types 6 and 22, did not show sensitivity towards any of the typing phages.

When 31 replicate isolates were examined from 12 patients whose initial isolate had proved to be typable with this phage set in the case of only seven patients did the replicate isolates show a similar phage pattern to that of the original isolate; however, when these replicate isolates were re-examined after storage for one week three of these cases now showed differences in the patterns obtained with the various isolates. Of the three cases originally showing a variation from the original isolate two showed further discrepancies a week later; in one case a change in eight reactions, involving 50 or more plaques, and in the other a gain of two such reactions.

Eighteen strains of Ps. pyocyanea isolated from 10 patients in a small rural hospital where sporadic outbreaks of urinary tract infection due to Ps. pyocyanea had occurred over an 18-month period and all of which had proved to be of pyocine type-8 varied

Table 26. Phage sensitivity patterns obtained with 19* strains of Ps. pyocyanea of known pyocine type.

| Pyocine type | Phages of the typing set | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | D | K | O | Q | R | S | T | U | V | W |
| 1/2 | | 50 | | | | | | c | | | | |
| 1/3 | sc | | 100 | | sc | | c | | | | | sc |
| 1/8 | | sc | | | | | | 50 | | c | | |
| 3 | | 4 | | | | | | c | | sc | | |
| 3 | 2 | sc | | 100 | 100 | | sc | | | | | |
| 3 | | | | | | | 100 | c | | 50 | | |
| 3 | | | | | | | | c | | | | |
| 6 | | | | | | | | c | | | | |
| 16 | | 100 | 2 | 6 | 50 | | 100 | sc | 15 | c | 15 | |
| 16 | sc | | 40 | sc | 100 | | 100 | 100 | | 50 | | sc |
| 16 | c | sc | | 50 | 20 | 100 | c | c | | | 100 | c |
| 16 | c | sc | | 100 | 20 | 100 | c | c | | | sc | c |
| 16 | | | | | | | | 50 | | | | |
| 10 | | | | | | | | 100 | | 10 | | |
| 22 | | 10 | | | | | | 50 | | | | |
| 25 | 6 | 3 | | | | | | 50 | | | | sc |
| 29 | | | | | | | | | | sc | 20 | |
| ut | | | | | 50 | | | sc | | | | |
| ut | 100 | | | | 100 | | | | | | | sc |

*One strain only from each patient represented.
ut = untypable i. e. no pyocine production noted.
c = confluent lysis, sc = semi-confluent lysis.
numerals = number of phage plaques.

in their sensitivity to the set of 12 typing phages.

Phage typing of the standard indicator strains.

Since it is possible that the inhibitions observed on pyocine typing plates might in some instances be due to lysogeny of the test strain rather than pyocine production the activity of the typing phages against the eight standard indicator strains was investigated. Only indicator strains nos. 2, 4 and 5 showed sensitivity to one or more of the typing phages and the patterns of sensitivity obtained, together with the pyocine types of the lysogenic strains from which the phages were originally isolated, are shown in Table 27.

Indicator strain no. 2, a pyocine type-16 strain, shows a sensitivity pattern similar to several of the type-16 strains already phage typed (Table 26). From these results, however, there seems little evidence that lysogeny of a test strain is responsible for the inhibitions observed on pyocine typing plates. Phage A, isolated from a strain which showed no activity against any of the indicator strains when examined for pyocine production produces confluent lysis of the indicator strain no. 2. One might argue that this was the result of the high concentration of the phage preparation (routine test dilution, p. 63), however, strains of pyocine type-16, responsible for phages T, U and V, under pyocine typing conditions attack indicator strains nos. 1, 3, 4, 7 and 8 and

Table 27. The activity of the 12 typing phages against 3 of the standard indicator strains used in pyocine typing.

| Indicator strain | Phages of the typing set | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | D | K | O | Q | R | S | T | U | V | W |
| | Pyocine type of strain of <u>Ps. pyocyanea</u> from which phage was originally isolated. | | | | | | | | | | | |
| | ut | 19 | 11 | uc | 11 | 3 | 1 | 3 | 16 | 16 | 16 | 1 |
| 2 | c | sc | | 100 | 20 | c | sc | | | | sc | c |
| 4 | | | | | | | | 100 | | sc | | |
| 5 | | | | | | | | | | | | c |

ut = untypable i.e. no pyocine production noted.
uc = unclassifiable i.e. uninterpretable typing pattern.
c = confluent lysis, sc = semi-confluent lysis.
numerals = number of phage plaques

yet their phages, even when concentrated, attack in one instance none of the indicator strains in another case only indicator strain no. 4; phage V attacks only indicator strain no. 2 which is completely unaffected when the lysogenic parent strain is examined for pyocine production using the standardised technique. In addition, the phages produced by the strains of pyocine type-1 attack only one or two of the indicator strains whereas the lysogenic parent strains, when tested for pyocine production, attack all the indicator strains save no. 6. The phages of the easily distinguishable pyocine types 1, 3, 16, 19 and the untypable and unclassifiable

strains all produce the same pattern on the indicator strains namely lysis of indicator strain no. 2. Finally phage S, isolated from a lysogenic strain of Ps. pyocyanea which on pyocine typing inhibited indicator strains nos. 1, 3, 4, 7 and 8, failed to show any activity against these indicators on phage typing yet produced at least 100 phage plaques against indicator strain no. 4 which is completely unaffected by such a strain when tested for pyocine production.

# CHAPTER XII

## CHARACTERISATION OF INDIVIDUAL PYOCINES

Fredericq (1948) was able to demonstrate the existence of 17 distinct colicines using such criteria as the morphology of inhibition zones, the presence and properties of resistant variants, the effect of proteinases and etc. Preliminary experiments were therefore carried out to ascertain whether these criteria might also be employed to characterise individual pyocines.

The effect of proteinases on pyocines.

1) TSBA plates were prepared incorporating 250 µg. per ml. of trypsin; the plates were used to examine 10 pyocine-producing strains of types 1, 3, 5, 8, 9, 10, 11, 16, 29 and 31 by the standardised method. In no instance did the type pattern differ from that of control tests using TSBA without trypsin.

2) Each of the above 10 pyocine-producing strains of *Ps. pyocyanea* was inoculated on to two TSBA plates and processed in the normal manner up to the point of treatment with $CHCl_3$. Then, sterile filter paper strips which had been soaked either in a solution of trypsin or pancreatin, each at a concentration of 250 µg. per ml., were placed on the surface of the medium at right angles to the original inoculum site and the strips left in position for one hour. After the strips had been removed the surface of one plate of each

pair was flooded with a 4 hr broth culture of indicator strain no. 5 and the surface of the second plate in each pair was flooded with a similar broth culture of indicator strain no. 7. After removal of excess fluid the plates were dried at room temperature and then incubated at 37°C. overnight. These two indicator strains were chosen since either or both were normally sensitive to all of the 10 producer strains.

Regardless of the pyocine-producing strain involved and in the case of both proteolytic preparations there was no evidence of inactivation of the pyocines by either trypsin or pancreatin since there was complete inhibition of the indicator strains over the entire area where the producer strain had grown.

Resistant variants.

It had been frequently noted during pyocine typing that a few colonies of resistant variants of the indicator strains appeared in all or some of the inhibition zones e.g. Fig. 10, p. 91. When these resistant variants were retested either as 4 hr broth cultures or as suspensions in sterile physiological saline, to the same density as such broth cultures, they could frequently be shown to be as sensitive to the producer strain as had been the original indicator strain.

Thirty-eight such resistant variants were picked from the inhibition zones resulting from the activity of pyocine-producing strains of types 1, 3, 5, 8, 9, 10, 11, 16, 29 and 31; with five

exceptions all of these apparently resistant variants again displayed sensitivity to the corresponding producer strain when retested. Cross-resistance was observed in the case of three variants from indicator strain no. 5 produced by the activity of pyocine types 5, 9 and 29 and similar cross-resistance in the case of two resistant variants of indicator strain no. 7 to pyocine types 9 and 31. However, each of these five variants which displayed cross-resistance, retained their sensitivity towards all of the other pyocine-producing strains.

Since the inhibition of indicator strain no. 7 appears frequently in pyocine type patterns (Table 4, p. 94) resistant variants of this indicator strain resulting from the pyocine activity of 14 strains of Ps. pyocyanea, representing a similar number of pyocine types, were inoculated directly into broth and after incubation at 37°C. for 4 hr were examined for cross-resistance to each of the pyocine-producing strains (Table 28).

In the majority of cases neither direct nor cross-resistance occurred; cross-resistance was observed among the variants derived from the activity of strains of pyocine types 9, 17 and 31. Strangely perhaps, the resistant variants obtained by the activity of types 9 and 31 also showed resistance towards the pyocine type-10 producer strain although the variant resulting from the activity of

Table 28. Resistant variants of indicator strain no. 7 to the activity of 14 different pyocine types and retested as indicator strains against all 14 pyocine-producing strains.

| Pyocine producer strain of type | Indicator strains: Resistant variants resulting from activity of pyocine-producing strains of type | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 3 | 8 | 9 | 10 | 11 | 16 | 17 | 18 | 22 | 24 | 25 | 31 | 37 |
| 1 | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 3 | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 8 | + | + | + | + | + | + | + | + | + | + | + | + | + | - |
| 9 | + | + | + | - | + | + | + | - | + | + | + | + | - | + |
| 10 | + | + | + | - | + | + | + | + | + | + | + | + | - | + |
| 11 | + | + | + | + | + | + | + | + | + | + | + | + | + | - |
| 16 | + | + | + | + | + | + | + | + | + | + | + | + | + | - |
| 17 | + | + | + | - | + | + | + | - | + | + | + | + | - | + |
| 18 | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 22 | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 24 | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 25 | + | + | + | + | + | + | + | + | + | + | + | + | + | - |
| 31 | + | + | + | - | + | + | + | - | + | + | + | + | - | + |
| 37 | + | + | + | + | + | + | + | + | + | + | + | + | + | - |

+ = inhibition, - = no inhibition.

this latter producer strain, itself failed to show resistance to any of the 14 pyocine-producing strains. In addition, the resistant variant resulting from the activity of the pyocine type 37 strain showed resistance when retested against the said strain and also when tested against producer strains of pyocine types 8, 11, 16 and 25; the variants resulting from the activity of these four types themselves were found to be sensitive to all 14 pyocine-producing strains.

The patterns of inhibition displayed by strains of pyocine types 31 and 9 involve only indicator strain no. 7 in the case of type 31 and nos. 5 and 7 in the case of strains of type 9. Since genuinely resistant variants of indicator strain no. 5 had also been obtained through the activity of pyocine-producing strains of types 5 and 9 a further investigation of these three pyocine types was pursued.

## Temperature dependence of pyocine production with certain pyocine types

Pyocine-producing strains of type-5 normally inhibit only indicator strain no. 5 when tested by the standardised method. However, producer strains of type-5 show extreme sensitivity towards the temperature necessary for pyocine production; primary incubation of such strains at 35.5° or 37°C., regardless of the duration of incubation, produces no detectable pyocine activity against any of the eight indicator strains. When strains of pyocine types 5, 9 and 31 were examined for pyocine production by the

standardised technique (32°C) and also with primary incubation temperatures of 35.5° and 37°C. the effects on indicator strains nos. 5 and 7 were as in Table 29.

Table 29. Activity of pyocine types 5, 9 and 31 against indicator strains nos. 5 and 7 after assay for pyocine production using the standardised technique and also primary incubation at 35.5° and 37°C.

| Producer strain of type | Inhibitory activity after primary incubation at | | | | | |
|---|---|---|---|---|---|---|
| | 32°C. | | 35.5°C. | | 37°C. | |
| | Indicator strain no. | | | | | |
| | 5 | 7 | 5 | 7 | 5 | 7 |
| 5 | + | - | - | - | - | - |
| 9 | + | + | - | + | - | + |
| 31 | - | + | - | + | - | + |

+ = inhibition, - = no inhibition.

It would appear from the Table that the pattern of inhibition displayed by the pyocine type-9 strain of Ps. pyocyanea, when assayed under the standardised conditions, results from the activity of two distinct pyocines one active against indicator strain no. 7 and whose production is independent of the primary incubation temperature and the other attacking indicator strain no. 5 and whose production is apparent only after primary incubation at 32°C. In addition, a similar temperature dependence can be noted in the case of the type-5 producer strain but no such effect with the producer strain of type 31.

Diffusion of pyocines and morphological appearance of inhibition zones.

Six strains, of pyocine types 1, 3, 9, 10, 17 and 31 were inoculated on to the surface of a plate containing TSA by means of separate, sterile, calibrated pipettes so that the inoculum was circular and measured 0.75 cm. in diameter. After incubation at 32°C. for 14 hr the plate was processed in the usual manner up to the stage of treatment with $CHCl_3$. Subsequently, the surface of the plate was flooded with a 4 hr broth culture of indicator strain no. 7 and after removal of excess fluid the plate was dried at room temperature and then incubated at 37°C. overnight. The diameter and morphology of each of the inhibition zones was then noted (Table 30).

Table 30. Diameter and morphological appearance of inhibition zones produced against indicator strain no. 7 by strains of six different pyocine types.

<table>
<tr><th>Strain of pyocine type</th><th>Diameter of inhibition zone (cm.)</th><th>Morphological appearance of the inhibition zone</th></tr>
<tr><td>1</td><td>1.0</td><td>No resistant variants; abrupt edge.</td></tr>
<tr><td>3</td><td>1.4</td><td>Slight central resistant growth; abrupt edge.</td></tr>
<tr><td>9</td><td>2.0</td><td>Narrow band of resistant variants immediately within inhibition zones; abrupt edge.</td></tr>
<tr><td>10</td><td>1.2</td><td>Few resistant variants; diffuse edge.</td></tr>
<tr><td>17</td><td>2.0</td><td rowspan="2">Identical with that resulting from pyocine type-9 activity</td></tr>
<tr><td>31</td><td>2.0</td></tr>
</table>

It can be noted from the Table that both the diameters and appearances of the inhibition zones are identical in the case of those pyocine types which also share resistant variants of indicator strain no. 7 i.e. types 9, 17 and 31 (p. 153). When the experiment was repeated using indicator strain no. 5 and producer strains of types 1, 3, 5, 9 and 10 the zones of inhibition produced by the strains of pyocine types 5 and 9 were identical and differed from those resulting from the activity of the other producer strains (Fig. 18).

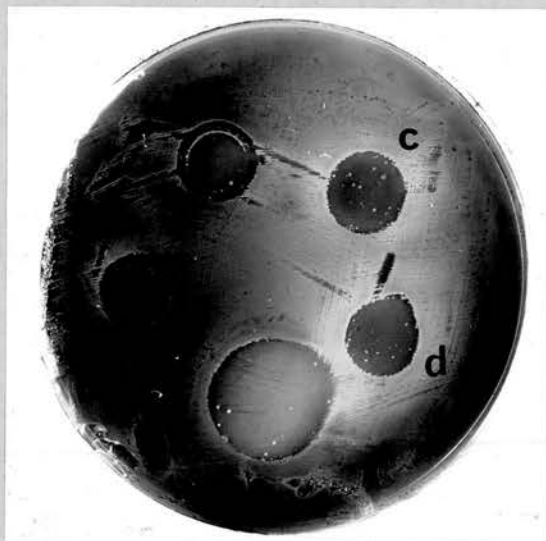



Fig. 18. Zones of inhibition, with an abrupt edge, produced against indicator strain no. 5 by the activity of strains of pyocine types a- 1; b-10; c-9; d-5 and e-3.

A standard inoculum of each producer strain was applied to the surface of the medium (TSA) and the plate incubated for 14 hr at 32°C. Macroscopic growth was then removed, the medium sterilised by exposure to $CHCl_3$ vapour and the medium flooded with a broth culture of indicator strain no. 5 (4 hr, 37°C.). Excess fluid was removed, the plate allowed to dry and then reincubated at 37°C. for 18 hr.

# CHAPTER XIII

## PRODUCTION AND EXTRACTION OF PYOCINES

1) from solid media.

