STAPHYLOCOCCAL INFECTIONS IN MAN AND ANIMALS

A thesis presented for the degree of

Doctor of Philosophy

by

WILLIAM CHARLES NOBLE, M.Sc.

Boots Research Fellow
The Wright-Fleming Institute of Microbiology
St. Mary's Hospital Medical School,
Paddington, London, W.2.

Staphylococcal infections in man and animals

SUMMARY

This thesis describes experimental work which led to the evolution of a technique whereby subcutaneous abscesses could be produced in mice using small doses of staphylococci inserted on plugs of cotton dust.

Staphylococci collected during studies on cross-infection in surgical wards were used to produce abscesses by this technique and the severity of the lesions produced compared with the infectivity of the strain for man (as observed in the wards), with the *in vitro* production of toxins and enzymes and with other characteristics of the staphylococci. There was a correlation between the 'virulence' of the staphylococci for mice, the 'epidemicity' for man and the production of toxins such as the alpha and beta haemolysin and enzymes which split proteins.

The production of abscesses was used to investigate the relative infectivity for mice of 'fresh' and 'dried' staphylococci. It was found that, although no significant difference in virulence could be detected between fresh and dried staphylococci using this technique, the dried staphylococci appeared to have a longer lag phase *in vivo* than did the fresh cocci.

Finally, using various techniques which affected the reaction of the host to staphylococci, an attempt was made to

determine some of the factors which influenced the production or severity of staphylococcal lesions.

Because of the inherent variability of this technique of producing staphylococcal lesions (the assessment of severity is to some extent subjective) statistical methods have been used throughout the work to help assess the significance of observed differences in behaviour.

The experimental work is preceded by a discussion on part of the extensive literature on the virulence of staphylococci.

ACKNOWLEDGEMENTS

I am deeply indebted to my supervisor Professor R. E. O. Williams for help and encouragement throughout this work. I am also indebted to Professor P. Armitage and Miss W. Grant of the Department of Medical Statistics and Epidemiology at the London School of Hygiene and Tropical Medicine for discussion on the statistical analysis of experimental work. Others who have given me help on specific points are mentioned in the text. Except where specifically stated in the text, all the experimental work recorded in this thesis was carried out by me with the technical assistance of Miss D. A. Place to whom I am very grateful.

TABLE OF CONTENTS

Table of Contents (continued)

STAPHYLOCOCCAL INFECTION IN MAN AND ANIMALS

INTRODUCTION

In recent years there has been a great deal of interest in the staphylococcus in its role as the most frequent cause of cross-infection in hospitals. In the wards, transfer of staphylococci from patient to patient has sometimes led to widespread disease. Staphylococci have been isolated from almost every part of the human body and from every inanimate object in the wards.

One of the problems that has been given close attention is the search for the mechanism by which the staphylococcus causes disease. Some bacteria, such as the diphtheria bacillus, possess toxins which are clearly responsible for a major part of the symptoms of disease and although infection with non-toxigenic strains may occur, the disease is then not diphtheria. No such simple mechanism can explain staphylococcal disease, however, except in staphylococcal food poisoning where a stable toxin can be implicated.

It has long been known that strains of staphylococci which cause disease generally possess the ability to coagulate human plasma, but within the category of coagulase positive strains are some which appear to be able to cause disease.

regularly and others which appear unable to do so. Staphylococci have been examined by a number of biochemical tests and the cocci or their products injected into a variety of animals, from rabbits to goldfish, without finding a single factor unequivocally distinguishing the more virulent from the less virulent strains. Many of the tests resolve into methods of distinguishing between coagulase positive and coagulase negative cocci rather than between strains of coagulase positive cocci.

Staphylococci cause disease in man, but apart from cattle, cause little natural disease in animals. Laboratory animals are, in the main, fairly resistant to staphylococci and experimental studies have shown that, even in man, large doses of cocci may be needed to cause disease regularly. A technique which requires the intraperitoneal injection of 10^8 cocci into an experimental animal is clearly unsatisfactory if relatively fine differences are being sought between strains.

This thesis describes experimental work leading to a technique whereby subcutaneous lesions can be produced in mice using small doses of staphylococci. The uses of the method are explored in an attempt to distinguish between the degrees of pathogenicity (the virulence) of a large group of staphylococci isolated during studies of cross-infection in hospital wards;

to determine whether dessication affects the virulence of staphylococci; and to explore some of the factors which affect the outcome of experimental infections.

DEFINITIONS

Before proceeding to discuss published work on staphylococci it is desirable to define some of the terms to be used.

When the term 'staphylococcus' is used without qualification, it is used to mean the Gram positive, catalase positive, coagulase positive coccus Staphylococcus aureus.

The term 'micrococcus' is used without qualification as a general term for the Gram positive, catalase positive, coagulase negative cocci variously known as Staphylococcus albus, S. epidermidis or Micrococcus.

It is most convenient to use the terms virulence and pathogenicity in the way that Miles (1955) defined them: "Pathogenicity is best regarded as an attribute of a species, a genus or some other group of parasites". "Virulence is conveniently reserved for the pathogenicity of a given stable homogeneous strain of a microbe, as determined by observations of its action on the host in relation to which the statement about virulence is made."

REVIEW OF THE LITERATURE

METHODS OF DETERMINING PATHOGENICITY AND VIRULENCE IN VIVO

In order to pursue the experimental work on which this thesis is based, it was necessary to use a test which would distinguish degrees of pathogenicity among a large number of strains of staphylococci collected during studies of cross-infection. Further, since the survival of staphylococci on drying had been studied previously (Noble, 1961; M.Sc. Thesis) it seemed natural to look for a technique which would also enable the investigations to be extended to a study of the virulence of dried organisms. As will be seen, many of the published tests distinguish between coagulase-positive and coagulase-negative cocci rather than between strains of coagulase-positive cocci, but it is this latter distinction which is of interest in the context of this thesis. From a study of published work it seemed unlikely that gross differences in behaviour would be encountered between strains of staphylococci and so the technique, to be useful, would need to give results which were susceptible of analysis by statistical methods. These various criteria impose restrictions on the tests available and it is convenient to bear them in mind in the discussion of methods of determining virulence.

TABLE A Routes of injection of staphylococci.

Route of injection	Host	Dose of cocci	Evidence of disease	References
intra-peritoneal	guinea pig	10^7	infection of viscera	Foster (1962a)
	mouse	10^9-10^{10}	death	Christie, North & Parkin (1946) Lack & Wailling (1954) Anderson (1956) Fisher & Thompson (1956) Grogan & Artz (1961) Cohn (1962) Fisher & Goshi (1963)
	mouse) rabbit) rat) guinea) pig)		growth in sacs	Gladstone & Glencross (1960) Lam et al., (1963b)
intra-venous	mouse	10^7-10^{10}	death	Gorril (1951) Lack & Wailling (1954) Smith & Dubos (1956a) Grogan & Artz (1961) D. D. Smith (1962) (1963)
		10^7	infection of viscera	Gray et al., (1957)

Experimental infection in animals

The various routes of infection, the infecting doses and the animals used in published work are shown in Table A. This list is not exhaustive but presents examples drawn from the extensive literature on staphylococci; within each group the references are given in order of date. A thorough review of this subject prior to 1958 is given by Elek (1959).

Intraperitoneal infection. Infection intraperitoneally with large doses of staphylococci, but not micrococci, has been found to lead to death of the experimental animal within 6 to 9 hours (e.g. Cohn, 1962); the main cause of death in these circumstances appeared to be the alpha haemolysin (Christie, North & Parkin, 1946; Fisher & Thompson, 1956; Fisher & Goshi, 1963) but relationships to other staphylococcal products have been described (Lack & Wailling, 1954; Anderson, 1956; Grogan & Artz, 1961).

Studies of the production of various substances *in vivo* have been carried out using intraperitoneal sacs from which growth products could be withdrawn (Gladstone & Glencross, 1960; Lam *et al.*, 1963b).

Intravenous infection. Death of the animal may also follow the intravenous injection of large doses of staphylococci (e.g. Lack & Wailling, 1954) or more prolonged

TABLE A continued

Route of injection	Host	Dose of cocci	Evidence of disease	References
intra-dermal	rabbit	10^7-10^8	pustule	Burke & Miles (1958) Shilo (1962) Johnson, Cluff & Goshi (1961) Simon (1963) Elek & Conen (1957)
	guinea pig			
	man			
intra-muscular	guinea pig	10^5	abscess	Taylor et al., (1962)
	mouse	10^7	swelling and ulceration	Selbie & Simon (1952) Howard (1954) Gorrill & McNeil (1963)
sub-cutaneous suture	mouse	10^2-10^7	stitch abscess	James & MacLeod (1961) Rountree (1963a) Taubler, Kapral & Mudd (1963) McDade & Hall (1963)
	guinea pig			Taylor et al., (1962)
	rabbit			Robertson (1958)
	man			Elek & Conen (1957)
surface lesions	man	10^1-10^6	pus, erythema	Foster & Hutt (1960) May (1961)
	chick embryo	10^1-10^6	death	Jones (1946) Knothe (1952) McKenna & Taylor (1953) Frappier & Sonea (1953) (1954) Kienitz & Preuner (1958) Wiley (1961) McCabe (1962)

disease of the kidneys may result (e.g. Gorrill, 1951). Biochemical changes in the tissues may be followed if doses of 10^7 cocci are given, when death tends to be delayed for a few days (Gray et al., 1957).

There were usually very clear cut differences in the behaviour of the coagulase positive and the coagulase negative cocci on both intraperitoneal and intravenous injection; only the coagulase positive cocci caused disease (e.g. Smith & Dubos, 1956a). Observation of death in animals as a criterion of infection has two major disadvantages however. (1) The factors which cause death in an experimental animal may not be those which enable the staphylococcus to cause disease, such as wound infection, in man. (2) The death rate is closely dependent on the dose of cocci administered and large groups of animals must be used to overcome random fluctuations in dose and response. Against these must be set the advantage that observation of death is not influenced by subjective assessments made by the investigator.

Intradermal infection. The production of pustules in animals by intradermal injection of staphylococci appeared to require a dose of about 10^7 cocci unless prior inflammation had been induced (Burke & Miles, 1958; Johnson, Cluff & Goshi, 1961; Simon, 1963). Similar doses were needed to produce

pustules in man (Elek & Conen, 1957) and this topic is discussed more fully in the section on disease in man.

Intramuscular infection. Intramuscular infection of guineapigs with doses of up to 7×10^4 cocci failed to produce lesions (Taylor et al., 1962). However, mice injected intramuscularly in the thigh with doses of more than 10^6 staphylococci developed severe swelling and sometimes ulceration (Selbie & Simon, 1952; Howard, 1954). Measurement of the diameter of the infected thigh and comparison with the uninfected thigh enabled Selbie and Simon to draw up a table of virulence based on a graded response to the cocci. Using this technique Gorrill & McNeill (1963) found only a very small gap between the dose needed to cause 100% infection (5×10^6 cells) and the dose causing no infection ($1{\cdot}5 \times 10^6$ cells).

Infections produced using subcutaneous sutures. Stitch abscesses were produced using as few as 10 viable units of staphylococci when these were introduced subcutaneously into mice on suture material (James & MacLeod, 1961). Different materials varied in their ability to pick up the inoculum but cotton and silk were found to be satisfactory. Although James and MacLeod did not claim to detect differences other than those between staphylococci and micrococci by this technique, there appeared to be some differences between the response to

staphylococci lysed by phages 80/81 and 52/80/81 and the response to the other staphylococci tested.

Elek & Conen (1957) used this method to produce lesions in man and recently a number of workers have used this technique to study the pathogenisis of staphylococcal infections in animals (see Table A).

The growth of staphylococci in embryonated eggs

The use of chick embryos to study staphylococci has shown that very low doses of cocci, of the order of 10 viable units, cause death although large doses of micrococci may leave the embryo unharmed (Knothe, 1952; and others see Table A). Although at least the alpha haemolysin is produced in eggs (Jones, 1946) no relation has been found between death of the embryo and the in vitro production of alpha and beta lysins, quantitive production of coagulase (Kienitz & Preuner, 1958) or the presence of a capsule on the coccus (Wiley, 1961).

Experimental infections in man

The production of superficial lesions in man has required techniques very similar to those used in animals and references to infection in man are given under the appropriate methods in Table A. When staphylococci were injected intradermally into man (Elek & Conen, 1957) pus was formed consistently only when the dose was greater than 10^6 viable units of cocci.

Subcutaneous injection failed to produce lesions as did staphylococci injected into skin incisions or on to scarified or intact skin.

Elek and Conen found little or no difference in response on intradermal testing of 6 strains of staphylococci except at high dose levels (10^7 viable units) when differences were seen in the appearance of lesions caused by staphylococci from a stye and those from an outbreak of cross-infection in a nursery. No potentiation of virulence was found when mucin, plasma or starch was added to the inoculum as adjuvant. When the staphylococci were inserted on suture material however, marked potentiation of 'virulence' was seen. Severe stitch abscesses were produced using as few as 10^2 cocci. If the suture was stitched in and then removed, no lesion resulted, although it was shown that 80% of the inoculum remained in the skin.

Investigations of surgical sepsis (Clarke, 1957) showed that the incidence of wound infection was statistically significantly higher in lesions which had been closed with stitches than in those closed with clips and this was thought to be due to the presence of the foreign material in the wound.

Foster and Hutt (1960) found that experimental lesions made by scraping the epithelium from a small area of the forearm

of man produced lesions of a constant size which did not bleed but in which the capillary loops were visible. An overnight broth culture of staphylococci was used as the inoculum and the lesions were sealed with a coverslip held on with adhesive tape. Infections were produced with inocula as small as 15 viable units of cocci, a sero-purulent exudate being present with erythema at 24 hours; however no difference was detected between 5 strains of staphylococci using this test. Uninoculated lesions gave a serous exudate and an inoculum of micrococci gave a seropurulent exudate but neither caused erythema. If the lesions were allowed to dry, no exudate formed and after removal of the coverslips all the lesions dried up and healed in about a week. Even from an inoculum of 15 viable units the staphylococci multiplied to about 10^7 viable units within 24 hours. The coverslips were stained and examined and although evidence of phagocytosis was seen on all slides, most of the cocci were extracellular.

Similar studies, though of limited extent, were reported by May (1961), strains of staphylococci from bullous impetigo and from a boil were put on the forearm. When dry, the inoculum, a loopful of an 18 hour broth culture, was enclosed with a plastic curtain ring and covered with a cellophane window held on with adhesive tape. Lesions developed close to, or under the ring and tended to resemble the lesions from which the staphylococcus had been isolated.

Comparison of routes of infection

The effects of introducing staphylococci into mice by a variety of routes has been studied by several authors. Staphylococci given subcutaneously or intramuscularly were far less often lethal, except at high dose levels, than those injected intraperitoneally or intravenously. Some staphylococci were found to be more lethal by the intravenous route of infection than by the intraperitoneal route but for others the reverse was true. The fact that a strain was 'virulent' by one route of infection but not by another suggested that each route might measure a different attribute of the staphylococcus (Smith, Hale & Smith, 1947; Dutton, 1955; Bass & Higginbotham, 1960; Smith et al., 1960; Gorrill & McNeil, 1963).

Discussion and Summary

This thesis has the title "Staphylococcal infections in man and animals" and experimental work of this nature always has the disadvantage that the interest in the virulence of the staphylococcus stems from its behaviour in man, whilst the experimental work must usually be carried out in animals. It should be remembered that virulence or pathogenicity for one animal species does not necessarily imply virulence for another. There are some infective agents, for example the poliomyelitis virus, whose host range is almost completely restricted to man, and others, for example the salmonella species, where the host

range includes man, rodents, fowl, cattle, sheep and pigs. It is equally important to consider the route of infection; it has been demonstrated that some strains of staphylococci may be virulent by one route of infection but relatively avirulent by another and there is no evidence that the factors that cause death in a mouse following intraperitoneal infection are necessarily those involved in producing a boil or stitch abscess in man.

The number of deaths in man due to staphylococci is small compared to the amount of disease (e.g. Public Health Laboratory Service, 1960). It would seem therefore that techniques leading to the production of non-fatal disease in an animal would be more likely to give a result which had some relevance for man. Against this must be set the advantage that death is an absolute measurement and does not depend on subjective assessments.

As has been seen, very large doses of staphylococci are needed to cause skin infection by simple injection, but the use of cotton sutures has been found, by several workers, to reduce the dose needed to cause infection in both man and animals. It seems unlikely that, in the production of natural staphylococcal disease, more than a few staphylococci are introduced initially to the site at which the lesion is to occur. Potentiating agents such as sutures or crushed tissue may therefore be of considerable importance in the aetiology of staphylococcal disease.

From the experimental point of view, the most useful of the techniques described seem to be those employing infected sutures or the infection of surface lesions. Both these methods give rise to non-fatal infections in man and animals and in addition permit the use of dry inocula.

FACTORS WHICH MAY INFLUENCE THE VIRULENCE OF STAPHYLOCOCCI

In the following sections are discussed those factors peculiar to the staphylococcus which may be connected with the initiation of infection and whose variation might influence the degree of pathogenicity of the organism. Factors which may predispose the host to infection are discussed later.

A considerable number of factors have been described as influencing the virulence of the staphylococcus. These may be divided into two basic groups referred to here, for convenience, as 'markers' and 'virulence factors'.

MARKERS

Amongst the characteristics of a staphylococcus which may be regarded as 'markers' rather than as 'virulence factors' are sensitivity to phage, resistance to antibiotics and mercury salts and the production of pigment.

The relation between phage-type and virulence

The relation between sensitivity to phage and virulence is complex. There is no doubt that a great many epidemics of staphylococcal disease are caused by a few phage-types of staphylococci. For example, analysis of staphylococci sent to the Staphylococcus Reference Laboratory in the years 1954-1960 showed that 4 types were responsible for 50% of the maternity ward epidemics and 3 types for 50% of the epidemics in surgical wards. Since these types were responsible for only 25% and 21% of all lesions in the material submitted, it is clear that they had greater epidemic propensities than other strains (Williams, 1959; Williams & Jevons, 1961).

It seems reasonable to regard these 'epidemic' strains as more virulent than 'non-epidemic' strains although epidemicity might simply imply greater ease of transmission. If there were a direct relationship between phage lysis and virulence it might be expected that all staphylococci lysed by one particular phage would be virulent. However Williams and Jevons (1961) found for example that staphylococci lysed by phage 80 only (81 was not considered) caused 10.5% of the sepsis in hospitals and those lysed by phages 52/52A/80 and 52/80 caused 7·1% of the infection. Staphylococci lysed by phages 29/52/80 however caused only 0·5% of the sepsis. Thus it seems that lysis by one particular

phage cannot be correlated with the ability to cause disease. There is a general correlation of virulence with phage pattern however and it may be that multiple pathways for phage absorption and lysis exist.

The type 80 staphylococcus was first isolated in Australia (Rountree & Freeman, 1953) and soon spread to other countries but did not become common in Britain until 1955 (Rountree, 1963c). Staphylococci lysed only by phage 80 can be produced from similar strains by lysogenic conversion (e.g. Rountree & Asheshov, 1961), but it is impossible to determine whether type 80 appeared in this way more or less simultaneously in several countries or whether it spread from Australia by a form of world-wide cross-infection. Similar changes in the incidence of other phage-types of staphylococci have been observed (Parker & Jevons, 1963).

In summary, some phage-types of staphylococci are more frequently causes of epidemics than others. It seems possible that exceptionally virulent mutants might arise which could spread through a large population; whether this could happen on a world-wide scale is not known. Virulence is probably not related directly to susceptibility to a particular phage or phages but both may be related to some other characteristic of the strain.