Eight strains of Ps. pyocyanea, of pyocine types 1, 3, 5, 9, 10, 11, 16 and 31, were grown at 32°C. for 14 hr on TSA and after freezing and thawing (p. 59) the resultant extracts were tested for activity against the eight standard indicator strains in the following manner; 4 hr broth cultures of the latter were sown as lawns on plates of nutrient agar medium and allowed to dry at room temperature before applying the pyocine extracts with sterile pipettes. The nutrient agar plates were then incubated overnight at 37°C The patterns of inhibition which resulted were identical to those obtained with these same producer strains using the standardised technique.

Thereafter quantitative estimates of pyocine activity were obtained from freeze-thawed preparations of Ps. pyocyanea strain R. 21, the standard pyocine type-1 producer strain, after growth on either nutrient agar, 1 per cent. peptone water agar, an agar version of the medium of Kageyama and Egami (p. 56) or TSA. Regardless of the medium employed the conditions of incubation were those of the standardised method i. e. 32°C. for 14 hr. Thus four separate pyocine extracts were available and these were assayed (p. 62) against agar lawns sown with indicator strain no. 8; in the case of the pyocine

obtained from the nutrient agar the titre was only 1 in 8, whereas a titre of 1 in 32 resulted with the preparation from peptone water agar. Identical titres, 1 in 64, were found with the preparations from the modified medium of Kageyama and Egami and also from TSA.

In contrast with the titration of phage preparations (Fig. 19) in which complete lysis of the bacterial lawn is followed by semi-confluent lysis then by the appearance of individual phage plaques as the test dilution is increased, pyocine preparations (Fig. 20) showed a gradual extinction of inhibitory activity.

When 100 ml. of freeze-thawed extract of strain R21, exhibiting a titre of 1 in 64, was purified (p. 61) and thus reduced to a twentieth of the original volume the resulting product showed the same titre of activity as the original crude extract.

2) <u>in fluid medium</u>.

The spontaneous production of pyocine by strain R21, when grown in the broth medium of Kageyama and Egami at 32$^{o}$C. was assayed quantitatively after various periods of incubation (Table 31).

Prior to assay, the broth cultures were centrifuged at 3,000 r.p.m. for 15 min., the supernatant fluid transferred to a fresh, sterile container and $CHCl_3$ added to a final concentration of 5 per cent.; the contents were then thoroughly agitated for a few minutes and then allowed to stand until the $CHCl_3$ had again separated to form a bottom layer. The chloroform-sterilised,

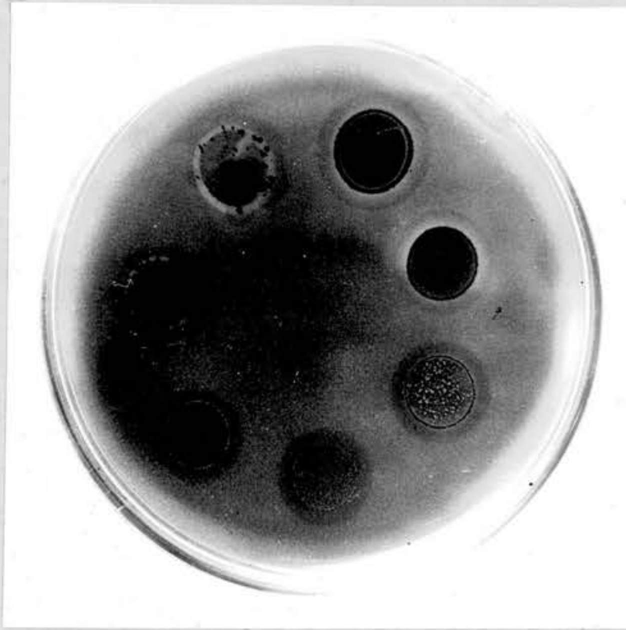


Fig. 19. Tenfold dilutions of a phage preparation from Ps. pyocyanea assayed, against indicator strain no. 4, and showing confluent, semi-confluent and discrete phage plaques as the test dilution is increased.

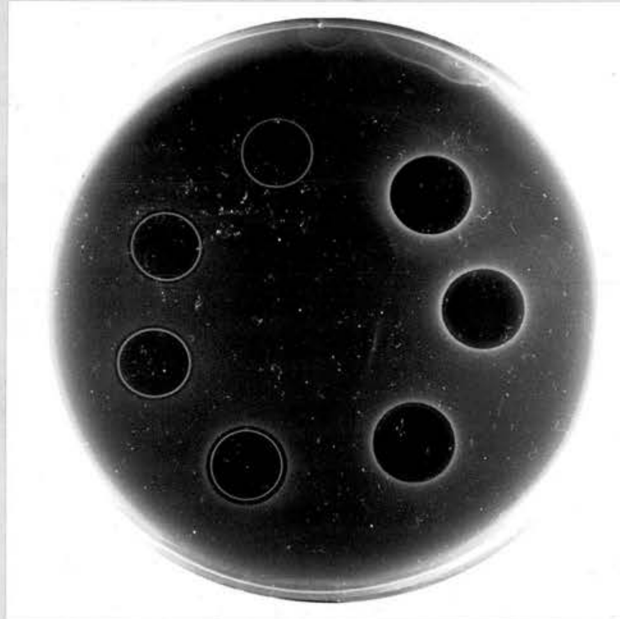


Fig. 20. Doubling dilutions of a pyocine preparation from Ps. pyocyanea assayed, against indicator strain no. 8, and showing gradual extinction of inhibitory activity.

Table 31. Spontaneous production of pyocine by Ps. pyocyanea strain R21 using the medium of Kageyama and Egami (1962).

| Period of incubation (hr) | Turbidity* | Titre of pyocine activity† |
|---|---|---|
| 4 | 1 | 1 in 4 |
| 6 | 7 | " |
| 8 | 22 | 1 in 32 |
| 10 | 36 | " |
| 12 | 43 | 1 in 64 |
| 16 | 52 | " |
| 20 | 58 | " |
| 24 | 67 | " |
| 28 | 67 | " |
| 32 | 64 | " |

*Turbidimetric measurements were made with 1 in 5 dilutions of sample.

†Against indicator strain no. 8.

crude pyocine preparation formed the supernatant which was finally removed to a fresh sterile screw-capped bottle.

It can be noted from the Table that despite the continued growth of the pyocine-producing strain, as measured turbidimetrically, pyocine activity does not increase after 12 hr incubation.

This pyocine-producing strain of Ps. pyocyanea exhibits temperature dependence when examined for pyocine production using the standardised method (Fig. 14, p. 110); a similar dependence was noted when the strain was grown in broth medium in that assay of a preparation, harvested from a culture grown at 32°C. for 12 hr, gave a titre of 1 in 64 whereas a parallel broth culture, incubated at 37°C. for 12 hr, showed no detectable pyocine activity.

3) by induction.

Pyocine preparations from strain R21, together with those from strains of pyocine types 3, 5, 9, 10, 11, 16 and 31, showed relatively low titres i. e. not greater than 1 in 256 when assayed quantitatively by the above methods.

Since it was intended to investigate the use of pyocines for prophylactic and/or therapeutic purposes an attempt was made to obtain preparations of greater potency and the following method of induction of pyocine production using mitomycin C gave vastly increased yields of pyocine. Kageyama (1964) had already described the use of this agent but did not give exact details as to the optimum conditions for induction.

Static broth cultures.

Two 100 ml. aliquots of the broth medium of Kageyama and Egami, each in a 500 ml. conical flask, were inoculated with Ps. pyocyanea strain R21 and were then incubated at 32°C. In the

case of one of these broth cultures mitomycin C was added, to a final concentration of 1 µg. per ml., after 12 hr incubation and the other broth culture was similarly treated after incubation had continued for 24 hr. In both instances incubation was then continued and samples of the cultures were taken every hour and assayed for pyocine activity. In both cultures the maximum titre of pyocine activity, 1 in 128, was reached 3 hr after the mitomycin C had been added; a control test using mitomycin C alone showed no inhibitory activity.

Rotated broth cultures.

To a 12 hr static broth culture of strain R21, grown at 32°C. in the same medium, was added mitomycin C to a concentration of 1 µg. per ml.; incubation was then continued on a drum rotating at 17 r.p.m. After 3-4 hr the culture lysed and when a cell-free extract was assayed for pyocine activity a titre of 1 in 8,000 was noted. A control sterile broth preparation, containing mitomycin C at the same concentration, showed no inhibitory activity on the indicator strain.

In Table 32 are summarised the results of further experiments using mitomycin C for the induction of pyocine production.

From Table 32 it appears that mitomycin C, at a final concentration of 1 µg. per ml., is most effective when added to 8-12 hr rotated broth cultures, incubated at 32°C.; lysis of the

Table 32. Induction of pyocine production in Ps. pyocyanea strain R21 with mitomycin C.
Rotated broth culture : 32°C.

| Mitomycin added after growth for | Duration of contact (hr) | Total period of incubation (hr) | Turbidity* | Titre of pyocine activity+ |
|---|---|---|---|---|
| 4 hr | 0 | 4 | 1 | 1 in 4 |
| " | 8 | 12 | 6 | 1 in 4,000 |
| " | 20 | 24 | 2 | 1 in 8,000 |
| 6 hr | 0 | 6 | 7 | 1 in 16 |
| " | 2 | 8 | 23 | 1 in 256 |
| " | 3 | 9 | 18 | 1 in 1,000 |
| " | 4 | 10 | 13 | 1 in 8,000 |
| " | 5 | 11 | 10 | " |
| " | 6 | 12 | 9 | " |
| " | 18 | 24 | 3 | " |
| 8 hr | 0 | 8 | 22 | 1 in 32 |
| " | 2 | 10 | 28 | 1 in 512 |
| " | 3 | 11 | 17 | 1 in 8,000 |
| " | 4 | 12 | 6 | 1 in 16,000 |
| " | 16 | 24 | 4 | " |
| 12 hr | 0 | 12 | 35 | 1 in 64 |
| " | 2 | 14 | 43 | 1 in 1,000 |
| " | 5 | 17 | 25 | 1 in 8,000 |
| " | 7 | 19 | 19 | 1 in 16,000 |
| 16 hr | 0 | 16 | 54 | 1 in 64 |
| " | 2 | 18 | 65 | 1 in 1,000 |
| " | 5 | 21 | 69 | 1 in 1,000 |
| " | 7 | 23 | 60 | 1 in 2,000 |
| 24 hr | 0 | 24 | 63 | 1 in 64 |
| " | 2 | 26 | 65 | 1 in 1,000 |
| " | 5 | 29 | 63 | 1 in 2,000 |
| " | 7 | 31 | 63 | " |

*Turbidimetric measurements were made using a 1 in 5 dilution of each sample.

+ Against indicator strain no. 8.

producer strain normally occurred 3-5 hr after adding the agent. The inoculum used for each culture (200 ml. broth) had been 0.5 ml. of an overnight static broth culture of R21 in the same medium. Further investigations revealed that even higher concentrations of pyocine e.g. of titre 1 in 500,000 could be obtained if 1) the original inoculum was increased to 2.5 ml. per 200 ml. of broth or 2) sterile broth was heavily inoculated with strain R21, previously grown on nutrient agar at 32°C. This suspension was then incubated, with rotation, for several hours at the same temperature before adding mitomycin C, to the usual concentration, and thereafter incubating for a further 3-5 hr. The synthetic medium of Kageyama and Egami was more time-consuming to prepare than Tryptone Soya broth (p. 54) and, in addition, it was found that the latter medium gave equally high yields of pyocine.

Mitomycin C was also used, as above, to obtain high-titre pyocine extracts from strains of pyocine types 3, 10 and 16; when such extracts, together with those from strain R21, were purified, however, no increase in pyocine activity was noted.

# CHAPTER XIV

## SOME CHARACTERISTICS OF PYOCINES

### The stability of pyocine preparations.

The pyocine preparations obtained by freeze-thawing agar cultures of the eight producer strains (p. 158) were stored for four months at 4°C.; after this period there was no alteration in their activity against the eight standard indicator strains.

Using a high-titre, purified pyocine preparation obtained from the producer strain R21 a quantitative estimate of it's stability was made; three aliquots of 10 ml. were stored either at 4°C., room temperature (c. 21°C.) or 37°C.

After two weeks storage, the preparations, held at 4°C. or room temperature, showed a drop in titre from 1 in 16,000 to 1 in 8,000; however, no further reduction was noted after a further 10 weeks storage under these conditions. The preparation which was held at 37°C. showed a rapid reduction in pyocine activity; after two weeks there was a drop in titre from 1 in 16,000 to 1 in 1,000 and after a further four weeks the titre had dropped to 1 in 32. After three months storage at 37°C. no pyocine activity could be detected.

It will be remembered that Ps. pyocyanea R21 does not spontaneously produce detectable pyocine when incubated at 37°C. in

either solid (Fig. 14, p. 110) or fluid media (p. 162).

When the experiment was repeated using a pyocine preparation obtained from a strain of type-3 and whose original titre was also 1 in 16,000 the results were similar to those noted with the product of strain R21; this pyocine type-3 strain had already been shown to produce pyocine spontaneously when incubated at 37°C. (Fig. 7, p. 80).

The specificity of pyocine.

The activity of particular bacteriocines is normally considered to be restricted to the same or closely related species; it was considered therefore that the widespread sensitivity of strains of Shigella and Escherichia to pyocinogenic and apyocinogenic strains of Ps. pyocyanea, which was noted in the search for suitable indicator strains, could have been due to products other than pyocines.

Proof that the inhibitory activity of strains of Ps. pyocyanea against members of other genera was of a non-specific nature and not due to pyocine activity was obtained by using a purified pyocine extract.

Forty-four strains of bacteria, representing seven genera (Table 33), were tested for sensitivity to Ps. pyocyanea R21 (pyocine type-1) by the standardised typing technique. These same 44 strains were also assayed for sensitivity to a purified pyocine preparation from strain R21 by applying this preparation, at a titre

of 1 in 4,000, to bacterial lawns of these 44 strains.

Table 33. Nature of 44 strains of bacteria examined for sensitivity to Ps. pyocyanea R21 (pyocine type-1) and to a purified pyocine preparation from this strain.

| 1. Salmonella | 2. Vibrio | 3. Staphylococcus |
|---|---|---|
| S. dublin | V. Ojana | Staph. aureus, Oxford 'H' and |
| S. tennessee | V. Wag 2 | four other strains of this species. |
| S. anatum | V. Inaba | |
| S. of group b | V. El Tor | 4. Escherichia |
| S. of group c | | |
| | | Esch. coli Row (Fredericq) and |
| | | five other strains of this species. |
| 5. Six strains of: Enterococcus, Klebsiella, Proteus and Shigella. | | |

None of the five strains of Salmonella showed sensitivity under either condition of testing. Thirty-seven of the remaining 39 strains, representing six genera, were eminently sensitive to the activity of strain R21 when tested by the standardised typing technique; the two strains which were not inhibited under these conditions belonged to the genera Klebsiella and Proteus. When these 39 strains were assayed for sensitivity to the purified pyocine preparation all were completely resistant.

A further aspect of the specificity of pyocine activity was demonstrated by the following observation; When a high-titre extract from strain R21 (a type-1 strain i. e. one which inhibits all indicator strains except no. 6) was tested against indicator strain no. 6, no

inhibition was noted despite the fact that the extract had a pyocine titre of 1 in 16,000.

Resistant variant colonies.

It has already been noted that no genuine resistant variant colonies to the pyocine from strain R21 could be isolated; it was considered, therefore, that such resistance might only arise stepwise as in the case of variants to the action of penicillin or chloramphenicol. Genuine resistant variants might result by a gradual exposure of a sensitive strain to increasing concentrations of the bacteriocine; for this purpose, a gradient plate technique (Sybalski and Byron, 1952) was used to prepare pyocine-gradient plates (pp. 64-65).

Second-step resistant variant colonies of indicator strain no. 8 were obtained (Fig. 21); three such variants were used to inoculate the same number of sterile broths and incubated at 37°C. for 4 hr. When, however, a bacterial lawn was prepared on nutrient agar with each culture and exposed to the original pyocine preparation the latter exhibited the same titre of activity against each of the variant colonies as it did against a control lawn of indicator strain no. 8, namely 1 in 8,000.