The relation of antibiotic resistance to virulence

Many of the staphylococci which cause sepsis in hospitals are resistant to antibiotics, particularly penicillin and tetracycline (see for example Williams et al., 1962). It seems possible that this is due to the selective advantage which resistance to antibiotics confers on the staphylococcus, for Barber et al., (1960) showed that if the use of particular antibiotics was restricted, sepsis occurred with antibiotic sensitive rather than resistant strains. Antibiotic resistant strains also appear to persist less well in carrier sites in persons discharged from hospital than do the sensitive strains (Noble et al., 1964). There seems to be no reason why resistance to antibiotics as such should affect the virulence of a strain except in a hospital where many of the potential hosts of the coccus are under treatment with antibiotics. It may be, however, that strains showing resistance to antibiotics are those which are most active biochemically or those with the highest mutation rates (Barber & Whitehead, 1949; Barbour & Edwards, 1953).

Thus in the absence of antibiotics we might expect the production of sepsis to depend on the virulence of the staphylococcus and the susceptibility of the host; whilst in the presence of antibiotics, resistance to antibiotics is also necessary. Communicability, the ability of the staphylococcus to become

established in carrier sites such as the nose or perineum rather than the ability to cause septic lesions, may well be influenced by resistance to antibiotics, e.g. among many other authors Williams et al., (1962) showed that the nasal carrier rate of staphylococci resistant to penicillin and tetracycline rises with the length of stay in hospital.

Communicability. In a discussion of the communicability of a staphylococcus it is important to take account of the fact that some patients (dispersers) have an ability to disseminate staphylococci which is markedly greater than normal. This appears normally to be a function of the patient rather than of the staphylococcus and although it can contribute to the spread of staphylococci in a hospital ward, it may have no importance in a consideration of virulence (Hare & Ridley, 1958; Noble, 1962; Davies & Noble, 1962, 1963 and in preparation). Papers relating to this work are to be found in Appendix 3 of this thesis.

Resistance to mercury salts as an index of virulence

Strains of staphylococci which might, on the basis of phage-type, be regarded as potentially epidemic for man were found by Moore (1960) to be more often resistant to mercuric chloride than strains which were 'non-epidemic'. Moore found that 76% of phage-group I strains and 40% of group III strains, but only 6% of group II strains, were able to grow in the presence

of mercury. Staphylococci lysed by phages from more than one group were all sensitive to mercury but 37% of non-typable strains were resistant. Within the phage-groups strains of 80/81 and 52/52A/80/81 were almost uniformly resistant to mercury but strains of 52A/79 were normally sensitive. Hence the relation was with the staphylococci which spread in hospitals rather than those which spread in a non-hospital population.

Moore noted that some coagulase negative strains were resistant to mercury and this was confirmed by P. B. Smith (1962). Moore found no relationship between sensitivity to mercury and either the egg-yolk reaction (Gillespie & Alder, 1952) or the serum opacity test (Tomlinson & Parker, 1956).

Using Moore's technique, Green (1962) tested 717 strains of staphylococci from patients and from the hospital environment. The distribution of resistance in relation to phage-type was the same as in Moore's series except for local changes in epidemic type. Turner and Willis (1962) agreed with Moore's finding that staphylococci associated with hospital infections tend to be mercury resistant, but suggested that the value of the test lies only in its ability to detect strains resistant to many antibiotics. This view is also held by Akinlade (1962) and Jessen et al., (1963).

It seems likely that, taken in conjunction with the coagulase test, Moore's test may give some idea of the

epidemic potential of a staphylococcus for there is a strong correlation of mercury resistance with resistance to more than one antibiotic; it is these strains which are most often responsible for outbreaks of staphylococcal disease in hospitals.

Pigmentation and virulence

The production of a golden pigment was the character which led Rosenbach to give the pathogenic staphylococcus its specific name. The species is now delineated by the production of coagulase and although the majority of coagulase positive strains produce the golden pigment, the correlation is not perfect (Elek & Levy, 1950; Shaw, Stitt & Cowan, 1951).

Barber (1955) found that strains with 'aureus' pigment isolated from a parent strain with 'albus' pigment, whilst resembling the parent in the production of alpha lysin, free coagulase and sensitivity to phage, differed in the production of bound coagulase and in being more resistant to antibacterial agents. White mutants from aureus strains generally resembled the parent except that they had lost the ability to produce bound coagulase. It was thus tempting to suggest that a series existed from the golden pigmented 'virulent' strains to the white pigmented 'avirulent' strains.

The production of pigment on tryptose phosphate agar as a means of distinguishing between staphylococci (Sompolinsky, 1962)

showed that the intensity of pigmentation diminished when the strains were stored and that the production of pigment was not always the same in otherwise identical strains.

Recently there have been suggestions that production of yellow pigment (probably distinct from the 'aureus' pigment) on glycerol monoacetate agar is related to the resistance of the staphylococcus to penicillin and tetracycline (Willis & Turner, 1962; Turner & Willis, 1962). It has been shown that the yellow pigmented 'epidemic' strains are physiologically distinct from the orange strains (Jacobs, Willis, Ludlam and Goodburn, 1963; Willis, Jacobs & Goodburn, 1964).

It is not clear to what other factors, if any, the production of pigment on glycerol monoacetate agar is related. There appeared to be no direct relation to coagulase or haemolysin production or to the ability to cause opacity on egg-yolk or human fat agar. On ordinary media, pigment production has been related to the presence of fermentable carbohydrate (Serag & Green, 1944; Brown & Harris, 1963) or to milk (Christie & Keogh, 1940; Chapman, 1943).

VIRULENCE FACTORS

The general concept of a virulence factor, as applied here, is of a product of the staphylococcus which in some way modifies the host tissue or otherwise helps the staphylococcus

to become established in host tissue. As will be seen, the search for a single factor which would explain differences in virulence between staphylococci has led to a great many paths being explored. Yet in 1948 Blair wrote:

" ... the pathogenicity of staphylococci involves their capacity to produce toxins and their ability to become established in body tissue. Usually the pathogenic capacity of a given strain represents the total effect of both factors working together; ... Finally the ability of staphylococci to cause infection is conditioned by the relative susceptibility of the host and the efficiency of his defense mechanisms."

In the following sections the various factors are treated separately but the view is taken that the 'virulence' of a strain is the observed result of the sum of a number of abilities.

The role of the haemolysins

Haemolysins have a deleterious effect on some red blood cells and have thus attracted a great deal of attention. Confusion arose in early work because the selective action of the various haemolysins on red cells was not appreciated; Elek (1959) considered however that only 3 haemolysins need be postulated to explain observed haemolysis. The alpha lysin acts best on rabbit, sheep, cow and goat red cells and not at

all on human, guineapig or horse cells. The beta lysin is characterised by the 'hot/cold' lysis of sheep red cells and has no action on rabbit cells. The delta lysin acts on many cells including horse and rabbit.

Alpha haemolysin

There are two aspects of the production of alpha lysin which are of interest in the context of this thesis. One is the frequency with which this lysin is produced by strains of staphylococci from clinical sources, although this must of necessity be confined to 'retrospective' studies; and the other is the direct observation of the effect of the lysin on experimental animals.

Comparison of sources. There is a very close correlation between the production of alpha lysin and coagulase. It has variously been reported that from 82% to 100% of coagulase positive strains of staphylococci from human sources produce the alpha lysin but these reports differed, not only in the source of the strains, but also in the methods used to demonstrate the presence of the lysin. Grading of staphylococci according to the site from which they were isolated showed that strains from lesions, presumably the most virulent, produced more alpha haemolysin than those from carrier sites (Brown, 1960). However, correlation does not imply causation and Forssman (1938) has expressed the

opinion that alpha haemolysin might be harmless for man despite its action on rabbit erythrocytes in vitro.

Experimental observations. The intravenous injection of alpha lysin in sufficient quantities causes death of an animal in a very short time and immunization against the lysin prevents or delays the death of animals injected in this way (e.g. Burnet, 1929; Conti, Cluff & Scheder, 1961; Lithander, 1961; for other early papers see Elek, 1959). The toxin has been described as acting on smooth muscle (Thal & Egner, 1961), on the vascular system in general (Thal & Egner, 1956; Brown & Scherer, 1958) on the vascular system of the kidney (e.g. Rigdon, Joyner & Rickets, 1934; Thal, 1955) and on the lungs (Jackson, Gibbons & Magner, 1958). The pathology of the lungs was said to resemble that of the human lungs following acute staphylococcal pneumonia.

Injection of alpha lysin intradermally in animals results in necrosis (e.g. Goshi, Cluff & Johnson, 1961) unless the animal's serum contains antibody against the alpha lysin, when only erythema results. Smith, Goshi, Norman and Cluff (1963) found, however, that the reactions in man to the injection of alpha lysin were not like those seen in animals. The reaction was biphasic and consisted of a red wheal and a flare. The histology on biopsy was indistinguishable from that seen following

the injection of pollen antigens; marked infiltration of the area with leucocytes had occurred. Smith and his colleagues noted however that none of the human sera that they examined were entirely free of anti-alpha lysin and this may have been responsible for differences in reaction.

Injection of mice with very large doses of staphylococci intraperitoneally also causes death in a very short time and it has been suggested that this is due to alpha lysin. However the addition of large quantities of antitoxic serum gave little protection to mice so challenged (Fisher & Goshi, 1963) and it was concluded that either the lysin was absorbed on to tissue or that some other toxin was responsible for the death of the mice. Sellers and LeMaistre (1960) found that alpha lysin was a constant feature of 'virulent' staphylococci but suggested that the rapid death observed in animals when 10^9 cells are injected intravenously, may be due to competition between the host and bacterium for some enzymes or substrates. Animals may die from staphylococcal infections despite the presence of anti-alpha-haemolysin in the blood (Smith, 1937; Ekstedt, 1963).

Intraperitoneal infection of mice with staphylococci from clinical sources showed that virulence, measured in terms of the death rate (LD_{50}), correlated best with those strains which manufactured a wide range of extracellular products

including the alpha lysin (Lack & Wailling, 1954; Howard, 1954; Anderson, 1956). D. D. Smith (1962), using well established laboratory strains of staphylococci, found no correlation between the various haemolysins, the number of antigen/antibody flocculation lines and the LD_{50} for mice measured by intravenous infection. As discussed earlier, however, 'virulence' measured by one route of infection may not be the same as by another and Smith's investigations differed from those of Lack and others not only in the source of the staphylococci, but also in the method of challenge. Foster (1963) failed to find any relation of in vitro production of alpha lysin to infection of guineapigs.

Apart from such rare incidents as the Bundaberg Disaster, however, it seems unlikely that large doses of staphylococci or toxic products of staphylococci are ever introduced into man or animals (staphylococcal food poisoning is not considered here). The more relevant role of the alpha lysin is the part, if any, that it plays in the infection of, for example, surgical wounds.

The virulence of staphylococci injected intramuscularly into mice has been found to be correlated with the production of alpha lysin, and the presence of coagulase, fibrinolysin and beta lysin appeared to enhance the 'virulence' (Selbie & Simon, 1952; Howard, 1954). Other studies have indicated that strains

possessing alpha and delta lysins were more virulent than strains possessing either lysin alone (Marks & Vaughan, 1950; Johanovsky, 1958).

Using infected sutures in mice Taubler, Kapral and Mudd (1963) found that alpha lysin appeared to be necessary for the production of a lesion, for mutants which produced little lysin gave fewer lesions than the parent strain. Active immunization with toxin gave protection against challenge, but mice passively immunized with anti-alpha lysin were not completely protected. This suggests that whilst alpha lysin may be necessary for the production of lesions, it is not the only factor involved. Lambert (1960) reached similar conclusions using intradermal infection of guineapigs.

The beta haemolysin

In contrast to alpha lysin, the beta haemolysin is not so commonly produced by strains of staphylococci of human origin, although it is more commonly produced by strains from animal sources. Beta lysin was found to be less toxic for mice and rabbits than the alpha lysin (Bryce & Rountree, 1936) and was thought not to influence the virulence of staphylococci for animals (Howard, 1954; Lack & Wailling, 1954; Derbyshire, 1963). As discussed earlier, however, strains showing a broad spectrum of extracellular products are more virulent than those with a narrow spectrum. The beta lysin could well be regarded as being unnecessary for lesion production but may act synergistically

with other coccal products. Beta and delta lysins have been reported as acting synergistically on human red cells (Elek, 1959). Christie and North (1941) reported the same for alpha and beta lysins and one may speculate whether their staphylococci also produced delta lysin or whether both the alpha and delta lysins are able to act synergistically with the beta lysin. It has been reported that strains producing beta lysin do not produce fibrinolysin (Rountree, 1947), but this has been denied (Levy, 1952).

The delta haemolysin

Williams and Harper (1947) described a third haemolysin which was not neutralised by alpha or beta antitoxin. This 'delta' lysin is produced by most strains of staphylococci isolated from human sources and, on qualitative grounds, the correlation with virulence is as good as that of the alpha lysin. Delta lysin is known to be lethal to human leucocytes (Gladstone & van Heyningen, 1957).

The role of coagulase in the virulence of staphylococci

The production of coagulase has long been accepted as the marker of potential virulence among the Gram-positive cocci, but it is not proposed here to deal with the various pieces of evidence which have led to this, for they are summarized by Elek (1959). The test for coagulase may be performed either by adding human plasma to a thick suspension of staphylococci on

a microscope slide (Williams & Harper, 1946) or by incubating a culture of staphylococci in broth or saline containing 10% human plasma (Fisk, 1940). A positive result with either test indicates coagulase production for clinical purposes, but Duthie (1954) suggested that staphylococci possess two coagulases and that the slide test measures 'bound' coagulase or 'clumping factor' whilst the tube test measures 'free' coagulase. Strains of staphylococci may be encountered which lack either one of these coagulases, but most specimens isolated in clinical practice possess both. Recent work has suggested however that 'bound' coagulase may not act on the fibrinogen complex as does the 'free' coagulase (Brown & Faruque, 1963).

It has been suggested that only those species of animal whose plasma is coagulated by staphylococci are susceptible to infection with this organism (Smith & Hale, 1944; Hale & Smith, 1945; Smith, Hale & Smith, 1947). Staphylococci which coagulated guineapig plasma were found to be more virulent for guineapigs than those which failed to coagulate guineapig plasma. Staphylococci injected into mice in coagulable plasma appeared to be more virulent than when injected in serum, and phagocytosis of staphylococci was inhibited in the presence of plasma but not serum, although phagocytosis of other organisms was not affected.

The variation in coagulability of plasma from different animal species by staphylococci is a result of the presence or absence of an 'activator' or 'coagulase reacting factor'. There is less activator in human serum than in human plasma (Madoff & Weinstein, 1962) and similar variations in content or the presence of anticoagulants might have had some effect on the differing reactions observed when staphylococci were injected into animals suspended in serum or plasma.

MacLeod, Hall and Frohman (1963) observed that citrate inhibited the growth of staphylococci and that heparin prevented the bactericidal action of serum; Sanders (1963) reported that in vitro the action of coagulase was inhibited by heparin. It would therefore seem unwise to assume that plasma differs from serum solely in the fibrinogen content. Mouse plasma is weak in activator and addition of human serum to mouse plasma was found to enable staphylococci to clot the plasma (Fusillo & Weiss, 1963). Human plasmas too may differ in coagulability and it has been suggested that mixed human and bovine plasma should be used in routine tests (Illes, 1964).

Lack and Wailling (1954) challenged mice intraperitoneally with staphylococci suspended in either serum or plasma from the same human donor, but failed to find any relationship between the menstruum and virulence. They point out, however, that they used lower doses of staphylococci than did Smith, Hale and Smith.

Possession of both free and bound coagulase was found by Alami and Kelly (1960) not to be important in the initiation of infection when staphylococci were introduced intraperitoneally or intravenously. They found that the addition of mucin to the intraperitoneal injection increased the virulence of free coagulase positive, bound coagulase negative cocci but not that of the other combinations of coagulases.

Foster (1962a and b) injected mixtures of Staph. aureus which were coagulase positive or negative for guineapig plasma intraperitoneally into guineapigs, but could find no difference in the rate or degree of phagocytosis of the cocci by the reticulo-endothelial system, when the animals were examined at intervals after infection. There was some difference in the ability of the strains to multiply in the kidneys following intracardial injection, but this was not related to the coagulase ability of the cocci for guineapig plasma.

Gorrill (1951) found an almost complete correlation between the ability of staphylococci to coagulate human and mouse plasma by both the slide and tube test, on a total of 12 coagulase positive and 8 coagulase negative strains. When injected intravenously in a dose of about 40 million cells, the coagulase positive strains proved the most virulent for mice as

judged by the death rate. In this instance the ability of staphylococci to kill mice was strongly correlated with the ability to coagulate plasma from species other than that used in the virulence test and no species difference was demonstrated.

By contrast only 1 of 75 strains coagulating human plasma tested by Selbie and Simon (1952) gave a typical clot in the tube test using mouse plasma, although slide coagulase tests using mouse plasma gave results indistinguishable from those observed using human plasma. Those strains that gave a strong coagulase reaction with mouse plasma generally caused intramuscular lesions in mice which were larger on the first day, more progressive on the second and more regressive on the fourth and seventh days. The effect of coagulase on virulence was additive to that of the alpha lysin. A similar conclusion was reached by Coutinho and Nutini (1963).

Production of coagulase was found to be of prime importance in the initiation of infection by intraperitoneal staphylococci by Anderson (1956). Using laboratory strains of staphylococci of human origin, D. D. Smith (1962, 1963) found no relation of coagulase (for mouse or human plasma) to virulence as measured by death of mice following intravenous infection. However, staphylococci isolated from sheep coagulated mouse plasma in the tube test more firmly than did strains of human origin.

The sheep strains were also more 'virulent' for mice than the human strains; bovine strains occupied an intermediate position in both tests.

Some studies on staphylococcal mutants which possessed either free or bound coagulase (Alami & Kelly, 1960; Kapral & Li, 1960; Li & Kapral, 1962; Karas & Kapral, 1962) indicated that either of these factors could be absent without altering the virulence, as compared with the parent strain, when staphylococci were introduced by a variety of routes into mice and rabbits. Further, Kapral and his colleagues isolated an 'avirulent' strain which possessed both bound and free coagulase and like all the other mutants, possessed all the biochemical properties of the parent strain. This would seem to suggest that none of the extracellular products investigated had any effect on virulence either, unless quantitative differences in enzyme production existed.

The use of mutants, however, involves the danger that some other factor may change unnoticed. In another study on mutants it was found that the 'mutant' which possessed neither bound nor free coagulase had also lost its susceptibility to phage and changed its sensitivity to 6 antibiotics (Iam et al., 1963a). It seems difficult to accept this strain as a mutant of a parent Staph. aureus and it is clear that a great deal of care is needed in interpreting the results of such studies.

The suggestion of Hale and Smith (1945) that staphylococci were protected from phagocytosis by the presence of coagulable plasma aroused the interest of many workers. Comparison of the results is made difficult by the fact that various workers have used a variety of leucocytes from different animals. Some workers found that a specific serum factor was necessary for phagocytosis of staphylococci but not micrococci. Others found that *in vitro* the presence of fibrin had no effect on phagocytosis and that staphylococci can multiply in leucocytes under some circumstances. In general all this work has swung away from the role of coagulase and has concentrated on the specific serum factors, the opsonins, which enhance the readiness with which staphylococci are phagocytosed. A detailed discussion is outside the scope of this thesis, but see for example Rogers & Tompsett (1952), Cohn & Morse (1959), Kapral & Shayegani (1959), Rogers & Melly (1960), Melly, Thomison & Rogers (1960), Mackaness (1960), Rogers (1963).

The growth of micrococci was found to be inhibited in serum but that of staphylococci was not (Ekstedt & Mungester, 1955; Ekstedt, 1956a and b; Yotis & Ekstedt, 1959, 1960; Ekstedt & Yotis, 1960; Fletcher, 1962). Differences in nutrition in the serum were ruled out but it was found that partially purified coagulase added to the system stimulated the respiration of both

coagulase positive and coagulase negative cocci, although the stimulation was twice as great for the coagulase negative species. The inhibitory effect of serum on growth appeared to be due to a water soluble fraction. When this was purified it was found to inhibit the oxidation of glucose by Bacillus subtilis and Micrococcus lysodeikticus and to inhibit the growth of micrococci. This activity could be reversed by the addition of coagulase to the micrococci and B. subtilis but not to M. lysodeikticus. Mice challenged intracerebrally with coagulase negative cocci were found not to be killed unless coagulase was added to the inoculum but the virulence of streptococci was not enhanced by the addition of coagulase nor was the coagulase effective if given intravenously when the micrococci were inoculated intracerebrally.