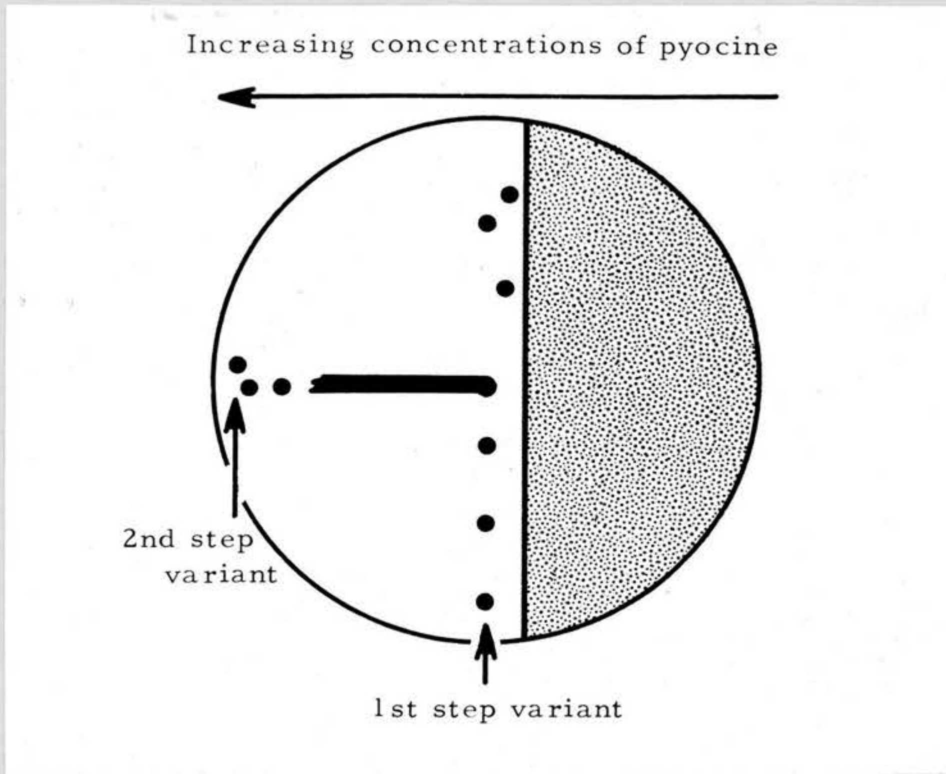



Fig. 21. Gradient plate with highest concentration of pyocine R21 on the left. After seeding with indicator strain no. 8 and incubating at 37°C. a resistant variant situated beyond the area of confluent growth, was streaked towards the left and the plate re-incubated overnight. Second-step resistant variants are obtained in the area to the left of the horizontal streak.

Strain R21 was streaked diametrically across the surface of a plate of TSBA and incubated at 32°C. for 14 hr; the plate was then treated in the standardised manner up to the point of treatment with $CHCl_3$. A broth suspension of indicator strain no. 8, turbidimetrically similar to a 4 hr broth culture, was then streaked across the original inoculum area; a similar suspension of a

second-step resistant variant, derived from this same strain, was also streaked out and the plate re-incubated at 37°C. overnight. As expected the standard indicator strain showed eminent sensitivity to the pyocine of R21, however, in the case of the variant of this same strain no inhibition was noted (Fig. 22).

Fig. 22. Strain R21 streaked diametrically across the surface of TSBA; the plate incubated at 32°C. for 14 hr and then processed in the standardised manner up to treatment with $CHCl_3$. Two broth suspensions, one of indicator strain no. 8 and the other of a second-step resistant variant, derived from this strain, were inoculated across the original inoculum area; the plate was then incubated overnight at 37°C.

To determine whether resistance was dependent upon continuous contact with pyocine the following experiment was performed: Inocula from points a and b, i. e. with organisms within

and outwith areas containing pyocine respectively, were used to prepare broth suspensions as before and tested against strain R21 as above. Irrespective of their origin, both suspensions retained resistance to the pyocine of R21.

Stability of resistant variants.

Inocula from points a and b were plated out on nutrient agar and incubated overnight at 37°C. Resistance was once again noted when several discrete colonies were examined for sensitivity to strain R21 as before. However, when a second subculture was made from such colonies and the process of testing repeated resistance was no longer maintained and all showed sensitivity to strain R21.

Toxicity of pyocine.

Liu et al.(1961) investigated the toxicity of Ps. pyocyanea and showed that the bacterial slime, followed by haemolysins and extra-cellular enzymes e.g., lecithinase, protease and lipase, are the primary agents of pathogenicity in this species; they based their observations on in-vitro tests using HeLa tissue cell cultures and in vivo by injection into animal hosts. Bacterial cell suspensions showed little toxicity and the pigment pyocyanine, though toxic towards mammalian cells when cultured in vitro, exhibited no toxic effects when injected intracutaneously in rabbits or intraperitoneally in mice.

In the course of the purification of pyocines the slime fraction is removed either by centrifugation, in the case of cell-bound slime or by precipitation with manganese ions when occurring in culture extracts; in addition, pyocyanine is removed by absorption into the $CHCl_3$ layer during sterilisation. Since Liu et al. (1961) did not investigate pyocines it was decided to determine whether or not these were toxic since it is considered that pyocines might be of therapeutic value.

Enzymatic activity in pyocine preparations.

Pyocine preparations from strain R21, obtained either from spontaneous or induced cultures in Tryptone Soya broth or the medium of Kageyama and Egami (1962), rarely contained haemolysins, lecithinases or lipases and even then only in the undiluted preparations or at a dilution of 1 in 4 at most. Gelatinase activity was exhibited by both crude and purified pyocine preparations from R21 but never exceeded a titre of 1 in 64. Preparations obtained by freeze-thawing cultures of strain R21, grown on either TSA or an agar-modification of the medium of Kageyama and Egami for 14 hr at 32°C., produced gelatinase titres of less than 1 in 64 but in addition, the TSA extracts exhibited lecithinase and lipase activity to a titre of 1 in 32 and 1 in 8 respectively.

Cytopathogenicity of pyocine preparations.

Three lines of tissue culture cells were exposed to both crude

and purified pyocine preparations from strain R21 and in concentrations from 1 in 2 to 1 in 3,200 (pp 67-68). No rounding nor dissolution of the cells occurred except in the presence of pyocine preparations obtained by freeze-thawing cultures of strain R21 after growth on TSA; the maximum final concentration of pyocine in the latter test was 1 in 12, however, cytopathogenicity occurred to a titre of 1 in 20.

Animal toxicity.

Crude and purified preparations of pyocine R21, with a titre of 1 in 8,000, were used to inject 30 adult mice by the following routes; subcutaneous, intramuscular and intraperitoneal in doses of 0.1, 0.25 and 1.0 ml. respectively. Five mice were injected in each instance. These pyocine preparations contained no detectable extracellular enzymatic activity except gelatinase at a titre of 1 in 8 (crude) and 1 in 32 (purified).

The animals were subsequently observed over a period of 14 days and showed no evidence of ill effect regardless of whether the pyocine preparation administered was in the crude or purified state and regardless of the route of administration.

When drinking water was replaced with a crude, pyocine R21 preparation (titre 1 in 8,000) five adult mice consumed 2120 ml. over a six week period without ill effect. Three of the five mice were female and these had litters of 4, 6 and 10 offspring; when rehoused

separately with their offspring, these three families of mice went on to consume a further 900, 905 and 928 ml. of the same pyocine preparation in the next seven weeks. In the same period the two remaining male mice consumed a further 870 ml. of the crude pyocine preparation. Both adults and offspring thrived during this period and no deaths occurred.

In a later episode a single adult mouse, over a period of 32 days, consumed 221 ml. of highly potent, crude pyocine from strain R21 (85 ml. of titre 1 in 250,000 and 136 ml. of titre 1 in 500,000) without ill effect.

A therapeutic use of pyocine.

Two adult mice, chronic intestinal carriers of Ps. pyocyanea, were used in a preliminary investigation into the possible use of pyocine preparations as therapeutic agents.

The mice were housed separately and transferred daily to clean autoclaved cages. One animal, the test mouse, had it's supply of drinking water replaced with a pyocine preparation (titre 1 in 8,000) and the other animal, the control mouse, which was excreting the same pyocine type of Ps. pyocyanea was maintained under identical conditions except that it had a normal supply of drinking water.

Faecal pellets were collected and cultured daily (pp. 68-69) from the inside of the cages and if necessary from paper towels placed under the open wire grid which comprised the floor of the

cage. Food pellets, drinking water, unused paper towels and fresh bedding, though tested regularly, never revealed Ps. pyocyanea. The pyocine preparation offered to the test mouse was changed daily, although a 10 ml. sample, left for several weeks in the mouse-room (temperature c. 21°C.), showed no drop in titre.

During the first treatment period of ten days duration, 50 ml. of purified, and 150 ml. of crude pyocine preparation (titre 1 in 8,000) were consumed by the test mouse. From Table 34 it can be noted that there was a substantial reduction in the number of colonies of Ps. pyocyanea isolated from the faeces of the test mouse; however, when the pyocine preparation was withdrawn and replaced with normal drinking water parasitisation of the test mouse with the strain of Ps. pyocyanea soon regained it's original level. When treatment was resumed, 14 days later, a similar reduction occurred; the control mouse, during the same period, continued to excrete large numbers of the organism except for a spontaneous lapse during the middle of the experiment. In the case of the treated mouse, however, the periods of partial clearance of Ps. pyocyanea from the faeces closely followed the oral administration of the pyocine preparation.

Table 34. Faecal excretion of Ps. pyocyanea by two mice one of which received a pyocine preparation orally for two separate periods and the other which did not receive any such preparation.

| Duration (days) | No. of colonies of Ps. pyocyanea isolated from faeces of | |
|---|---|---|
| | Test mouse | Control mouse |
| 1 | 10+ | 10+ |
| 2 | 50+ | 10+ |
| 3 | 50+ | 10+ |
| 4 | 0 | 10+ |
| 5 | 1 | 10+ |
| 6 | 1 | 10+ |
| 7 | 5 | 50+ |
| 8 | 0 | 10+ |
| 9 | 1 | 50+ |
| 10 | 0 | 50+ |
| 11 | 0 | 50+ |
| 12 | 1 | 10+ |
| 13 | 1 | 10+ |
| 14 | 3 | 6 |
| 15 | 3 | 0 |
| 16 | 10+ | 10+ |
| 17 | 10+ | 50+ |
| 18 | 50+ | 50+ |
| 19 | 10+ | 50+ |
| 20 | 50+ | 50+ |
| 21 | 50+ | 10+ |
| 22 | 50+ | 50+ |
| 23 | 10+ | 10+ |
| 24 | 10+ | 10+ |
| 25 | 50+ | 50+ |
| 26 | 10+ | 10+ |
| 27 | 6 | 50+ |
| 28 | 2 | 50+ |
| 29 | 5 | 10+ |
| 30 | 4 | 10+ |
| 31 | 6 | 50+ |

10+ = more than 10 but fewer than 50 colonies isolated.
50+ = more than 50 colonies isolated.
Figures = no. of colonies isolated.
Vertical bars indicate periods when a pyocine preparation replaced the water supply for the test mouse.

Since no other mice were available which were naturally parasitised with Ps. pyocyanea an attempt was made to artificially parasitise otherwise healthy mice so that the effect of orally administered pyocine preparations could be evaluated quantitatively.

In the first instance, six adult mice were fed a peptone water culture containing Ps. pyocyanea ($10^6$ organisms per ml.) for 48 hr; this strain was of pyocine type-16. However, five days after the peptone water culture was replaced with a normal supply of drinking water three of the six mice were no longer excreting Ps. pyocyanea and after a total of ten days only one mouse was still excreting the strain with which it had been hoped to parasitise the mice.

The same group of six mice were again fed a similar culture of the same strain of Ps. pyocyanea but on this occasion for six consecutive days. Twelve days after the bacterial culture was replaced with drinking water two of the six mice were still excreting the pyocine type-16 strain of Ps. pyocyanea. One of these two mice had it's drinking water replaced with a crude pyocine preparation which had a titre of 1 in 8,000 against this pyocine type-16 strain.

This pyocine preparation was administered for 14 days and except on the fourth, fifth and sixth days the faecal excretion of Ps. pyocyanea by the test mouse was similar to that of the control

mouse i. e. c.50 colonies per test (pp. 68-69); on these three days the number of colonies isolated was respectively 3, 0 and 2. When the drinking water supply of the test mouse was replaced with a pyocine preparation, but of a titre of 1 in 16,000, within four days there was complete eradication of the strain of Ps. pyocyanea and this state of affairs continued for a period of two weeks. However, during this latter period the control mouse also ceased to excrete the strain of Pseudomonas.

# CHAPTER XV

## A CLOSER LOOK AT PYOCINES

### Pyocines and agar slide cultures of *Ps. pyocyanea*

The activity of pyocines towards sensitive strains of *Ps. pyocyanea*, observed macroscopically with the standardised pyocine typing technique, was also observed microscopically using an agar slide-culture technique (p. 69).

The strain under test was exposed to pyocine in two ways: 1. A crude pyocine preparation (titre 1 in 64) obtained from a freeze-thawed culture of a producer strain was mixed with an equal volume of a 4 hr broth culture (37°C.) of the strain under test; a drop of this mixture was inoculated on to the agar-block. 2. A sterile block of TSA was inoculated with a pyocine-producing strain and incubated at 32°C. for 14 hr; the macroscopic growth was removed and the agar sterilised by exposure to $CHCl_3$ vapour. The surface of the agar was then seeded with the test strain (4 hr broth culture, 37°C.) and re-incubated at 37°C.

Fig. 23 (a) shows individual cells of indicator strain no. 3 immediately after inoculation on to an area which had previously supported the growth of producer strain R21, a pyocine type-1 strain. After 4 hr incubation at 37°C. (Fig. 23, b) these cells failed to multiply and indeed it is difficult to discern the original cells: these

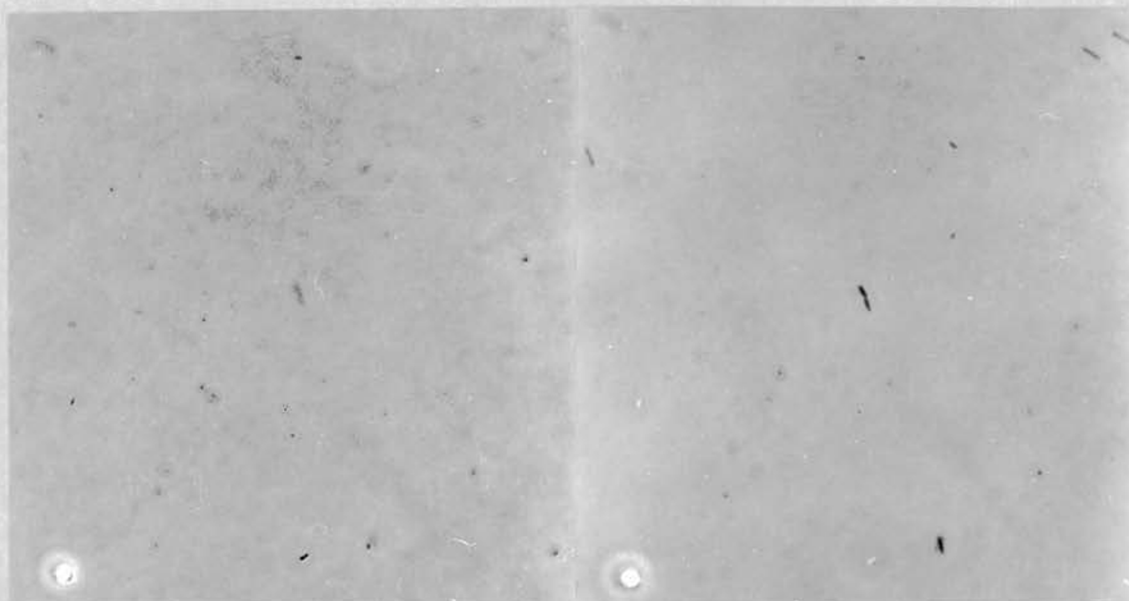


Plate a 0 hr    Plate b 4 hr

Fig. 23. Plates a and b show the microscopic appearances of Ps. pyocyanea (indicator strain no. 3) on agar slide-cultures which had previously supported the growth of a strain of pseudomonas which produced pyocine activity against strain no. 3. Plates c and d (below) show the equivalent appearances of the same indicator strain on slide cultures devoid of pyocine. Plates a and c were photographed immediately after inoculation of the agar with Ps. pyocyanea (indicator strain no. 3) and plates b and d after the media had been incubated at 37°C. for 4 hr. Phase-contrast X 1,200.

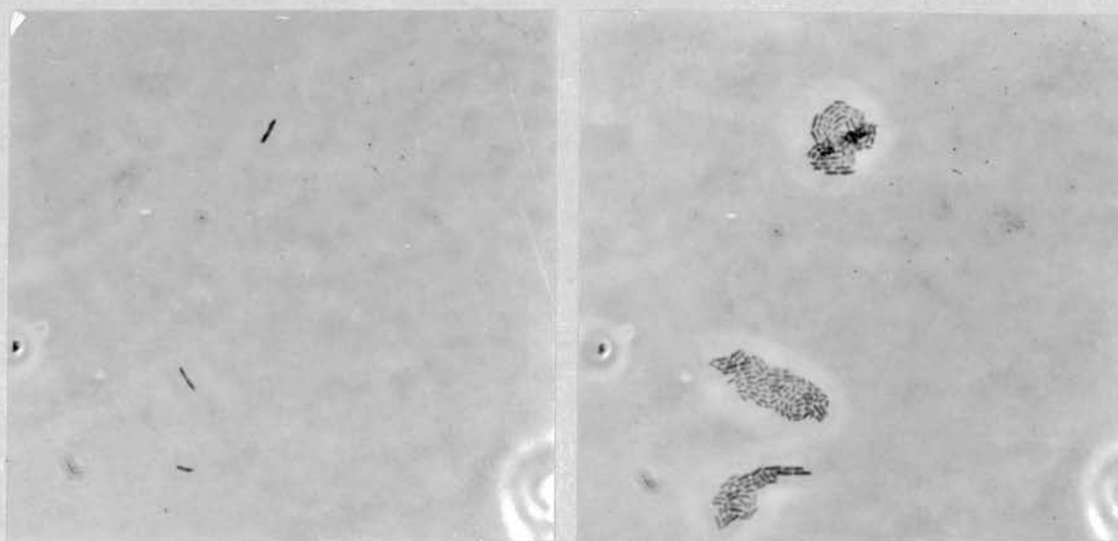


Plate c 0 hr    Plate d 4 hr

appear as faint ghost-like images. Plates c and d show the clear-cut growth of similar cells on TSA which had not previously supported the growth of R21. An examination of indicator strains 1, 2, 4, 5, 7 and 8 which are all inhibited by R21, using the standardised technique, also revealed inhibition when examined microscopically. Indicator strain no. 6 which is not inhibited by the type-1 strain (R21) in the standardised typing method was also not inhibited when examined by the agar slide-culture technique.

These tests were repeated with several strains and in all instances an indicator strain failed to grow in the presence of a pyocine to which it was sensitive, as judged by the standardised typing method and similarly when the indicator strain was challenged with a pyocine to which it was resistant, by the macroscopic method, that strain grew in an uninhibited fashion (Fig. 24).

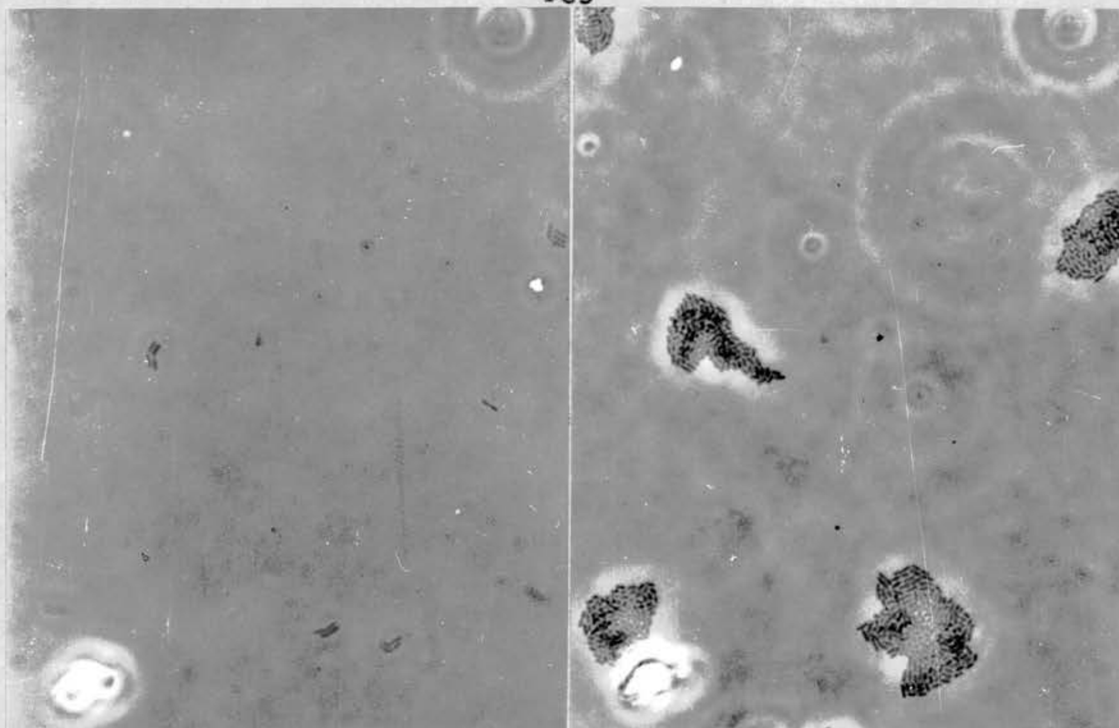


Plate a 0 hr Plate b 4 hr

Fig. 24. Plates showing the microscopic growth of Ps. pyocyanea (indicator strain no. 3) on agar slide-cultures which had previously supported the growth of a strain of pseudomonas (type-9) which produced pyocine inactive against strain no. 3. Plate a was photographed immediately after inoculation of the agar with strain 3 and plate b after the medium had been incubated at 37°C. for 4 hr. Phase-contrast X 1,200.

## Pyocines and phages of Ps. pyocyanea.

The close similarity of bacteriocines and phages is widely recognised and a few properties shown by these are listed in Table 35.

An important difference between the two agents, however, is that bacteriocines cannot replicate themselves on sensitive strains. Despite numerous attempts, with various pyocines, propagation of pyocine activity was never achieved. When an agar-block from

Table 35 Properties common to both bacteriocines and phages.

| |
|---|
| 1. Limited spectrum of activity, normally restricted towards strains of the same, or closely related, species. |
| 2. Lethality follows adsorption on to receptor sites situated on the cell wall of a sensitive strain. |
| 3. Both agents appear spontaneously in cultures of bacteriocinogenic or lysogenic strains following lysis of a small percentage of cells. |
| 4. Procedures inducing bacteriocine production e.g. irradiation with ultraviolet light, also induce the release of phage and vice-versa. |

within the zone of inhibition created by pyocine activity was cut out from the surrounding medium, added to a 4 hr culture of a known sensitive strain and incubated overnight no propagation of the bacteriocine could be demonstrated. When a single phage plaque, however, was cut from the agar by means of a Pasteur pipette and added to a similar broth culture subsequent incubation at 37°C. revealed a millionfold increase in the concentration of phage particles. As shown in Fig. 19, titration of a phage preparation against a sensitive strain results in zones of confluent lysis followed by the appearance of discrete phage plaques as the test dilution is increased; at intermediate dilutions large or numerous plaques merge to form zones of semiconfluent lysis. On the contrary, a pyocine preparation (Fig. 20) shows a gradual diminution of activity against the sensitive

strain as the test dilution increases.

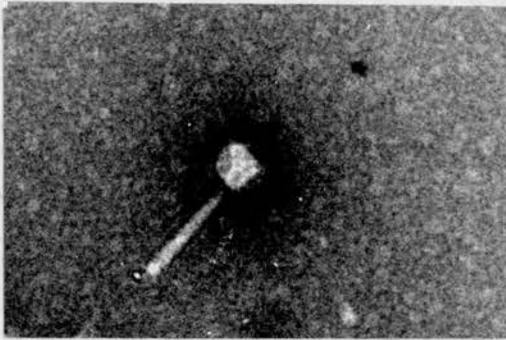
A great deal is known regarding the nature and ultrastructure of phages, including those associated with the genus Pseudomonas (Bradley, 1963). As with phages from other genera various morphological forms exist e.g. the contractile variety consists of a geometrically constructed head which acts as a reservoir of deoxyribonucleic acid (DNA), a sheath consisting of striated or helically arranged subunits and which on contraction reveals a narrow, hollow tailpiece; several thin fibres can sometimes be seen, attached to the tail tip. Some of these features can be seen in Fig. 25.

Phages, therefore, are biological units of sophisticated morphological structure. Bacteriocines, on the contrary, have long been regarded as chemical substances, devoid of any genetic continuity *per se* and few reports exist regarding their ultrastructure.

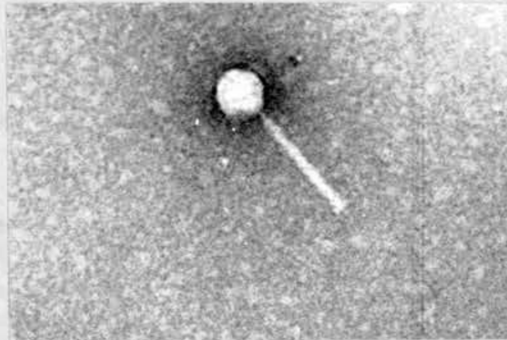
Therefore studies of pyocine preparations were made with an electron microscope (p. 71).

Fig. 26 shows the appearance of a crude pyocine preparation from a type-10 strain of *Ps. pyocyanea* after negative-staining with phosphotungstic acid. The field is filled with a myriad of microscopic particles of two types, one a bullet-like component resembling an extended phage sheath and the other similar to a headless phage with the sheath contracted.

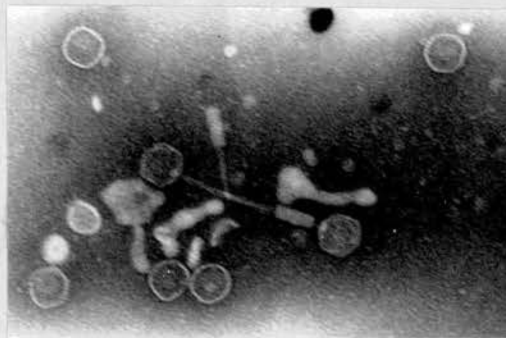
a



b



c



d

Fig. 25. Electron micrographs of a contractile phage of Ps. pyocyanea stained with phosphotungstic acid. Intact phage may be seen with tail sheaths contracted (a) and extended (b and c); phage heads and a headless tailpiece can also be seen (d). X 100, 000.

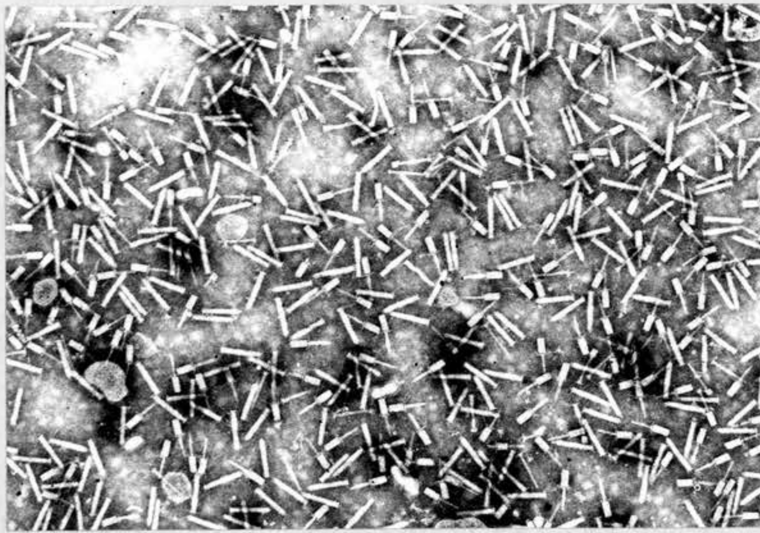


Fig. 26. Electron micrograph of crude pyocine preparation from a type-10 strain of Ps. pyocyanea (no. 3823) stained with phosphotungstic acid. Numerous contracted and extended rod-like particles are visible, X 50,000.

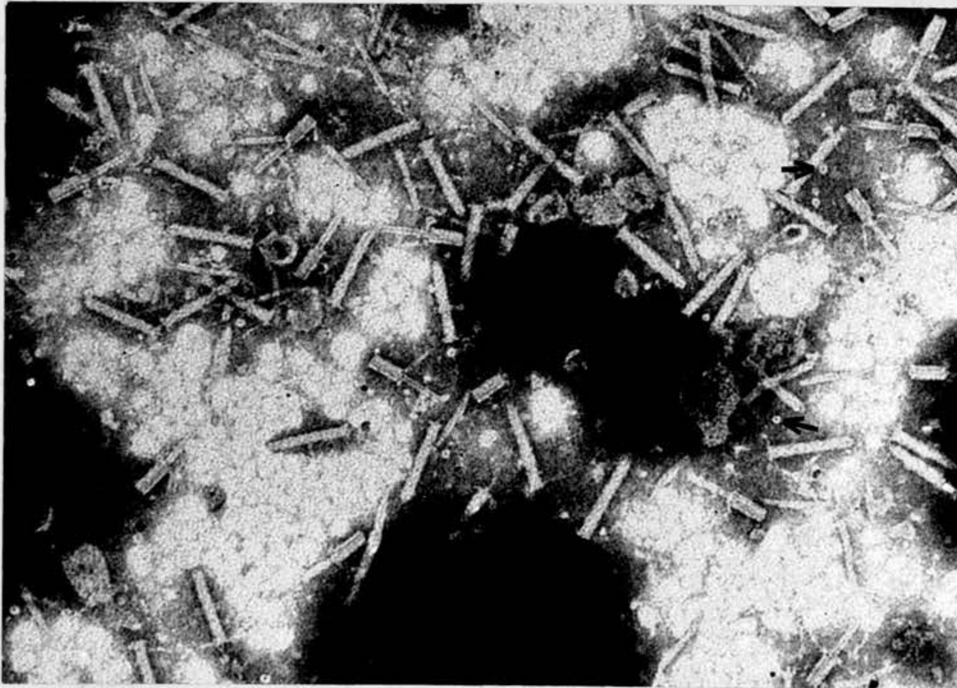


Fig. 27. Electron micrograph of crude pyocine preparation from Ps. pyocyanea strain 112, a type-10 strain, stained with phosphotungstic acid. The tail pieces can be seen to possess an inner core and end-on projections of these are suggested by the ringlets (arrowed). X 100,000.

Examination of both crude and purified pyocine preparations, from nine strains of Ps. pyocyanea, of pyocine types 1, 3, 9, 10 and 17, revealed similar structures in every case; in no instance was there evidence of intact phage units or phage heads.

At a higher magnification (Figs. 27 and 28) it can be noted that the bullet-like particles appear to show a helical substructure and that the other unit consists of two hollow cylinders in the form of a sheath and tailpiece. The majority of particles measured approximately 100 mμ x 10 mμ in the case of the bullet-like structures, and the tailpieces, measuring 100 mμ x 7 mμ, were contained in sheaths 40 mμ x 15 mμ and enclosed a central core of diameter to that of a tailpiece. Minute ringlets are also discernible suggesting end-on projections of the hollow cylinders.