Boake (1956) and Lominski et al., (1962) found that anticoagulase increased the resistance of animals to intravenous challenge with staphylococci, but not micrococci, if the antigen was prepared from the same phage-group of staphylococcus as the challenge strain. Others have shown that different phage-groups of staphylococci possess antigenically different coagulases (Barber & Wildy, 1958; Henderson & Brodie, 1963; Harrison, 1964).

Human serum may contain antibodies to many staphylococcal products such as haemolysin, coagulase and leucocidin (Lack & Towers, 1962) and has been shown to confer some resistance in

mice to infection with lethal doses of staphylococci (Fisher, 1959). Fisher's results might possibly underestimate the efficacy of the serum since he challenged the mice with the Smith strain of staphylococcus which possesses a very distinct capsule which contributes to its pathogenicity. But, equally, human serum has been shown to possess antibody to this type of material and these opsonins might explain why the protective effect was observed (Rogers, 1963).

Coagulase itself may have a pathologic effect on tissue; coagulase injected intravenously produced fibrin depletion in rabbits but not in mice, which lack activator (Smith & Johnson, 1958). Coagulase was demonstrated in precipitated material by Blobel and Berman (1961) using fluorescein labelled anticoagulase.

In summary then, cocci which cause disease usually possess the ability to produce coagulase (or some factor very closely linked to coagulase). No satisfactory studies have been carried out on mutants which lack both bound and free coagulase but are nevertheless recognisable as strains of Staphylococcus aureus. The action of the coagulase may be to cause mechanical blockage of the tissue but coagulase also appears to act as an antagonist to the opsonins which are essential for the phagocytosis of staphylococci.

The egg-yolk reaction

The production by some staphylococci of opacity in broth containing egg-yolk was first noted by Gillespie and Alder (1952). The reaction was neutralised by high dilutions of commercial antitoxin and appeared to be due to a lipase. Many strains of staphylococci gave a reaction but none of the micrococci did so. It was found that strains from lesions were more often egg-yolk positive than were strains from nasal sources (Alder, Gillespie & Herdan, 1953; Alder, Gillespie & Thompson, 1955).

However, Willis and Turner (1962) who compared lipolytic activity on egg-yolk agar, tributyrin agar and human fat agar, found that the results were the same on all three media, showing that the enzyme was a lipase and that coagulase negative strains gave similar results to the coagulase positive strains. They did not agree with Parker's (1958) finding that phage-type 71 staphylococci were egg-yolk negative. Parker had used Gillespie and Alder's broth method. Willis and Turner concluded that egg-yolk could not be used to distinguish between staphylococci.

These apparently conflicting results were explained by Shah, Russell and Wilson (1963) on the grounds that the agar plate method was far more sensitive than the broth method. They compared the two methods and observed that 89·2% of 204 strains

were positive by the plate method compared to only 59·8% by the broth technique. About 7 times as much enzyme appeared to be produced by those strains which were positive on both media as by those positive on agar only.

The mortality rate in patients with staphylococcal bacteraemia was found by Rosendal (1963) to be highest in those infected with egg-yolk negative strains (measured by the broth technique). It was suggested that the virulence factor may be related to an unknown enzyme which destroys the lipase in the egg-yolk negative cocci.

There is thus conflicting evidence regarding the relation of virulence to the egg-yolk reaction. Strains from lesions produced more lipase than strains from carrier sites, but the existence of an enzyme which destroys lipase has been postulated.

The importance of hyaluronidase

The importance of hyaluronidase as a spreading factor was discussed by Elek (1959) who came to the conclusion that the association was not with the 'invasive cocci' but with the group of cocci which also produced coagulase, haemolysin etc. Schwabacher et al., (1945) found that, whilst the production of hyaluronidase was no more frequent in strains from lesions than in strains from carrier sites, it was more abundant in the strains from lesions.

Strains of staphylococci causing impetigo were found by Jessen et al., (1959a and b) generally to produce more hyaluronidase than strains from bacteraemia or furunculosis, whilst nasal strains produced little by comparison; production of hyaluronidase was associated with strain rather than with source however. For example, phage-type 71 strains produced much hyaluronidase; they are also associated with impetigo and tend to have other features in common such as the ability to inhibit the growth of corynebacteria (Parker, 1958; Faber & Rosendal, 1960).

The importance of fibrinolysin

It has not proved possible to find any direct relation between the production of fibrinolysin and virulence (up to 80% of coagulase positive strains may produce fibrinolysin). It is another character, however, which appears more frequently in those strains considered 'virulent' than in those considered 'avirulent' (Selbie & Simon, 1952; Lack & Wailling, 1954; Jacobs, Willis & Goodburn, 1963).

The role of leucocidin

Leucocidins appear to be produced by about 50% of staphylococci isolated from human lesions. Leucocidins have been described as having a negative chemotactic effect on leucocytes and, in higher concentrations, of killing the

leucocytes of some species but not of others (Gladstone & van Heyningen, 1957). This appears to be another factor which has an obvious 'use' in the production of infection but which is not essential for lesion production.

Recently there has been a revival of interest in leucocidin. Delta haemolysin kills leucocytes and its presence would therefore interfere with the direct determination of leucocidin, but the presence of serum may be used to prevent the action of the delta lysin (Gladstone & van Heyningen, 1957). The most satisfactory method of determining leucocidin appears to be that of Woodin (e.g. Woodin, 1961) which separates the leucocidins and estimates the two components separately, but this method is not easily applicable to surveys of staphylococci.

Studies have shown that patients with staphylococcal infections show a rise in antileucocidin titre (e.g. Lack & Towers, 1962; Gladstone, Mudd, Hochstein & Lenhart, 1962) and Bänffer (1962) found that puerperal mastitis occurred significantly more frequently in mothers with low titres of antileucocidin although no relation to the anti-alpha-haemolysin was found.

Encapsulation and virulence

Various studies have been reported suggesting that encapsulated staphylococci able to resist phagocytosis are more virulent, measured by the lethal effect of intraperitoneal

infections, than unencapsulated variants (Gilbert, 1931; Lyons, 1937). As emphasised elsewhere, however, the use of variants is not without danger. More recently, immunization of animals with capsular material was found to result in protection against challenge with encapsulated strains (e.g. Stamp, 1961; Morse, 1960, 1962), but not necessarily against other strains (Fisher, Devlin & Erlandson, 1963). Measurement of the lethality of encapsulated staphylococci for embryonated eggs indicated that although the capsule protected the staphylococcus against phagocytosis, some other factor was responsible for the death of the embryo (Wiley, 1961).

Price and Kneeland (1954, 1956) reported that a capsule could be demonstrated by capsular swelling techniques on most strains of staphylococci, but not on micrococci, and that the presence of a capsule bore no relation to virulence. Although it has been shown that a 'pseudocapsule' composed of growth products, but chemically unlike the polysaccharide capsule of the Smith strain, can be produced on staphylococci (Sall, Mudd & Taubler, 1961) this does not necessarily explain Price and Kneeland's observations. In vitro the polysaccharide capsule appears to be confined to a few strains of staphylococci all of which closely resemble the Smith strain (Fisher, Devlin & Erlandson, 1963; Rogers, 1963). Rogers believed that these represented the few

strains whose capsule is stable *in vitro* and that *in vivo* many staphylococci may exhibit a capsule, for most human sera are capable of opsonising phagocytosis of the Smith strain. This belief has been challenged; Morse (1963) failed to detect any Smith surface antigen on 25 strains of staphylococci from clinical sources, although this approach fails to provide any information on *in vivo* conditions.

Warren and Gray (1963) found that the incorporation of subinhibitory levels of antibiotics into media with subsequent heating of the staphylococcal culture released polysaccharide which resembled chondroitin sulphate into the medium. They suggested that the action of the antibiotic may be to prevent incorporation of the material into the mucopolysaccharide component of the cell wall; the heat acting merely to bring about release of the material from the cell. We may speculate that if antibodies present in human serum were also able to interfere with cell wall synthesis, polysaccharide might be released *in vivo* and this might explain the presence of opsonins to staphylococci such as the Smith strain in human sera, without the staphylococci necessarily possessing an actual capsule.

Chemical analyses of staphylococcal capsules have yielded a variety of results, perhaps because there is a variety of capsules. Wiley and Wannacott (1962) found an encapsulated

strain of staphylococcus to have a capsule including 8% phosphorus and no nucleic acid. This is unlike the Smith polysaccharide capsule investigated by Morse (1962) which had no phosphorus but moderate amounts of amino acids. Further, Clausen and Rosenkart (1962) reported the presence of hyaluronic acid, a well known capsular material, on some staphylococci.

Respiration rates

Gellenbeck (1962) found that the general respiration rate of bacteria from tissue was higher than that of the parent staphylococcus grown on laboratory media. The endogenous respiration rate of the staphylococci on media was about 50% higher than the tissue staphylococci however. The substrate for endogenous respiration appears to be the free-pool amino acids (Ramsey, 1962; Kedzia, 1962a). Kedzia also found that the endogenous respiration rate of staphylococci isolated from lesions was higher than that of staphylococci isolated from carrier sites. Studies on mutants with impaired respiration (Gause, Kuchetkova & Vladimirova, 1961) suggested that metabolism of the flavins was disturbed when the respiration was abnormal.

It seems reasonable to suppose that the metabolism of 'virulent' staphylococci is different from that of avirulent cocci, but very precise techniques may be needed to demonstrate this difference.

Other tests for virulence factors

Many of the following tests have a very strong correlation with coagulase production and appear to reinforce the distinction between coagulase positive and coagulase negative cocci rather than to distinguish between strains of the coagulase positive species. They are included under one heading for the sake of convenience.