Fig. 29 shows a unit from a pyocine preparation obtained from a type-9 strain of Ps. pyocyanea which appears to be on the point of disengaging, the partial separation of the narrow tailpiece revealing the hollow nature of the surrounding sheath.

Are such particles responsible for pyocine activity ?

The activity of pyocine towards sensitive strains of Ps. pyocyanea has already been demonstrated both macroscopically and with the light microscope;using the electron microscope additional features of this activity may be noted.

One ml. of crude pyocine preparation from R21, a type-1

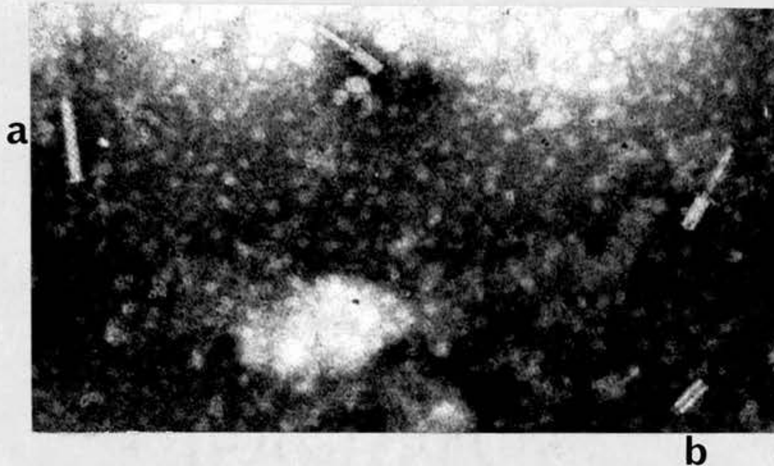



Fig. 28. Electron micrograph of crude pyocine preparation from a type-3 strain of Ps. pyocyanea stained with phosphotungstic acid. The extended particle (a) appears to have both flattened and tapered extremities; a helical substructure is also suggested. An empty sheath (b) is also visible. X 100, 000.

Fig. 29. Electron micrograph of crude pyocine preparation from a type-9 strain of Ps. pyocyanea stained with phosphotungstic acid. The hollow nature of the sheath is revealed by the semi-detached tailpiece. X 160, 000.

strain, and exhibiting a titre of 1 in 8,000 was mixed with 7 ml. of a peptone water culture of indicator strain no. 8 (4 hr at 37°C.). After incubation at 37°C. for two hours the contents were fixed in 0.25 per cent. formaldehyde and examined in an electron microscope (p. 71).

In Figs. 30-32 it can be seen that attachment of the particles to a bacterial cell occurs and the entire surface of a sensitive cell is smothered with the rod-like units. In the vicinity of the cell, one can also observe a number of empty sheaths and detached tailpieces. In Figs. 30 and 31 the surface of the bacterial cell is grossly irregular as if disruption of the cell was imminent and indeed, when cells were examined after 18 hr. contact with the pyocine preparation, more than 90 per cent. were found to be completely disrupted.

When the system was re-examined after contact at 0°C. attachment was observed as before; however, at this temperature little or no lysis occurred.

When this same pyocine preparation was added to indicator strain no. 6, resistant to R21 when tested macroscopically or by the agar slide technique, neither attachment nor lysis occurred even after 24 hr incubation at 37°C. This strain, however, did not lack pyocine receptors sites for when it was exposed to a pyocine preparation (titre 1 in 8,000) from a type-10 strain of <u>Ps. pyocyanea</u>

and to which it was macroscopically sensitive, both attachment and lysis resulted. When this same pyocine preparation was left in contact with indicator strain no. 8 for two hours at 37°C. (Fig. 32) the attachment of the pyocine particles, via the narrow tailpieces, sometimes assumed a rosette-like formation surrounding spherical detachments from the cell surface. In this case there is less evidence of hollow sheaths or discarded tailpieces; this cell was perhaps observed at an early stage of parasitisation.

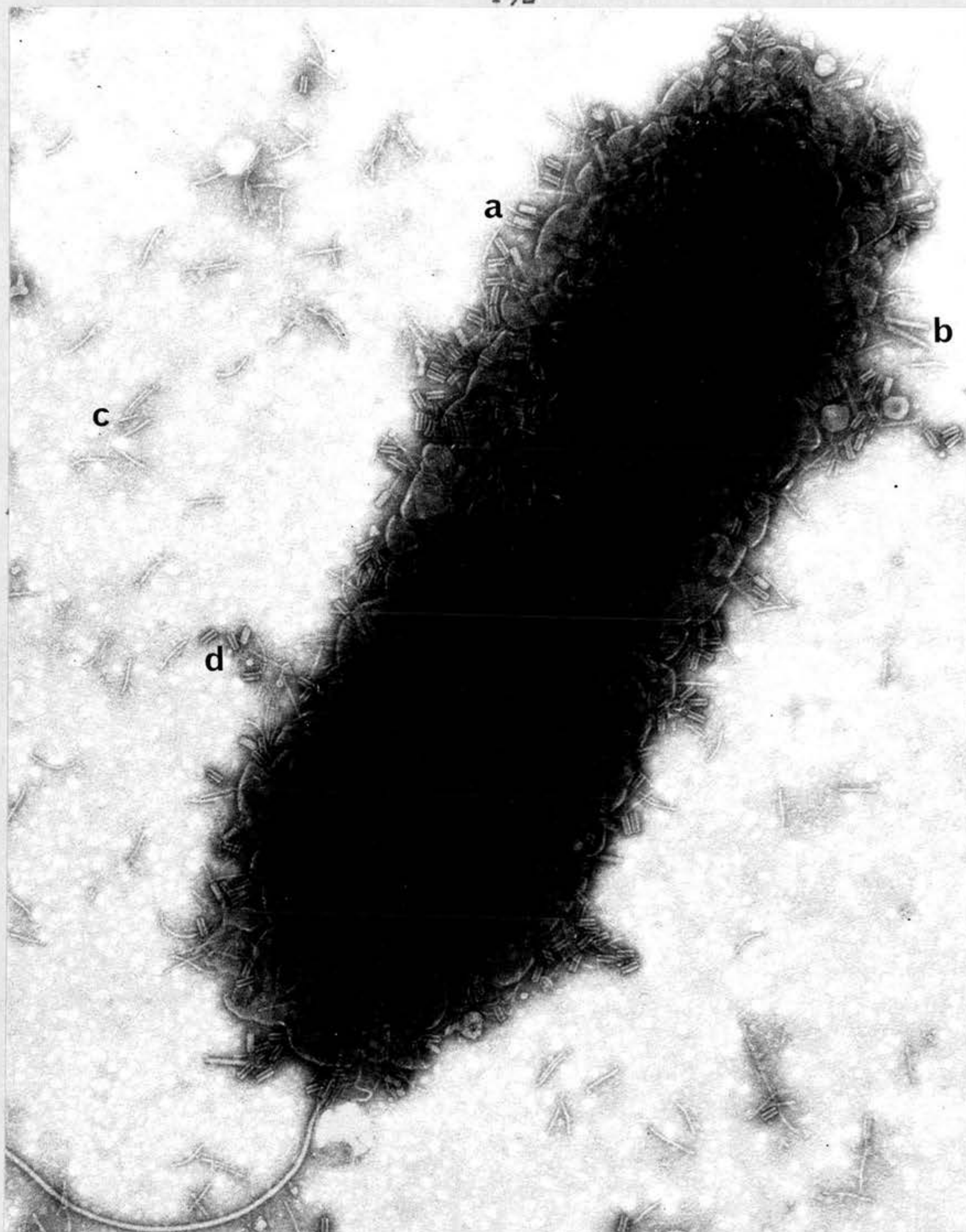



Fig. 30. Electron micrograph of Ps. pyocyanea, indicator strain no. 8, after 2 hr contact (37°C.) with crude pyocine R21 preparation; stained with phosphotungstic acid. The cell surface appears convoluted and completely surrounded by pyocine particles. Intact, contracted units are visible (a) attached to the cell surface and several extended particles (b) can be seen close to the cell surface. Many discarded tailpieces (c) and empty sheaths (d) are present in in the background. X 80,000.

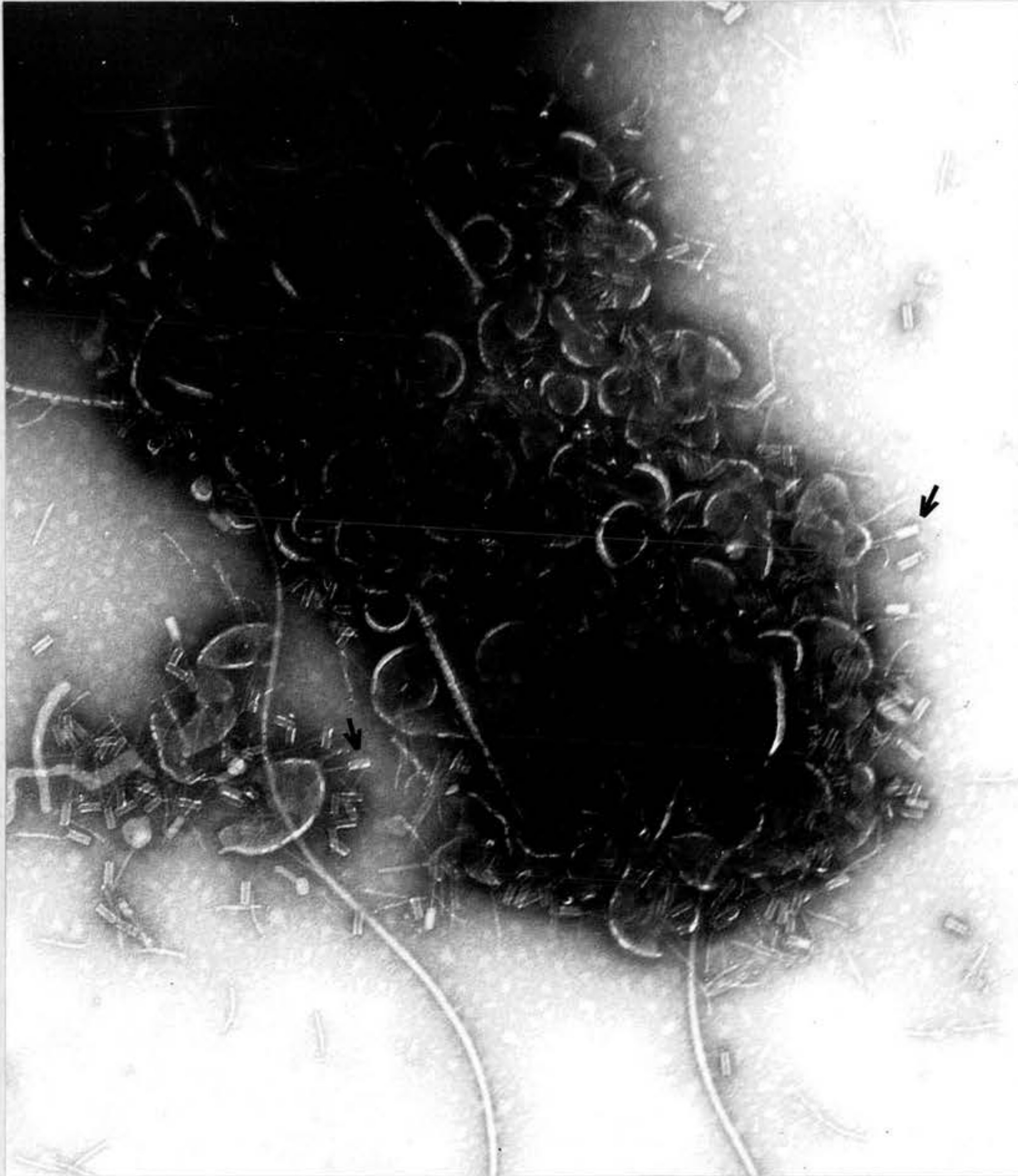


Fig. 31. Electron micrograph of Ps. pyocyanea, indicator strain no. 8, after 2 hr contact (37°C) with crude pyocine R21 preparation; stained with phosphotungstic acid. The cellular debris is surrounded with empty sheaths and discarded tailpieces; intact units (arrowed) can also be seen attached to the cellular material. X 80,000.

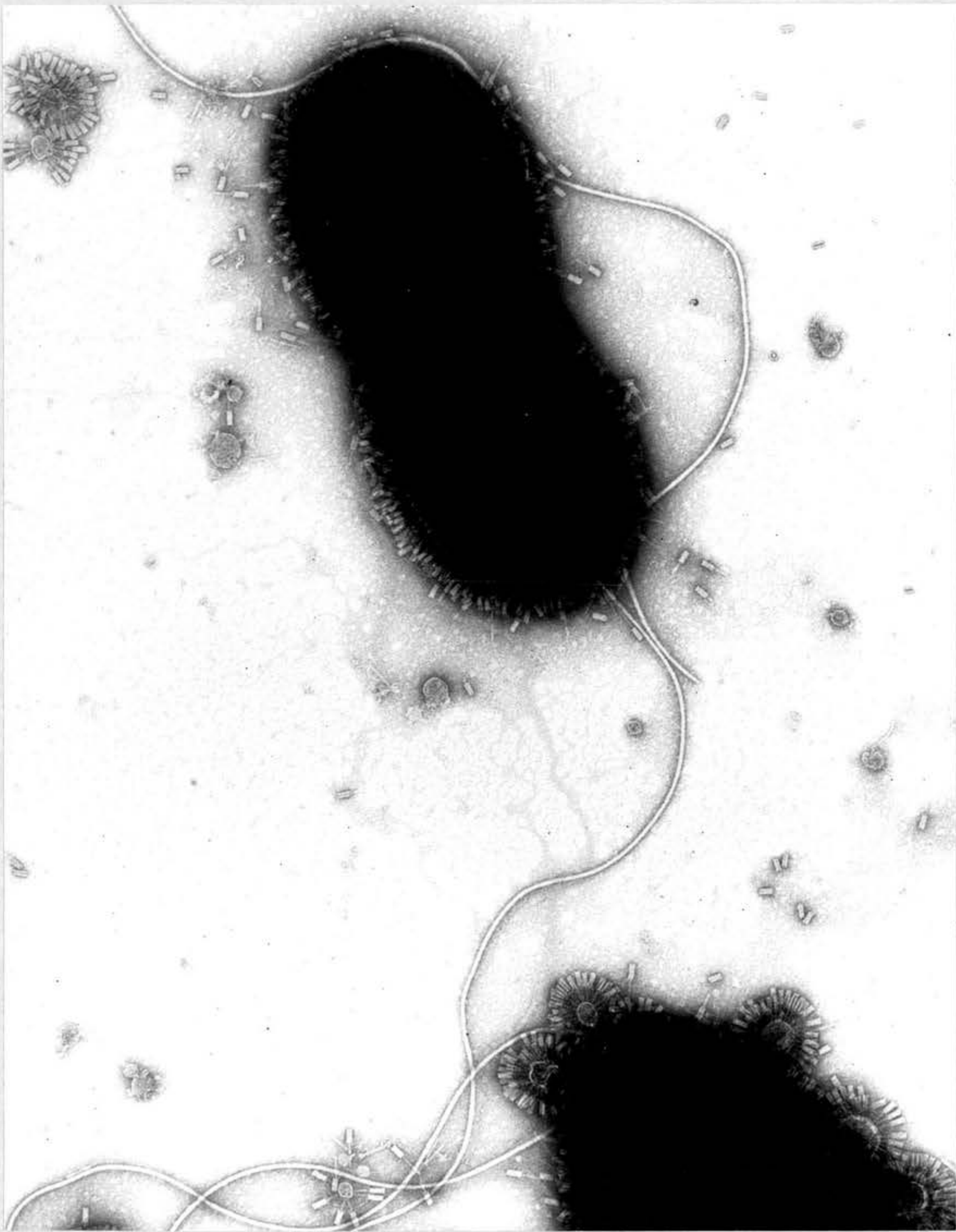


Fig. 32. Electron micrograph of Ps. pyocyanea, indicator strain no. 8, after 2 hr contact (37°C.) with a crude pyocine preparation from strain 430, a type-10 strain; stained with phosphotungstic acid. Many intact, contracted units can be seen attached to the cells, some forming rosettes around possible cellular material; empty sheaths, tailpieces and intact units are visible in the background. X 50,000.