There is a very good correlation between phosphatase production and coagulase production (Barber & Kuper, 1951). During a survey of staphylococci from nasal sources it was found (Noble, unpublished) that only 3 of 618 coagulase positive cocci did not give a phosphatase reaction after 18 hours incubation. The proportion of coagulase negative species which gave a positive result was about the same (0·5%). Willis, Jacobs and Goodburn (1964) reported that about 80% of coagulase negative cocci produce phosphatase. However, these differences might be explained on the basis of 'observer variation' in the level at which a positive result is read.

Recently it has been suggested that there are quantitative differences in phosphatase production which are related to antibiotic sensitivity and phage pattern (Gillissen & Ruda, 1958; Cannon & Hawn, 1963). A test for arginase production was positive in 69/70 coagulase positive strains and 4/25 coagulase negative strains (Soru et al., 1957).

There was a strong correlation between the production of desoxyribonuclease and coagulase (DiSalvo, 1958; Burns & Holtman, 1959; Jacobs, Willis & Goodburn, 1963).

Myers (1959a) described a test based on the ability of cocci to reduce ammonium molybdate and this appeared to be related to the pathogenicity of the strains for mice. Hertz and Waite (1961) however could find no advantage of this test over the coagulase test.

Reduction of tellurite was found to be common in strains which produced coagulase (Hoeprich, Croft & West, 1960). Unfortunately, the selective medium in which the tellurite was incorporated was too inhibitory to be used for the direct isolation of staphylococci; this is a feature shared by the many media based on tellurite reduction (Williams, Blowers, Garrod & Shooter, 1960).

Nordebring (1963) found that certain phage-group III strains of staphylococci, resistant to antibiotics, had an increased tolerance of gentian violet. This is reminiscent of the resistance to mercury discussed earlier.

Complete agreement between mannitol fermentation, which is itself correlated with coagulase production, and fluorescence under Woods light when staphylococci were grown on a medium containing fluorescein amine was demonstrated by Kimler (1961).

Thirty strains from each of three sources, septicaemia, boils and nasal carrier sites were compared for acetoin production by Kedzia (1962b). Acetoin production was greatest in the septicaemia group and least among the nasal strains and was not associated with differences in the growth rate.

Finally, Krynski et al., (1962) suggested that virulence was associated with higher respiration rates and greater biochemical activity as determined by a battery of tests but felt that individual strains could not be tested in this manner.

Immunity to staphylococcal infections

There are two aspects of immunity to staphylococci which are of interest here. One is the change in response on repeated infection and the other is immunity induced by inoculation of dead cocci or some fraction or product of the cocci.

Repeated infection of the skin of rabbits with serial doses of staphylococci was found by Panton and Valentine (1929) to lead to coexistent immunity and hypersensitivity. Whilst the reactions to pus-forming doses of staphylococci injected into the skin became less severe, the animals exhibited hypersensitivity to doses which had previously failed to cause a reaction. Associated with this was protection, by no means

complete, in the form of a delay of death following intravenous injection of lethal doses of cocci. Few of the rabbits developed antibodies to the alpha haemolysin. Hypersensitivity was also reported by Johnson, Cluff and Goshi (1961), but these authors do not mention immunity.

A number of reports can be found supporting the theories of either antitoxic or antibacterial immunity and those prior to 1958 are summarized by Elek (1959); for example Fisher (1957) and Lithander (1961) showed that mice and rabbits immunized subcutaneously with staphylococcal toxoid were protected against intraperitoneal challenge with large doses of staphylococci. Antitoxin levels were higher in animals given undiluted toxoid (they also survived better) than those given diluted toxoid. On the other hand, Stamp (1961) found that immunization with a strong coagulase producing, weak alpha lysin producing strain protected rabbits against challenge with this strain and that the protection was not related to the antitoxin levels.

Studies on the immunization of mothers and babies with alum precipitated staphylococcal toxoid containing leucocidins were carried out by Johanovsky and his colleagues (Schubert & Johanovsky, 1957; Sebek, Schubert & Johanovsky, 1958) who showed that immunization reduced the number of breast abscesses and other neonatal infections below that of the control group although protection was far from complete. Chlouverakis, Griffiths &

Swift (1963) could find no relation between anti-alpha haemolysin titres and staphylococcal infections in newborn infants. It is curious that these workers found the titre to be higher in the infants blood than in the mother's blood.

Brodie, Guthrie and Sommerville (1958) found that immunization with attenuated live vaccine protected rabbits against challenge with the same strain but not against challenge with staphylococci of a different serotype.

Immunity to challenge with the Smith strain of staphylococcus was related to the ability of the serum to promote phagocytosis of the cocci (Koenig, Melly & Rogers, 1962; Ekstedt, 1963). Immunization against the Smith strain appeared to be specific in that it did not protect animals against challenge with other strains.

If the suggestion made earlier, that the virulence of staphylococci is the result of the expression of a number of different abilities, be accepted, it is not difficult to reconcile these apparently conflicting results. Where the virulence of the strain is largely due to its resistance to phagocytosis, the production of opsonins will confer some protection against challenge. Where the strain produces much alpha lysin then antitoxin will confer protection and so on.

Inhibition of staphylococci by streptococci

Inhibition of staphylococci by viridans streptococci was reported by Myers (1959b) who suggested that exposure of staphylococci to 'antibiotics' produced by streptococci or other oral flora might influence the selection of resistant strains. This phenomenon does not appear to have been studied in different groups of staphylococci (a general review of antagonism between bacteria is given by Hoffman, 1957) and it might be of interest in considering the spread of staphylococci and frequency of nasal colonisation in hospital wards, although it is rare for viridans streptococci to be present in the nose.

Summary and Conclusions

To summarise this section, we may consider the evidence according to the main methods of determining virulence and see for what factors there may be any indication of importance.

Experimental infection in man. Owing to the difficulties inherent in carrying out tests in man, few strains of staphylococci have been tested, so that little evidence on virulence factors has emerged from studies of experimental infections in man, beyond the fact that the presence of foreign material in the lesion greatly potentiated infection (Elek, 1959). Although about 100 cocci are needed to cause disease regularly it is probable that disease can be produced less regularly with a lower dose than this. In addition, these studies have

been carried out on healthy volunteers and the dose needed to infect seriously ill patients may be lower than these results suggest.

Experimental work on the immunization of mothers and babies showed that staphylococcal toxoid containing leucocidin reduced the number of neonatal infections, although protection was far from complete (Schubert & Johanovsky, 1957; Sebek, Schubert & Johanovsky, 1958). Other workers have found that the antileucocidin titres in humans appeared to reflect the state of resistance to infection (Lack & Towers, 1962; Gladstone, Mudd, Hochstein & Lenhart, 1962; Bänffer, 1962). Chlouverakis, Griffiths and Swift (1963) found no relation between anti-alpha-haemolysin titre and staphylococcal infection in newborn infants. However, Smith, Goshi, Norman and Cluff (1963) showed that in humans the reaction to injection of alpha lysin was consistent with the idea that the lysin might act as a sensitising agent in the same manner as pollen.

These investigations therefore indicate the importance of potentiators, of which sutures would appear to be the most common in a surgical ward; and suggest that leucocidin and perhaps alpha-haemolysin are important in the initiation of infection.

Epidemiology of human infections. Few of the strains brought into hospital by patients are capable of causing much cross-infection and much of the disease is caused by a few strains (Shooter et al., 1958), usually of certain phage-types and resistant to 2 or more antibiotics. It is of course difficult to distinguish between communicability and virulence. It seems likely, however, that resistance to antibiotics, for example may contribute more to communicability than to virulence.

In regard to virulence, examination of clinical material has demonstrated that, although some of the strains may produce, say, alpha lysin only very weakly (Lack, 1956), in general strains isolated from lesions possess more enzymes or elaborate more toxins that do strains from carrier sites. Coagulase and alpha haemolysin appear to be the most important; the importance of leucocidin is difficult to judge owing to the leucocidal action of the delta lysin (Schwabacher et al., 1945; Elek & Levy, 1950; Anderson, 1956; Hinton & Orr, 1957; Knizhnikov & Kasatkina, 1957; Brown, 1960).

Infection in animals. Epidemiology of human infections suffers from the disadvantage that the 'virulence' of staphylococci from non-lesion sources may be underestimated. Whilst it is possibly rare for staphylococci from septic lesions to be those

of minimal virulence (they may be such if the patient is severely debilitated or is on massive doses of steroids), it is quite common to isolate a virulent staphylococcus from a carrier site where it is not causing clinical damage. There is thus the need to compare the characteristics of strains given equal opportunity to cause disease and animal experimentation permits this. It must be borne in mind, however, that this approach will ignore any characteristics of communicability which the strain possesses and also that the results obtained in animals may not be applicable to man.

The lessons learned from this work, however, have been very like those learned from a study of human infections. Some factors can be absent without apparently influencing virulence (Kapral & Li, 1960; Alami & Kelly, 1960) although it should be emphasised that these studies have usually been carried out on mutants from a parent strain especially picked for its virulence. In surveys of strains the additive effect of different characters has been noticed, those strains producing the most enzymes being, in general, the most virulent (Smith, Hale & Smith, 1947; Marks & Vaughan, 1950; Selbie & Simon, 1952; Lack & Wailling, 1954; Howard, 1954; Johanovsky, 1958; Coutinho & Mutini, 1963). Both in this group and in those relating to human infections there is the possibility that production of a toxin <u>in vitro</u>

may not be the same as that *in vivo* (Gladstone & Glencross, 1960; Gellenbeck, 1962; Beining & Kennedy, 1963; Foster, 1963).

Immunization against some particular product of the staphylococcus has been shown to protect the animal against challenge with strains having this product as the main virulence factor. These products have included the alpha haemolysin (e.g. Lambert, 1960; Lithander, 1961; Taubler, Sall & Mudd, 1963) capsular material (Morse, 1960, 1962; Fisher, Devlin & Erlandson, 1963) and coagulase (Boake, 1956; Lominski *et al.*, 1962).

Potentiators have been shown greatly to reduce the dose of cocci needed to set up infection (e.g. James & MacLeod, 1961).