# DISCUSSION

# CHAPTER XVI

## DISCUSSION

Primarily, the work reported here was undertaken in the hope that pyocine production by *Pseudomonas pyocyanea* would allow characterisation of such strains for epidemiological purposes; in addition, more academic investigations, were made into the properties of pyocines.

### The typing of Ps. pyocyanea by pyocine production.

It is essential that any laboratory method used to mark a microbial genus or species for epidemiological purposes should allow the recognition of a reasonable number of types or subtypes within that genus or species and similarly that the technique should give replicable results which are epidemiologically valid.

The standardised technique for the typing of *Ps. pyocyanea* by pyocine production, reported here, has these properties and in addition is simple to perform and requires neither sophisticated materials or apparatus; it can be used in the most humble of bacteriological laboratories. A large proportion of strains can be typed by the method (88.5 per cent. in a series of 5690 strains) which at present allows the recognition of 44 pyocine types (37 primary types and 8 subtypes of the common type-1 variety).

Retesting of strains after many months of storage has shown pyocinogeny to be a stable characteristic in vitro and evidence of the in-vivo stability of pyocine production has also been obtained by noting: 1. Whether replicate isolates obtained on different days from the same site in a patient were of the same, or of a different pyocine type, from the strain originally obtained from that patient. 2. The uniformity of pyocine type in strains from the same epidemic focus.

The results obtained during these investigations demonstrated the validity of the standardised pyocine typing technique and revealed that the method is more satisfactory than either serological or phage typing techniques.

The need for a satisfactory method of typing strains of Ps. pyocyanea has been reaffirmed by the many requests for the indicator strains following publication of the standardised technique (Gillies and Govan, 1966). The method is now used in at least 70 centres, situated in 21 countries; the Communicable Disease Center, Atlanta, Georgia acts as a satellite centre for the distribution of indicator strains as does the Microbiology Department in the University of Alberta.

It is hoped that such widespread use of the same indicator strains will enable world-wide correlation of the incidence of various pyocine types and facilitate international communication regarding

the identity of the various pyocines. Table 36 gives a few examples of the pyocine type incidence of strains of Ps. pyocyanea when the latter are examined by other workers using the standardised technique.

It was interesting to note that all three strains of pyocine type-9, examined in Toronto, originated from the same hospital; a similar restricted habitat for this somewhat rare type was also observed in strains isolated in the present series. From Table 36 it can be noted that the predominance of strains of types 1 and 3 is not restricted to this country but is probably a world-wide characteristic.

The sensitivity of the technique is greatly enhanced by the ability to subdivide strains of pyocine type-1 which allows the allocation of such strains into eight further types. In Uganda, Phillips et al (1968) found that 16 out of 21 strains of Ps. pyocyanea, isolated during a therapeutic trial, belonged to pyocine type-1. Use of the five additional indicator strains, however, revealed the presence of four distinct subtypes namely 1/2, 1/3, 1/4, and 1/8. Investigations presently being carried out locally indicate that a similar subdivision of pyocine type-3 strains might also be possible.

Table 36. Distribution of pyocine types in 446 strains of <u>Ps. pyocyanea</u> isolated and typed in four geographical centres.

| Pyocine type | No. of strains belonging to stated pyocine type in | | | | Total |
|---|---|---|---|---|---|
| | Toronto, Canada | Natal, South Africa | Dunedin, New Zealand | Sheffield, England | |
| 1 | 80 | 28 | 15 | 35 | 158 |
| 3 | 37 | 11 | 9 | 16 | 73 |
| 10 | 23 | 3 | 4 | 3 | 33 |
| 5 | 13 | 4 | 1 | - | 18 |
| 6 | - | 7 | 11 | - | 18 |
| 31 | 6 | 4 | 1 | - | 11 |
| 4 | - | 7 | - | 1 | 8 |
| 22 | 7 | - | - | - | 7 |
| 35 | 5 | - | 1 | - | 6 |
| 8 | 4 | - | - | - | 4 |
| 17 | 1 | - | 3 | - | 4 |
| 9 | 3 | - | - | - | 3 |
| 15 | 3 | - | - | - | 3 |
| 20 | 2 | - | - | - | 2 |
| 24 | 2 | - | - | - | 2 |
| 29 | 2 | - | - | - | 2 |
| uc | 3 | 18 | [illegible] | - | 21 |
| ut | 11 | 10 | 33 | - | 54 |
| Other types | 9 | 3 | 4 | 3 | 19 |
| Total | 211 | 95 | 82 | 58 | 446 |

One strain from each of the following pyocine types: Toronto - 13, 14, 16, 19, 21, 28, 30, 33 and 34; Natal - 2, 30 and 33; Dunedin - 13, 23, 25 and 26; Sheffield,- 11, 16 and 33.

uc - uninterpretable typing pattern or pattern not listed in Table 4.
ut - no detectable pyocine production.

The in-vitro stability of pyocine production, shown by strains after many months in storage, greatly facilitates the use of pyocine typing in laboratories where, either because of pressure of work or because strains are rarely encountered, typing is performed only at infrequent intervals. For this reason alone the technique must compare favourably with phage typing since Pavlatou and Hassikou-Kaklamani (1962) recommended that for reliable results phage typing must be carried out before the administration of antibiotics and immediately after isolation of the organism.

In an examination of over 6,000 strains of Ps. pyocyanea, in this country, ten pyocine types were commonly encountered namely; types 1, 3, 5, 9, 10, 11, 16, 27, 29 and 35. The percentage of typable strains, as shown in Table 9, remains remarkably constant for each centre from which strains were received, being 87, 86.8 and 86.7 per cent. in the case of Hospitals 1 and 2 and the University Diagnostic Laboratory respectively. The higher figure cited on page 93 is due to the even higher incidence of typable strains from centres not included in this table. This high proportion of strains, capable of characterisation by means of their pyocine production, confirms the findings of Holloway (1960) regarding the frequency of pyocinogeny in Ps. pyocyanea.

The influence of temperature on pyocine production.

From the many experiments carried out during the development of the standardised typing technique and when the method was compared with that of Darrell and Wahba (1964) it was apparent that the most important single factor in the production of pyocine was the temperature at which the potential producer strain was incubated; the culture medium and the period of incubation, though of some consequence, were of minor importance. Such temperature dependence is not unique to pyocinogeny since similar findings have been described in the typing of strains of Shigella sonnei by means of colicine production (Abbott and Shannon, 1958). Though such dependence has perhaps been offset in many investigations of bacteriocines by the use of induction e.g. with mitomycin C, it would seem pertinent for future investigators to bear in mind the possible influence of temperature. Many elaborate forms of media and cultural conditions are described in the literature but incubation during production is invariably at 37°C. regardless of the bacteriocine investigated.

Nevertheless pyocinogeny in Ps. pyocyanea is not always a temperature-dependent phenomenon; strains of pyocine types 3 and 31, for example, produce satisfactory and identical inhibition patterns after primary incubation at either 32° or 37° C., those of type 11 continue to produce an appropriate type pattern at 37°C. but the quality of inhibition is very poor.

It might be considered, because of the instability shown by pyocine preparations when held at 37°C. (p. 166), that the failure to detect pyocine production after incubation at 37°C. is due to the inactivation of pyocine already present. This hypothesis was disproven by incubating producer strains under the standardised conditions and, after preliminary processing up to the stage of sterilising with $CHCl_3$ vapour, the plates were held at 37°C. for 24 hr before applying the set of indicator strains. In all such experiments the normal pattern of inhibition resulted regardless of whether the producer strain was normally active only at 32°C. or also at 37°C.

Wahba (1963) suggested that the presence of resistant growth in areas where inhibition was expected e.g. 'D zones' was due to the inactivation of pyocine by proteolytic substances produced _pari-passus_ with the bacteriocine. Thus he used a culture medium incorporating a triad of chemicals each of which had previously been determined as an inhibitor of proteolytic enzyme production. It has been clearly demonstrated (Figs. 15 and 16) that in parallel experiments using Wahba's medium and TSBA the main determinants in giving a clear pattern of inhibition are the temperature at which the producer strain is incubated and, to a lesser extent, the duration of the incubation; Wahba's special medium has no

advantage over TSBA when the standardised typing technique is employed. It is still possible, however, that the lack or diminution of inhibition, when the producer strain is incubated at 37°C. is due to a 'phantom', destructive agent which functions or is only produced at 37°C.

Munier and Cohen (1959) noted that the incorporation of amino acid analogues e.g. p-fluorophenylalanine (FPA) into enzymatic protein provoked the synthesis of abnormal proteins which were biologically inactive; similarly Kang and Markovitz (1967) used FPA to induce capsular polysaccharide production, a phenotypic temperature-sensitive system, in Escherichia coli K12. A strain (AB259) which possessed the regulator genes R1 and R2 was non-mucoid when grown on minimal agar plates at 37°C. or 23°C. A wild type strain, however, possessing the same regulator genes produced non-mucoid colonies when grown at 37°C. but mucoid growth at 23°C. Growth of strain AB259 in minimal medium containing $10^{-5}$ to $10^{-6}$ M FPA resulted in depression of capsular polysaccharide synthesis and the emergence of mucoid colonies at 37°C; the simultaneous presence of FPA together with the natural amino acid, phenylalanine, led to the re-emergence of non-mucoid colonies at 37°C.

A similar enzymatic repressor system might be responsible for the effect of temperature on pyocine production observed in many

strains of Ps. pyocyanea. If enzymatic, such a repressor system would probably function best at 37°C. and explain the lack or diminution of pyocine production at this temperature; at lower temperatures e.g. 32°C. the biological regulator might not be so effective and thus explain the more frequent production of pyocine at temperatures below 37°C. Preliminary tests, using solid medium and with only 15 producer strains which were temperature-dependent indicated that such a repressor system might be involved when incubation was undertaken at 37°C It is intended to pursue this aspect of pyocinogeny at a quantitative level, using liquid media.

In-vivo stability of pyocine production.

Two indices have been employed in ascertaining the degree of in-vivo stability of pyocine production, namely the constancy of pyocine type in replicate isolates of Ps. pyocyanea obtained on different days from the same site in a patient and secondly, the uniformity of type in strains of Ps. pyocyanea isolated from the same epidemic situation.

Replicate isolates.

Five hundred and eleven patients were investigated and from these 2,510 isolates of Ps. pyocyanea were pyocine typed; the number of replicate isolates examined from any one patient varied from 1 to 54. The great majority of replicate isolates were of the same pyocine type as the original strain in a given patient but in some

instances more than one pyocine type was found in a patient.

Possible explanations of this latter finding are:

1. That the technique of typing varied from day to day; this may be discounted since control producer strains were always tested without showing any variation in results.
2. Genetic instability of pyocine production.
3. The simultaneous presence of more than one pyocine type of Ps. pyocyanea in the material examined.

When the findings were analysed it was noted that hospital patients showed a lesser constancy of pyocine type in replicate isolates than did patients treated at home (11.4 per cent. and 3.6 per cent. respectively). Such a discrepancy appeared worthy of further study especially since the early practice of typing only one colony from a diagnostic plate would mask the simultaneous presence of more than one pyocine type of Ps. pyocyanea.

The examination of several colonies (average, 6) from each of a series of primary diagnostic plates confirmed the difference in the findings between these two types of patient; not only was the incidence of more than one pyocine type of Ps. pyocyanea, on the same diagnostic plate, much higher in hospital patients but, whereas this incidence in hospital was independent of the nature of the specimen, such type multiplicity was restricted almost entirely to specimens of faeces in the case of patients treated at home.

This finding is further emphasised, for example, in the case of urine specimens. Seven instances of type multiplicity in such specimens were observed in 73 hospital patients compared to five occasions in 80 specimens submitted from patients, originally thought to be at home; however, two of these five patients were in fact undergoing treatment in hospital and their specimens were inadvertently sent to the University Diagnostic Laboratory which normally handles only specimens from general practice, another of these specimens was from an elderly man convalescing at home after prostatectomy and a fourth from a patient resident in a home for paraplegic patients. The fifth specimen of urine which gave multiplicity of pyocine type on one diagnostic plate was from a young woman suffering from a urinary tract infection due to Ps. pyocyanea who had been investigated as a hospital out-patient.

Therefore probably only the last of these five patients, at most, acquired their infection outside the hospital environment.

A similar investigation of strains of Ps. pyocyanea isolated from wounds, infected ears and a variety of other lesions, in 53 patients treated at home, revealed only one instance where more than one pyocine type was encountered; on the other hand, twelve instances of multiplicity of pyocine type were encountered in 131 hospital patients suffering similar lesions.

The following episode, which is one of many, illustrates the

need to examine several colonies from each diagnostic plate so that multiplicity of pyocine type in any one specimen can be determined.

In the case of patient A nine separate specimens were examined and on only three occasions was there an opportunity to submit more than one colony of Ps. pyocyanea to pyocine typing. The results of typing are given in Table 37.

Table 37. Patient A:
Pyocine types of strains of Ps. pyocyanea from both single colony subcultures and multiple colonies from primary diagnostic plates.

| Specimen | Date | No. of colonies examined | Pyocine type |
|---|---|---|---|
| Throat swab | 26th Jan. | 1 | 16 |
| " | 30th Jan. | 1 | 1/7 |
| Faeces | " | 1 | 1/7 |
| Tracheostomy wound | " | 1 | 1/7 |
| " | 1st Feb. | 1 | 1/7 |
| Nasal swab | " | 6 | 3 of 1/7; 3 of 16 |
| Throat swab | " | 6 | 3 of 1/7; 3 or 16 |
| Nasal swab | 2nd Feb. | 1 | 1/7 |
| Throat swab | " | 6 | 3 of 1/7; 3 of 16 |

It can be noted that although the single colony subcultures from throat swabs examined on the 26th and 30th of January revealed different pyocine types later examination of six colonies from each

of three primary diagnostic plates showed the simultaneous presence of both types concerned. In this particular case the bacterial colonies of the two pyocine types were also morphologically distinguishable.

Similar findings, to those noted above, have on occasion been associated with quite dramatic differences in the concentration of antibiotic required to inhibit different pyocine types in the same specimen and in the latter instance apparent success with agents such as gentamycin followed by relapse of the infection might be explained.

In regard to other species and genera several reports are available demonstrating mixed infections either with two or more members of the same genus or with species of different genera e.g. Hormaeche _et al_. (1943) in a study of 498 patients found that 32 (6.4 per cent.) of them were excreting more than one serotype of salmonella. They also encountered 34 instances (6.8 per cent.) of patients excreting both salmonellae and shigellae; finally, they noted 10 instances where patients were excreting two or more quite distinctive shigellae. More recently Juenker (1959) studied 75 patients and found that 13 (17.3 per cent.) were excreting two or more intestinal pathogens simultaneously.

After Sloan _et al_. (1960) reported that a single patient was found to be excreting three distinct phage types of _Salmonella_

typhimurium simultaneously, Bernstein (1960) examined these latter strains and concluded that phage mediated conversion was probably responsible but did not exclude the possibility of multiple infection.

Returning to infection with Ps. pyocyanea, Phillips et al. (1968) studied 21 cases of wound infection and found, using the Edinburgh technique, that two patients were each harbouring two distinctive pyocine types. This observation was made repeatedly on these two patients.

Unfortunately in other reports such as these mentioned above the bacteriological findings are not accompanied by any indication of the epidemiological background. However, clinical colleagues have been most generous in providing epidemiological and other facts regarding the patients from whom my series of strains of Ps. pyocyanea were isolated and the evidence thus accrued suggests that the isolation of more than one pyocine type of Ps. pyocyanea from a patient can result from mixed infections and does not necessarily reflect genetic instability of pyocine production. The greater frequency of isolation of more than one pyocine type in hospital patients as compared with those living at home (p. 115) probably reflects the greater density of the pseudomonas population in the hospital environment and the load of potential infection is, therefore, very much greater than if the patient had been treated at

home or in a hospital which is not under constant assault by such organisms.