Finally there remain the *in vitro* tests for virulence factors. Many of these tests do no more than help to delineate the species *Staphylococcus aureus*; for example, there is the very strong correlation between desoxyribonuclease and coagulase production. Some of the tests, such as resistance to mercury salts, indicate the strains likely to be resistant to antibiotics and may be useful as indicators of potentially pathogenic strains. In general greater biochemical activity appears to be exhibited by the virulent strains (e.g. Krynsky *et al.*, 1962) and it is perhaps this greater activity which enables the staphylococcus to utilise new metabolic pathways when the usual ones are blocked by some antibacterial agent.

Thus we might expect to find that those staphylococci which were 'epidemic' for man or 'virulent' for animals were those which possessed a battery of toxins and enzymes or were capable of mutating easily to meet changed conditions. Some of them may possess very little of one particular agent but may compensate by abundant production of others.

THE EFFECT OF DESSICATION ON INFECTIVITY

It has been suggested that the dried organisms found in the hospital environment are less capable of causing disease than those derived directly from a carrier. If this were so, environmental bacteria would be less important in considerations of cross-infection than those derived directly from a lesion or carrier site of a patient.

Staphylococci isolated on laboratory media from patients and from the environment by Laurell and Toth-Gyulai (1961) and then injected into animals gave no evidence of loss of virulence on the part of the environmental cocci. However, this does not answer the question asked in this thesis - 'are dried staphylococci as capable of causing disease as 'fresh' cocci?'

Dessication of strain Wood 46 on glass tubing (Maltman, Orr & Hinton, 1960) markedly reduced not only the number of viable bacteria but also the ability of the remaining bacteria to survive at 7°C in distilled water, saline and broth as

compared with fresh cultures. Correlated with this loss of viability and apparent damage to the cells which did survive, was an extended lag phase when the dried cocci were inoculated into broth, the extension being proportional to the length of time of drying. The dried organisms also required a longer time to coagulate human plasma in the tube coagulase test and survived less well in pooled human plasma than did fresh growths. The dried organisms were of course eluted from the glass but were not permitted to multiply before making the tests.

Fresh and dried preparations of staphylococci were compared for virulence in mice by intramuscular, intravenous and intracerebral challenge by Hinton, Maltman and Orr (1960). It appeared that the dried organisms were, viable unit for viable unit, less infective than fresh organisms. These authors do not mention the effect that clump-size, that is the number of viable cocci in a viable unit, might have on the virulence of the organism. It might be that in a fresh growth of staphylococci each viable unit contains many more viable cocci than is the case with the dried cocci.

However, this cannot be used to explain all the observations made by Maltman and his colleagues. The initial period of drying might be expected to have reduced the viable units to only one live coccus each (this will have occurred when

the viable count has dropped by 90%) and after this period the count would depend only on the viable count. Maltman's data however shows a progressive increase in the time needed, for example, to clot plasma even when the same viable count was used. The following table taken from Maltman, Orr and Hinton may serve to illustrate this.

Table 6

The effect of drying on the coagulase activity of Staphylococcus aureus.

Dry storage age (days)	Initial viable count per ml.	Clotting time (mins.)
0	$2{\cdot}3 \times 10^7$	60
2	$2{\cdot}0 \times 10^7$	120
9	$3{\cdot}3 \times 10^7$	145
12	$3{\cdot}7 \times 10^7$	185
15	$2{\cdot}4 \times 10^7$	280

Thus the initial increase in clotting time might be explained on the basis of a decrease in clump-size but the subsequent increases can not. Some decrease in the activity of the staphylococci had occurred.

Using sutures inoculated with broth culture of one strain of staphylococcus to set up subcutaneous lesions in guineapigs, Taylor et al., (1962) found that sepsis occurred in 50% of trials when 50 fresh cocci were introduced.

When the sutures were dried for 4 hours after being inoculated with culture, no decrease in the ability to cause sepsis was observed but drying for longer periods decreased the number of lesions produced. The effect could be overcome by increasing the inoculum on the sutures in a ratio proportional to the drying time. Taylor et al., felt that this was probably due to a reduction in the viable count of staphylococci but pointed out that the same effect might be obtained if a larger number of cocci was needed to overcome a drop in 'virulence' occasioned by dessication.

Staphylococci which had been dried on cotton threads for 2 weeks and then inserted under the skin of rabbits produced abscesses but similar sterile threads did not (Robertson, 1958). Robertson makes no mention of the dose of staphylococci inoculated however; he records that Colbeck (see also Colbeck, 1960) had rubbed the culture from one of these threads into a scarified area of skin and had developed a furuncle. Some years before, Colbeck had developed a cellulitis of the fingers after handling a tube in which cocci had been drying for 5 years. Again no indication of dose was given but these incidents do indicate that dried cocci are capable of causing disease in humans.

McDade and Hall (1963) found that by careful pairing of lengths of contaminated suture material it was possible to estimate the dose of dried cocci inserted under the skin of a

mouse. Preliminary experiments with a type 80/81 staphylococcus isolated from a patient, showed that virulence, as measured by the production of abscesses, fell only with the dose of cocci on the suture. There was a sharp drop in the infection rate as the dose fell, however, but although the data given in the paper is limited it seems that any difference in the virulence of dried cocci must be very small.

Dessication and storage was found not to affect the ID of staphylococci tested by Rountree (1963a); the infectivity, measured by the production of suture abscesses in mice, fell only in relation to the viable count of cocci inserted on the suture. Rountree (1963b) also found differences in the survival on drying of various strains. Strains which might, on the basis of phage type be considered epidemic for man (e.g. 80/81) were found to survive drying well and Rountree concluded that the differences in the death rate on drying were such as to give the 'epidemic' strains an ecological advantage over the non-epidemic strains.

Summary

In those tests in which the staphylococci have been rehydrated and then injected into the animal, there appears to be a drop in the 'virulence' as measured by the ability to infect the host. However, in tests in which the cocci have

been introduced dry into the animal and where sutures have provided a potentiator of infection, no drop in virulence has been observed. Differences in survival rates appear to exist and may give an ecological advantage to 'epidemic' strains.

FACTORS AFFECTING THE PRODUCTION OF LESIONS

There are a number of host and environmental factors which affect the production of disease. Some of these factors have been investigated in the experimental work which forms the second part of this thesis and a discussion of them is relevant here (for reviews see, for example, Kass & Finland, 1953; Elberg, 1956; Blair, 1958).

The effect of washing the inoculum. It might be supposed that when bacterial cultures are injected into animals by any route, the presence of preformed toxin in the menstruum might affect the outcome of infection. This can be controlled by using inocula which have been washed free of any toxin although this might damage the bacteria. Johnson, Cluff and Goshi (1961) could find no difference between 'washed' and 'unwashed' staphylococci however and Selbie and Simon (1952) could find no preformed alpha toxin in 24 hour broth cultures. Evans, Miles and Niven (1948) reported that skin infection in rabbits was produced by the growth of staphylococci rather than by any preformed material.

The use of antibiotics. Antibiotics have been used to demonstrate that the formation of skin lesions in animals was preventable, except when very large numbers of cocci were used, for up to 6 hours after challenge (Evans, Miles & Niven, 1948; Lambert, 1960).

Dineen (1961, 1962) found the optimum time for the administration of antibiotics in mice infected intravenously with large doses of staphylococci or klebsiella to vary for the different antibiotic/bacterial mixtures. He found, for example, that 4 mgm. of streptomycin given 1 hour after challenge with staphylococci was as effective as continued therapy with this antibiotic; for methicillin and staphylococci the optimum time was 2 hours after infection.

The effect of adrenaline on infection. Investigation of the effect of adrenaline on skin infections in guineapigs and rabbits showed that doses as low as 2 micrograms markedly increased the size of the lesions produced on intradermal inoculation of several species of bacteria (Evans, Miles & Niven, 1948). The potentiation only occurred however if the adrenaline was given within 2 hours of challenge and its action appeared to be to assist the bacteria to establish themselves.

As has been discussed earlier, the presence of a suture in the wound also markedly lowers the dose needed to

cause infection although it is not known how long the suture must remain in position.

These investigations all point to a critical period in the production of an infection. During the first few hours after inoculation the staphylococci appear to be particularly vulnerable to attack, either by the body's defences or by antibiotics. Any factor which protects the staphylococcus during this period potentiates infection.

Studies on inflammation. A long series of studies on inflammation was undertaken by Menkin (1929, 1930a, b, 1931a, b, c, 1932, 1933; Menkin & Menkin, 1930). When inflammation of the skin has occurred, substances such as trypan blue, iron compounds and foreign proteins such as horse serum accumulate in the inflamed tissue to a greater extent than in the surrounding tissue.

When staphylococci were used to produce the inflammation, trypan blue injected as early as 1 hour after challenge accumulated at the site of injection of the cocci, but when streptococci were used, fixation of the dye did not take place for up to 30 hours after challenge. Menkin suggested that a network of fibrin was laid down in the lymphatic ducts of the inflamed area; this might be a role for coagulase. Menkin's work has been confirmed in some respects and extended by Miles and co-workers, and by

Johnson and co-workers (Miles & Miles, 1943, 1958; Miles, Miles & Burke, 1957; Burke & Miles, 1958; Johnson, Cluff & Goshi, 1961; Goshi, Cluff, Johnson & Conti, 1961; Goshi, Cluff & Johnson, 1961). Miles et al., demonstrated that 'fixation' of the dye in the tissue was due to increased permeability rather than to blockage of the ducts by fibrin; they found that when either staphylococci or streptococci were injected no actual blockage of the channels was demonstrable for 5 hours after infection. The 'fixation' of dye was due to adsorption on to inflammatory protein or because the lymphatic drainage was quantitatively inadequate. It would appear that lymphatic occlusion is of little value in confining the infection, at least in the early stages, for staphylococci become widely spread throughout the tissue although inflammation occurs only at the site of injection (Young, 1954). Miles has suggested that distribution of the organisms through the tissue probably enables the body to eliminate them more easily and the potentiating effect of adrenaline and of sutures is perhaps due to the fact that the bacteria are confined in a greater concentration in one spot.