Uniformity of type in epidemics.

This index of in-vivo stability of pyocine production has been satisfactorily fulfilled by an investigation of 20 outbreaks involving from 5 to 90 patients in which the causal strain of Ps. pyocyanea retained it's type pattern throughout the course of each epidemic, some of which lasted several months. Complete uniformity was not observed in epidemic 4 (p. 121); this particular outbreak, however, was somewhat unique since strains of the three pyocine types involved were also isolated from the immediate environment.

Epidemic outbreaks of infection due to Ps. pyocyanea.

The use of pyocine typing in tracing the sources and methods of spread of epidemic outbreaks of infection due to Ps. pyocyanea has revealed two important points:

1. Environmental surveys demonstrated that the hospital environment usually harbours a multitude of different pyocine types of Ps. pyocyanea and that without such a method of characterising the organisms it is impossible to trace the reservoir of types involved in human infections.

2. The actual vehicle or reservoir of infection is usually limited e.g. anaesthetic equipment, imperfectly sterilised instruments etc. and so successful tracing and appropriate meas-

ures can normally terminate the outbreak; on the other hand when cross-infection occurs outbreaks are more difficult to control.

Other workers have found the Edinburgh pyocine typing technique useful in epidemiological studies, Fierer et al. (1967), in the first reported use of pyocine typing in the U. S. A., traced an outbreak of respiratory infection in 22 newborne babies of whom two died; the original source of the causal strain which was of pyocine type-3 was found to be delivery-room resuscitation equipment which had been contaminated via a wash-sink aerator. Their findings confirmed the statements made in 1. above and an additional interesting aspect of this outbreak was the use of pyocine and phage typing in parallel; the authors found the phage patterns of the type-3 strains to be similar but not identical and concluded that it would have been difficult to identify the epidemic strain of Ps. pyocyanea using phage typing alone. A suspected source of eye infection due to Ps. pyocyanea was also confirmed in the Philippines (Aragon, 1967) and workers at the Communicable Disease Center, Atlanta, Georgia have found the standardised method to be "a very useful epidemiological tool" (Caldwell, 1967). The technique has also proved useful in clinical trials of new therapeutic agents (Brumfitt et al., 1967; Phillips et al., 1967) and in laboratory studies of Ps. pyocyanea (Fedorko et al., 1967).

In the present series, the examination of 3, 730 strains of

Ps. pyocyanea isolated from 50 wards in a large general hospital (hospital 1) has produced ample evidence that infection can be acquired either endogenously or exogenously.

In some of these wards no one pyocine type predominated in patients suffering infection with Ps. pyocyanea and the possible endogenous nature of such infections is supported by the evidence summarised in Table 8 which shows the complete uniformity of pyocine type when replicate isolates were examined from different sites in the same patient including culture plates exposed at a patient's bedside.

Alternatively, the occurence of exogenous infection with Ps. pyocyanea is inferred from the results noted in Tables 19 and 20; here an almost complete monopoly was held either by pyocine type 35 or 29 in pseudomonas infections in two wards belonging to surgical units which are completely separate. In each ward this dynastic situation continued over a period of many months and it would seem highly unlikely that so many patients (72.2 per cent. in one ward and 69.7 per cent. in the other) could be admitted to these wards already infected with the respective and somewhat rare pyocine type of Ps. pyocyanea which predominated. It is interesting to note that in both units only the male wards were affected, the female wards remaining almost clear of infection with Ps. pyocyanea.

However, in a similar episode in yet another surgical unit (p. 124) in which infection with pyocine typee3 strains of Ps. pyocyanea predominated, both male and female wards were equally involved pointing to some mutual focus of infection e.g. the surgical theatre.

It is perhaps impertinent that I, without medical qualifications, make the following comment but it is made with sincerity, naimely that; the nature of certain reservoirs, found to be implicated in outbreaks of infection due to Ps. pyocyanea e.g. those cited in epidemics 1 and 2 (p. 116), reveals a disturbing laxity in the maintainence of hygenic principles. One is tempted to believe that were it possible to drink Oberon's potion and assume a cloak of invisibility, not a few disquietening practices, performed under the guise of hygiene, might still be observed even in this enlightened age.

Comparison of pyocine typing with phage and serological typing techniques.

The typing of Ps. pyocyanea by pyocine production has proved a more sensitive means of characterising strains than either phage typing or serological analysis; in practice it is also the most simple of the three to carry out.

The experiments reported on page 140 indicate that there are varying degrees of relationship between the pyocine types and serological or phage types of Ps. pyocyanea. A close linkage was observed between strains of serotypes 2 and 5 and pyocine subtypes

1/3 and 1/4 but on the other hand the 52 strains of serotype 6 could be allocated to nine categories of pyocine type. The potential relationship with phage types was obscured by the limited number of strains (52.4 per cent. of 82) which were found to be sensitive to one or more of the typing phages. Though it might have been possible to increase the number of typable strains by using a larger set of typing phages or by increasing the potency of the preparations used, sufficient experience of the technique was obtained to indicate that it is time-consuming and epidemiologically unreliable.

In contrast to the observation that dissociation gives rise to an alteration in serotype (Gaby, 1946) or phage type (Zierdt and Schmidt, 1964), the testing of colonial variants from the same culture revealed similar pyocine typing patterns throughout.

Further aspects of pyocine typing.

The essential difference between the pyocine typing technique of Darrell and Wahba (1964) and that developed in Edinburgh is that these authors incubate the potential producer strain at 37°C. for 24 hr whereas incubation at 32°C. for 14 hr, the standardised technique, gives infinitely better results. Excellent patterns of inhibition were obtained when the Darrell-Wahba indicator strains were used in conjunction with the standardised method of typing; more recently Darrell (1967) indicated that he had adopted the Edinburgh method.

Primary incubation of test strains at 37°C., regardless of the duration of incubation, seriously reduces the number of pyocine types which can be identified; in addition, when incubation is carried out at 37°C. and especially for 24 hr or more the macroscopic growth is often viscid and adheres to the medium so that it is very difficult to remove. This phenomenon is seldom if ever encountered after growth at 32°C.

It was perhaps this difficulty as well as the necessity for removing the macroscopic growth of the potential producer strain, which prompted Kohn (1966) to advocate the use of cellulose acetate strips; the latter were placed on the medium and inoculation of the test strain was on to the surface of the strips. It was assumed that any pyocines produced would pass through the membrane strip into the medium and the strip could then be easily removed along with the macroscopic growth; the indicator strains could then be applied in the normal manner. Typing of several hundred strains of <u>Ps. pyocyanea</u> was performed in parallel with an without the use of cellulose acetate strips and the deficencies associated with these strips have been noted (p. 138); in addition, test strains are very easily removed from the surface of TSBA after incubation at 32°C. and the inoculation of the dry cellulose acetate surface is even more time-consuming than the routine removal of macroscopic

growth with a glass slide.

Kohn used the typing technique of Darrell and Wahba (1964) and although he states that he obtained satisfactory results with cellulose acetate strips over a three month period, he does not give any detail regarding the number or pyocine type of strains tested. Since the typing method which Kohn used recognises only 12 pyocine types and since primary incubation is at 37°C. it would appear that the greater sensitivity of the Edinburgh technique has revealed disadvantages not apparent in Kohn's investigation. Nevertheless some pyocines are capable of spontaneous passage through such membranes (p. 136) thus they may be useful in the isolation of individual pyocines.

Pyocinogeny and pigment production in Ps. pyocyanea.

It is interesting to note that of 940 strains of Ps. pyocyanea 70 did not produce detectable pyocines but nevertheless 64 of these latter strains produced both pyocyanine and fluorescein and the remaining six strains produced only fluorescein. It is very probable, therefore, that the presence of untypable strains in this series, as judged by the standardised pyocine typing technique, reflects apyocinogeny in Ps. pyocyanea rather than errors in the identification of non-pyocyanine-producing strains.

The high frequency of pyocinogeny encountered in the entire series (more than 90 per cent. of strains) suggests that this

property might aid the identification of those strains which do not produce detectable pyocyanine under normal cultural conditions. 133 of 940 strains of Ps. pyocyanea produced neither pyocyanine nor fluorescein when grown on nutrient agar; of these 133 strains, 113 gave characteristic patterns of inhibition when subjected to pyocine typing.

The characterisation of individual pyocines.

Unfortunately the techniques employed by Fredericq (1948) which enabled the enumeration of 17 different colicines do not appear applicable in the case of pyocines. In general, resistant variants are difficult to isolate and maintain, proteolytic enzymes, e.g. trypsin do not destroy pyocines and the diffusion of most pyocines in agar is minimal. During the present investigations only two individual pyocines could be tentatively identified on the basis of whether or not they gave rise to mutually resistant variants, the temperature sensitivity for production and the morphology of their inhibition zones. One of the pyocines is produced by strains of type-5, the other by strains of type-31; the production of both of these pyocines by strains of pyocine type-9 suggests that as in the case of colicinogeny, a strain may be capable of producing several distinct pyocines simultaneously. Since pyocines are undoubtedly antigenic and capable of purification (Kageyama et al., 1964; Kageyama, 1964) their characterisation

by serological methods appears more promising. This suggestion was originally made by Hamon and Péron (1965) to apply to both pyocines and megacines (the bacteriocines from Bacillus megaterium) after these authors had noted the difficulty or impossibility of obtaining resistant variants to these bacteriocines. Following the isolation of genuine resistant variants to pyocine from the type-1 strain, R21, by means of a gradient plate technique which allowed stepwise exposure to increasing concentrations of bacteriocine it was noted that an essentially similar technique had been used successfully in the case of a megacine (Nagy, 1965); as with the pyocine-resistant variants, Nagy found that resistance was unstable and disappeared after subculture on a medium containing no megacine.

The use of serological techniques, therefore, seems the likeliest method of characterising individual pyocines. Such an approach, however, depends upon our ability to extract and purify these bacteriocines and some preliminary investigations were undertaken to this end.

The extraction, purification and properties of pyocines.

The extraction of pyocines from agar or broth cultures has revealed that spontaneous production is low; the highest titre of activity being approximately 1 in 256. Under suitable conditions,

however, induction with mitomycin C has enabled extremely high titres of several pyocines to be obtained. Though a similar induction can be brought about by irradiation with ultraviolet light (Jacob, 1954), the use of the carcinostat is both easier to carry out and more accurate to control quantitatively.

McGeachie and McCormick (1967) have reported that strains of Escherichia producing the colicines A, B, D, K and V showed considerable variation in the spontaneous production of their respective colicines even under standard conditions. With the exception of colicine K, obtained in very high yield, the concentrations of the various colicines were similar to those obtained with pyocines and seldom exceeded a titre of 1 in 200. The significance of variation in spontaneous production of various colicines was demonstrated when the standard set of colicine indicator strains (Abbott and Shannon, 1958) was examined against each of the colicines employing the range of concentrations in which each was spontaneously produced. A significant variation was noted in the patterns of inhibition obtained with colicines A and D and to a lesser extent with V using these different concentrations of bacteriocine. Such findings must have an important bearing on those typing techniques which depend upon the spontaneous production and detection of bacteriocines. It was interesting to note therefore, that when pyocine was extracted from strain R21, a type-1 strain, the titre

of activity remained remarkably constant at 1 in 64; furthermore when a high-titre pyocine preparation from the same type-1 strain was tested for activity against indicator strain no. 6 (which is resistant when challenged by the standardised method), no inhibition was noted despite the fact that the type-1 preparation had a pyocine titre of 1 in 16,000.

It remains to be seen whether similar results would be obtained with all pyocines since so far it has only been possible to study a few representatives, namely one each of strains of pyocine types 1, 3, 5, 9, 10, 11, 16 and 31; there was, however, little variation in the titres obtained with any one type when repeatedly tested after spontaneous production of it's pyocine. The activities against lawns of the indicator strains, of each pyocine preparation after extraction, were identical with those obtained by the standardised typing technique.

The remarkable specificity of pyocine activity was further demonstrated using a high-titre preparation from strain R21; although many strains of Shigella and Escherichia are completely inhibited when used as indicators in the standardised pyocine typing technique, this purified pyocine preparation, exhibiting a titre of 1 in 4,000, showed no activity against strains of Shigella, Escherichia, Salmonella, Proteus, Staphylococcus, Enterococcus and Vibrio. Thus the inhibition of such strains when tested by the

standardised technique is not due to pyocine activity but must be due to other factors, e.g. pyocyanine or extracellular enzymes.

When mice were fed orally with a similar high-titre pyocine preparation from strain R21, titre 1 in 8,000, they showed no alteration in their normal intestinal, bacterial flora and such a finding underlines the high degree of specificity of action of pyocine.

Toxicity of pyocines.

Both crude and purified preparations of pyocine obtained from liquid cultures of strain R21 have shown little evidence of the toxic extracellular enzymes lecithinase, lipase or haemolysin (Liu et al., 1961) and though gelatinase activity could be demonstrated in such extracts it did not appear to exert any toxic effect. Further encouraging evidence for the non-toxic nature, of this particular pyocine at least, was shown by the ability of tissue cell cultures (chick fibroblasts, Hep 2 epithelial cells and monkey kidney cells) to remain completely unaffected in the presence of both crude and purified pyocine preparations, exhibiting titres of 1 in 3,200. The final stage of this brief investigation into the biological properties of a pyocine provided the most satisfactory evidence so far of it's lack of toxicity. The in-vivo administration of potent, crude and purified pyocine preparations (exhibiting titres of 1 in 8,000) into adult white mice by a number of routes produced no harmful effects. Indeed, during the oral administration of many litres of pyocine, of similar potency, several litters were born, the young mice being

reared with the pyocine preparation as their sole source of fluid and in turn producing progeny themselves. Pyocine extracts of similar potency from strains of pyocine types 10 and 16 have also shown no toxicity after oral administration to mice. These observations have recently been confirmed by Higerd et al., (1967) who encountered neither morbidity after intraperitoneal injection of a pyocine in mice nor demonecrosis following subcutaneous injection in rabbits. Such findings serve to demonstrate a distinction between these pyocines and certain colicines since both colicine K (Goebel et al., 1955) and V (Hutton and Goebel, 1962) have been shown to exert potent toxic effects on certain mammalian species.

Pyocines as therapeutic agents.

The experiments described on page 175 indicate that intensive investigation of the possible therapeutic role of pyocines must be undertaken. It is perhaps not surprising that complete and prolonged eradication of Ps. pyocyanea was not achieved in the treated mouse since although one can autoclave the mouse cage it is quite a different matter to prevent re-infection via claws, hair etc. Under such conditions, any reasonable reduction in bacterial numbers is probably significant.

The natural resistance of Ps. pyocyanea to most antibiotics makes infections with such organisms extremely difficult to treat. The specificity of action shown by bacteriocines renders them

particularly attractive since their use would not result in the simultaneous elimination of the normal flora, together with the pathogen, a complication of modern broad-spectrum antibiotics and a primary reason for the appearance of many infections due to *Ps. pyocyanea*. Even if treatment of systemic infections with pyocines were not possible topical application to infected burns, ulcers or other surface lesions might be feasible.

This would require that the causal strain of *Ps. pyocyanea* be examined for it's sensitivity towards a number of pyocines in order to determine which particular pyocine should be used. At present, however, there appears no reason why a number of pyocines could not be pooled to provide a preparation with a wide spectrum of activity.

*A closer look at pyocines.*

Phages and bacteriocines such as pyocines show many similarities a few of which are summarised on page 184. An important difference between the two agents is the failure of bacteriocines to propagate on a sensitive strain. Titration of a phage preparation against a lawn of a sensitive strain leads on dilution to the appearance of discrete plaques resulting from the lysis of a single bacterial cell and the release and subsequent activity of further phage particles. A pyocine preparation, on the contrary, behaves

as a 'classical' antibiotic exhibiting on dilution a gradual diminution of activity as would be the case with any inhibitory substance which was not capable of propagation. Such a difference cannot be determined in the inhibition of indicator strains viewed macroscopically in the case of the standardised technique and microscopically using the agar slide-culture method. Proof that the patterns of inhibition obtained with the standardised pyocine typing technique were not due to the activity of virulent phages, simultaneously produced by pyocinogenic strains of Ps. pyocyanea, was obtained by showing that phage preparations from such strains frequently had no effect on any of the indicator strains.

Phages are often described as biological units, their adsorption on to sensitive bacterial cells leading ultimately to lysis of the cells and the release of further phage particles. The failure of temperate phages to bring about immediate lysis results from the incorporation of phage genetic material on to the bacterial chromosome which continues to function and replicate normally. This latter process, transduction, can endow the bacterial strain, parasitised, in this way, with genetic characteristics deriving from the bacterial strain from which the phage was isolated. Continuity is thus established either through lysis or transduction.

No such useful purpose is evident in the case of bacteriocines and their definition as antibiotics emphasises their apparent one and only function, namely lethality. One might ponder over the value

and specificity of such substances to the bacterial cells producing them.

Darwin (1859) observed that: "As the species of the same genus usually have, though by no means invariably, much similarity in habits and constitution, and always in structure, the struggle will generally be more severe between them, if they come into competition with each other, than between the species of distinct genera". Though no doubt Darwin had in mind larger forms of life than bacteria his observation seems no less applicable to the microbial world.

Reeves (1965) did consider the evolutionary significance of bacteriocines in regard to their unusual specificity of action and postulated that bacteriocines are "fertility recognition sites" which have evolved to perform a specialised antibiotic function.

A great deal is known regarding both the nature and ultrastructure of phages especially the T-even phages of Escherichia coli. Four morphological types of pseudomonas phage have been described (Slayter et al., 1964; Bradley, 1966; Takeya and Amako, 1966) including the common contractile variety (Fig. 25). The virion consists of a geometrically constructed head contaihing deoxyribonucleic acid (DNA), a contractile sheath enclosing a narrow, hollow tail section, and consisting of striated or helically arranged subunits. The sheath terminates in a somewhat ill-

defined base plate attached to which are several thin fibres.

Such ultrastructure resembles that already observed in T2 and T4 phages and suggests that attachment and penetration would follow a course already well-defined (Simon and Anderson, 1967), namely; after initial attachment of the virions to the bacterial surface is made by the distal ends of the long tail fibres the base plate is brought into close proximity with the cell surface. The tail sheath then contracts along the needle-like tail which simultaneously penetrates into the cell wall. Following injection of phage nucleic acid via this hollow tailpiece, the genetic material is either adsorped in the form of prophage or serves to bring about the production of further phage units leading ultimately to the disruption of the cell and the relaase of several hundred mature phages. Continuity is thus established.

Bacteriocines, on the contrary, have long been regarded as biological compounds, lacking the sophisticated ultrastructure of phages and devoid per se of the means of genetic continuity; the latter, however, must be inherent in the bacteriocinogenic strain to account for the stability of production and the specificity of the bacteriocines. In his review Reeves (1965) noted that bacteriocines differed from 'classical' antibiotics on account of their activity towards closely related species and also, when studied in sufficient detail, on account of their macromolecular size.

Kageyama (1964) succeeded in purifying a pyocine as a homogenous protein of high molecular weight (approximately 8, 800, 000) and published an electron micrograph of this preparation which showed many rod-like particles; however, no structural detail could be seen since he used shadowed preparations.

Using a negative staining technique employing phosphotungstic acid (Brenner and Horne, 1959), several pyocine preparations were examined electron microscopically and have shown a fascinating ultrastructure in this particular bacteriocine (Figs. 26-29).

These electron micrographs have demonstrated the presence in pyocine preparations of rod-like particles resembling the tail components of certain T-even or contractile pseudomonas phages. Two distinct structural forms can be seen, firstly a bullet-like component resembling an extended phage sheath and also one composed of two cylinders one partially enclosed within the other and similar in size and appearance to the needles and contracted tail sheaths of certain phages. Not dissimilar appearances have been noted by other workers e.g. Higerd et al. (1967) using a more restricted series of pyocines.

Similarly electron microscopy has demonstrated that the particles can adsorp to the surface of a sensitive cell and apparently bring about it's ultimate destruction (Figs. 30-32). The manner of attachment, with the sheath pointing outwards, despite the lack of

any structure resembling a phage head, would seem to indicate that the mechanism of attachment and possible penetration of these units is similar to that described in the case of the T2 and T4 phages of Esch. coli (Simon and Anderson, 1967). The components resembling extended phage sheaths seem to possess ill-defined base plates similar to those already described in a contractile pseudomonas phage (Slayter et al., 1964); no evidence of tail fibres, however, has yet been observed in pyocine preparations. In regard to the actual mechanism of lethality the configuration of the pyocine units suggests that a form of injection takes place following attachment and penetration.

Ikeda et al. (1964) noted the lack of DNA synthesis following the induction of a pyocine with mitomycin C and Kageyama et al. (1964) demonstrated the presence in induced lysates of a muramidase-like enzyme which acted at the time of lysis; immunological studies suggested that part of this enzyme was associated with pyocine itself. The specificity of this enzyme was wider than that of pyocine since pyocinogenic and pyocine-resistant strains were also lysed. Kaziro and Tanaka (1965a) noted that a pyocine caused a rapid and complete cessation of DNA, ribonucleic acid (RNA) and protein synthesis in sensitive cells. In addition, these same authors (1965b) showed that this activity occurred through inactivation of bacterial ribosomes; however, due to the macromolecular

size of the bacteriocine and on the basis of experimental evidence the authors excluded the possibility of direct contact between ribosomes and pyocine. They considered that some part of the pyocine might enter the cell but since they could find no evidence of a free inhibitor in extracts of treated cells they concluded that adsorbed pyocine might remain on the surface of a sensitive cell and exert it's activity therefrom through an unknown process.

It seems possible that some non-specific lethal agent might be injected into or at least brought into contact with the cell surface and be directly or indirectly responsible for destruction of the cell as shown in Fig. 31. The specificity of pyocine would then depend upon selective attachment, and indeed, attachment is not observed in resistant strains of Ps. pyocyanea.

Kageyama et al. (1964) demonstrated by measurements of radioactivity that a pyocine was adsorped to sensitive cells of Ps. pyocyanea at $0^{\circ}$C. but they noted that such cells remained viable even after 40 mins. contact at this temperature; at $37^{\circ}$C. the same cells lost their viability after 20 mins. contact.

Since electron microscopic studies have shown that the pyocine units can attach to sensitive cells at $0^{\circ}$C. their lack of lethality at this temperature would indicate a temperature dependence in the mechanism of injection or perhaps in the biological activity of the lethal principle.

Jacob (1954) examined the survival rates of sensitive cells of Ps. pyocyanea in the presence of various concentrations of a pyocine and defined a lethal unit of pyocine as the quantity necessary to kill a bacterium. Kageyama et al. (1964), following similar experiments, concluded that one particle of pyocine can kill a sensitive cell and estimated that one bacterium can elaborate about 200 particles of pyocine. Their experimental results, however, were not conclusive and further quantitative studies are required to confirm whether one pyocine particle can cause the death of a cell.

Since pyocines could never be propagated by passage through a sensitive strain it is significant that the electron micrographs of pyocine preparations never revealed structures similar in appearance to phage heads, the reservoir of viral DNA. A single phage plaque on the other hand, contains sufficient nucleic acid to bring about a millionfold increase in phage activity following passage through a sensitive strain. It has already been noted, on the contrary that no production of DNA can be observed following the induction of pyocines and that ribonuclease or deoxyribonuclease does not inactivate pyocine (Jacob, 1954; Hamon, 1956). Therefore, one can postulate that since pyocine particles appear to lack both a head and DNA and since RNA would also appear to be absent then propagation will not and, in the light of modern biological thought, cannot take place.

To some, no doubt, such morphological observations of pyocines will only serve to reassert the view that bacteriocines, or at least pyocines are merely defective forms of phage; phages are now being encountered which because of the lack of certain genes (Wood and Edgar, 1967) or the incorporation of certain amino acid analogues in the lysogenic bacterium (Cummings et al., 1967), display a wide range of structural and biological aberrations. Brenner et al. (1959) showed that treatment of intact T4 phages with duodecyl sodium sulphate resulted in structures almost identical in size and appearance to those units, shown in Fig. 27, which consist of apparently hollow needles surrounded by contracted sheaths.

It would be wrong to infer that the inhibition of a sensitive strain following the adsorption of phage always results in propagation nor indeed that the lack of a phage head indicates the absence of nucleic acid. An examination of such systems, however, normally reveals that when a phage is isolated which can kill but not infect a sensitive strain (Sekiguchi and Tanagi, 1960; Amati, 1962) phages with typical configuration can be seen to adhere to the cell surface and the lack of propagation is due to the biological inertness of the viral DNA. In regard to the second system Takeya and Amako (1966) have described a rod-shaped pseudomonas phage; the virion, however, contains DNA, can reproduce and displays an ultrastructure which is distinctly different from that observed in pyocine preparations.

Rod-like and spherical forms have also been encountered in RNA phages but once again the virions possessing these unusual configurations are capable of reproduction.

To complicate matters, however, Takeya et al. (1967) have also described a further rod-like entity varying in length from 50-400 mμ and which they refer to as a pyocine. No contractile sheath-like structures are visible in the published electron micrograph but the particles, apart from their variable length, are similar in appearance to the needle-like tailpieces shown in Fig. 27; few biological properties of this agent are reported, however, and so clarification of it's status must await further investigation.

In view of the several varieties of phages isolated from Ps. pyocyanea both in regard to ultrastructure and biological properties (Bradley, 1966) the remarkable uniformity of structure in the pyocines so far examined is significant.

In this age of genetic manipulation it seems immaterial to argue whether or not a bacteriocine, such as pyocine, is a defective form of phage or alternatively a primitive evolutionary form of such a virus. The answer may well lie in a future reappraisal resulting in a more comprehensive definition of that highly complex unit which we at present refer to as a phage. Apart from the ability of phages to replicate in sensitive bacterial cells the morphological similarity of the former with pyocines

certainly demands that their relationship must be even more closely examined.

The interpretation of electron micrographs in the first instance may reflect the attitudes, if not the desires, of the observer and one must constantly be on guard against a premature association of morphological appearances with biological activity. Nevertheless, the progression of events revealed by the several figures showing, firstly attachment and ultimately disruption of a sensitive bacterial cell by a pyocine preparation underlines the essential lethality of the latter regardless of the mode of action.

Prospects for the future.

Apart from it's value in characterising strains of Ps. pyocyanea for epidemiological or laboratory studies, the technique of pyocine typing has led to an interest in other aspects of pyocinogeny. Studies of the physical and biological properties of this bacteriocine reveal many exciting fields of future study especially in regard to the possible therapeutic use of pyocines and, more academically, the examination of their ultrastructure.

Infections due to Ps. pyocyanea are extremely difficult to treat because of the natural resistance of this species to most of the commonly used antibiotics. The apparent lack of toxicity coupled with the specificity of pyocines indicates that the potential therapeutic role of this bacteriocine must be investigated. If not

suitable for human use then one might bear in mind that the animal kingdom too is in need of such an agent. A wide variety of animals, domestic and otherwise, can suffer from infections due to Ps. pyocyanea; such infections in the case of fur-bearing animals e.g. mink or chinchillas, proving not inexpensive. At the last resort, the activity of pyocine preparations at room temperatures might indicate their ultimate use as specialised disinfectants.

Since pyocines are antigenic it would be interesting to observe electron microscopically, the interactions between sensitive cells of Ps. pyocyanea and pyocine preparations in the presence of their respective antisera. Serological studies might also elucidate the 'pluralite' of this bacteriocine and enable, as in the case of the colicines, a world-wide correlation of nomenclature.

## SUMMARY

## SUMMARY

1. The literature has been reviewed regarding the taxonomy of the genus Pseudomonas and the status of the species Ps. pyocyanea; other aspects of this species e.g. nomenclature, pathogenicity, and various attempts at characterisation of Ps. pyocyanea have also been considered.

2. The unsatisfactory nature of previous typing schemas prompted an investigation into the possibility of using pyocine production as an epidemiological marker of Ps. pyocyanea.

3. Preliminary studies on the production and detection of pyocine activity revealed that production, in particular, was dependent upon various factors during incubation of the producer strain. Optimum conditions for the production and detection of pyocines were determined.

4. A standardised pyocine typing technique has been developed and 37 pyocine types of Ps. pyocyanea have so far been recognised; it's reliability has been confirmed in vitro by typing strains after subculture or storage and in vivo by epidemiological studies.

5. Strains of pyocine type-1 were found to be those most commonly encountered and a successful subdivision of this type has enabled the recognition of eight distinct subtypes; this technique was also shown to give results which were epidemiologically valid.

6. Epidemiological investigations, using pyocine typing, have indicated the probability that a patient can be infected simultaneously with more than one pyocine type of Ps. pyocyanea. The technique has also proved useful in the investigation of epidemic outbreaks of infection due to this organism.

7. The Edinburgh method of pyocine typing has been compared with that described by Darrell and Wahba (1964) and found to have distinct advantages over the latter; the Edinburgh method has also been found to be reliable by workers in many countries.

8. The use of cellulose acetate strips in conjunction with the standardised technique has been shown to be unsatisfactory for typing purposes.

9. There was no correlation between lack of pigment production by a strain of Ps. pyocyanea and it's ability to produce pyocine. The majority of non-pigmented strains could be allocated to various pyocine types; strains producing the unusual pigment,

pyorubrin, could be subdivided by the standardised pyocine typing technique.

10. A comparative study of pyocine typing with serological and phage typing techniques has shown that it is a more sensitive means of characterising strains of Ps. pyocyanea; in addition pyocine typing is technically simple.

11. The pyocines studied were found to be resistant to the action of proteolytic enzymes. Genuine resistant variants to pyocine activity were seldom encountered and the diffusion of most pyocines in agar was found to be minimal.

12. The spontaneous production of pyocines in solid or liquid media was relatively constant but of low titre; high titres were obtained in liquid media following induction with mitomycin C. Pyocine preparations were purified using an ammonium sulphate precipitation technique.

13. Pyocine preparations were found to suffer little or no loss in activity after three months storage at 4° or 21°C. whereas there was a rapid diminution in activity during storage at 37°C. and no pyocine activity could be detected in preparations held at this temperature for three months.

14. Strains of Shigella, Klebsiella, Salmonella, Proteus, Enterococcus, Staphylococcus and Vibrio were found to be sensitive when used as indicators in the standardised pyocine typing technique but to be resistant when tested, as lawns, with a potent, purified pyocine preparation. Indicator strain no. 6, resistant to the pyocine of strain R21 when tested by the standardised typing method, retained it's resistance when challenged with a high-titre pyocine preparation obtained from this strain.

15. Resistant variants to the pyocine from strain R21 were obtained using a gradient plate technique which allowed exposure to increasing concentrations of this bacteriocine; the variants lost their resistance after several subcultures on a medium containing no pyocine.

16. Little evidence of toxic extracellular enzymes was found in pyocine preparations obtained from strain R21. No cytopathogenicity was noted when various tissue cell lines were challenged with high-titre preparations from this strain.

17. No toxic effects were observed in mice to which high-titre pyocine preparations from strain R21 were administered by a number of routes. A short therapeutic trial using such prep-

arations underlined the need to investigate further the possible therapeutic use of pyocines.

18. The inhibition or resistance of the standard indicator strains has been demonstrated at the microscopic level using an agar slide-culture technique.

19. The similarities of bacteriocines to phages have been discussed and electron microscopic studies of pyocine preparations have revealed structures which resemble extended or contracted tail sheaths of contractile phages.

20. Similarly, electron microscopic studies have shown that pyocine particles adsorp to sensitive cells of Ps. pyocyanea and apparently bring about their ultimate destruction. No attachment was observed with a strain which was resistant to the pyocine, as judged by the standardised technique or by the agar slide-culture method.

21. Finally, I have incorporated a discussion of the results in association with the contemporary literature.

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