Modification of the reticulo-endothelial system. Many substances, such as bacterial lipopolysaccharide (for a review see Shilo, 1959) or simple lipids (Stuart et al., 1960) may have an effect on the reticulo-endothelial system (R.E.S.). There are

numerous papers on this subject, but of interest in connection with the experimental work of this thesis is the work of Shilo (1962). Intravenous lipopolysaccharide given 24 hours prior to challenge increased the resistance of levan treated rabbits to necrosis following intradermal injections of staphylococci. The enhanced resistance lasted as much as 7 days; the effect of the levan was to prevent diapedesis and the injection of lipopolysaccharide appeared to inhibit the action of the levan.

Ethyl stearate and glycerol trioleate apparently specifically depress or stimulate the phagocytes of the R.E.S. (Stuart et al., 1960) and mice treated with these substances showed a decreased or enhanced resistance to intravenous infection with pneumococci. The use of these substances appears to offer a reliable means of altering the R.E.S. without affecting other tissue (Cooper & Stuart, 1962; Stuart & Cooper, 1962).

Other factors affecting the outcome of infection

There are a considerable number of factors external to the experiment which may affect the results. Except for the sex of the mice used, these have been kept constant throughout the following experiments, but a brief review may not be out of place.

Strain of animal. Differences between strains of the same species of animal may exist; C57 mice have been found

to be more resistant to intravenous infection with large doses of staphylococci than either ABC or Swiss No.1 mice (Gorrill, 1951), but no difference was found by D. D. Smith (1962) between W-Swiss and Porton mice. Differences in the gut flora appear to account for the greater susceptibility to infection of NCS mice, maintained free of mouse pathogens, than the parent strain (Dubos & Schaedler, 1960).

Female mice were more susceptible to small infecting doses of streptococci than males although with large doses the difference was obliterated (Wheater & Hurst, 1961). The same was true of infection with tubercle bacilli and the difference was enhanced by immunization with B.C.G. (Hoyt et al., 1957).

Diet. Rats fed on a protein deficient diet were more susceptible to infection with mouse pathogens than those fed on stock diet (Miles, 1951). The quality as well as the quantity of the protein markedly influenced the resistance of mice to infection with staphylococci, the poorer the diet, the greater the susceptibility (Dubos & Schaedler, 1958, 1959; Schaedler & Dubos, 1959). Starvation for 30-40 hours before challenge lowered the resistance of mice to infection with staphylococci (Smith & Dubos, 1956b; Boyer & Lamensans, 1961).

Addition of some concentrations of ethyl alcohol to the diet increased the susceptibility of mice to intravenous

challenge with staphylococci (Wasz-Höckert, Kosunen & Kohonen, 1958, 1959).

Temperature. The temperature at which animals are housed may also affect the outcome of infection. Rats and mice infected intravenously with staphylococci were more susceptible to infection if cooled and it was found that penicillin was of little therapeutic value at low temperatures (Jones & Campbell, 1962a and b).

When mice infected with Salmonella species were exposed to cold, micrococci, as secondary invaders, were recovered from the liver post mortem. The source of these organisms appeared to be the upper respiratory tract and the effect of cold seemed to be to lower resistance to infection with these organisms (Previte & Berry, 1962; Miraglia & Berry, 1962, 1963).

Time. Finally, it may be important to carry out all experiments at the same time of day since circadian rhythms have been demonstrated in the production of tumours, in mitosis, body temperature and the output of steroids (see for example Halberg, 1963).

RÉSUMÉ

The following sections of this thesis describe experimental work leading to a technique whereby subcutaneous lesions can be produced in mice using small doses of staphylococci.

The uses of the method are explored in an attempt to distinguish between the virulence of a group of staphylococci isolated during studies of cross-infection; to determine whether virulence of the staphylococci is affected by dessication; and to explore some of the factors which affect the outcome of experimental infections in mice.

What then are the factors which have emerged from a study of the literature and which should be borne in mind when considering the experimental work?

Firstly, that the route by which the staphylococci are introduced may affect not only the observed result of infection, but also which factors are responsible for the manifestations of 'virulence'. This being so, it seems essential to chose an experimental technique which is likely to have some resemblance to infection in man. For this purpose, the surface lesions produced by scraping the skin or by introducing sutures would seem to be the most convenient.

Secondly, that the production of a septic lesion by staphylococci probably depends on more than one factor; we cannot expect to find a simple relation between disease and some particular toxin. Further that, in a search for the factors related to virulence, it is essential to remember that production of a particular enzyme or toxin <u>in vitro</u> may not be paralleled

in vivo. Since the major interest lies in distinguishing between strains within the species *Staphylococcus aureus*, emphasis should probably be placed on the factors which vary qualitatively or quantitatively between the strains rather than on those which help to delineate the species *St. aureus*.

Finally, that care must be taken to provide as homogeneous a group of hosts as possible, since variation either of the host or of the conditions under which the host is kept can have a very great influence in the result of infection.

EXPERIMENTAL WORK

As has been described, previous workers have experienced some difficulty in devising a test *in vitro* or *in vivo* which correlated with the differences in virulence of different strains of staphylococci.

The response obtained in animals to intravenous and intraperitoneal injection of staphylococci varies greatly; large doses of staphylococci are needed to cause death of the animals and lower doses may have no apparent effect. Experiments showed that the Wright-Fleming Institute strain of mice was no more susceptible to infection with staphylococci by these routes than those used by other workers (Table 1).

Two techniques used by other workers and discussed earlier in this thesis appeared to be more promising; these were the use of cotton or silk sutures sewn into the skin and the surface lesion technique described by Foster and Hutt (1960). Investigations carried out on these lines are reported in the following pages. A full account of the technical methods, materials and the media used are given in Appendix 2.

EXPERIMENTS WITH LESIONS ON THE SKIN SURFACE

Foster and Hutt (1960) produced skin lesions in man by scraping away the epithelium with a scalpel until the

TABLE 1 Death rates in W.F.I. mice infected systemically with staphylococci.

Route of infection	Strain	Dose injected	Total mice injected	Number of deaths at day 1	2-7	8-20	Survivors at 3 weeks
intra-peritoneal	Smith	8×10^7	24	20	0	0	4
		3×10^7	6	2	0	0	4
		3×10^6	6	0	0	0	6
		3×10^5	6	0	0	0	6
		3×10^4	6	0	0	0	6
	PS 80	3×10^7	6	0	0	0	6
		3×10^6	6	0	0	0	6
		3×10^5	6	0	0	0	6
		3×10^4	6	0	0	0	6
intra-venous	Smith	6×10^7	6	0	0	1	5
		6×10^6	6	0	0	1	5
		6×10^5	6	0	0	2	4
		6×10^4	6	0	0	0	6
	PS 80	2×10^7	6	0	0	0	6
		2×10^6	6	0	0	0	6
		2×10^5	6	0	0	0	6
		2×10^4	6	0	0	0	6
	Wood 46	1×10^7	6	1	0	0	5
		1×10^6	6	0	0	0	6
		1×10^5	6	0	0	0	6
		1×10^4	6	0	0	0	6

capillary loops were visible. The staphylococcal inoculum, in fluid form, was placed on this lesion and the site covered with a cover-slip, or with the cap of a bijou bottle, held in place with adhesive tape. The method was originally used by Rebuck and Yates (1954) to examine the action of leucocytes *in vivo*, for leucocytes adhere to the coverslip.

It was felt that this method might prove useful in experimental infections of animals. It was found, however, both in this series of experiments and by Foster and Hutt, that if the dressing became loose and the lesion were allowed to dry no exudate formed and the organisms were difficult to recover. Difficulty was experienced in keeping the dressings on experimental animals and slight modifications in technique were made.

METHODS

The dressing for the lesion was made by placing a piece of polythene sheet about ½ inch square centrally on a piece of surgical plaster about 1 inch square. The polythene was kept in alcohol to clean and sterilize it before the dressing was made up.

A lesion about ½ inch square was scraped on the animal's flank about 1 hour after the area had been depilated and washed clean. The inoculum, about 1/100 ml. of fluid, was placed on the lesion using a standard wire loop and the area

covered with a dressing. Six individual lesions were made on each guineapig; each was covered with a small dressing and the whole covered with a lint pad held in place with 3" wide adhesive tape. This was usually enough to keep the dressing intact for up to 48 hours. Only one lesion was made on each mouse, but it proved extremely difficult to keep the dressing in place and small cages were made of a tube of perforated zinc ($\frac{1}{4}$" perforations) about 9 inches long and $1\frac{1}{4}$ inches in diameter. Food and water were introduced into these cages which, whilst giving the mouse freedom to run up and down did not permit it to tear at the dressing. The mice were retained in these cages for a maximum of 24 hours.

Organisms used in the study

Staphylococcus aureus	PS 80, Warren, Farrow.
Micrococci	NCTC 7944, Harrod.
Escherichia coli	E 56.
Streptococcus pyogenes	NCTC 8194.

For a description of these organisms see Appendix 2.

All the organisms except the streptococci were grown in nutrient broth and viable counts made on serum agar. The streptococci were grown in Todd-Hewitt broth and viable counts made on blood agar.

Estimation of clump-size

The clump-size (the number of viable cells in each clump or chain) was determined by drying a 10^{-1} dilution of the broth culture on a slide and counting the number of cells in each clump (see Appendix 2).

Examination of the exudates

The dressings were transferred serially from slide to slide, leaving a film of exudate on each slide. The majority of the slides were strained with Gram's stain in a search for organisms but some of the slides were stained with Leishman's stain to facilitate recognition of the cells.

Examination of the lesions

The lesions were normally examined after 24 hours. The dressing was removed and stuck down on a labelled slide and a swab taken from the lesion, all the exudate being removed. After swabbing, the flanks of the guineapigs were cleaned with alcohol. No late infections were seen and the lesions healed within 10 days; within 3 weeks the fur had grown again and the animal could be re-used. The mice were killed after each experiment and in addition to the lesion, the heart blood was sampled using a swab. The kidneys were examined by homogenizing them in a M.S.E. blendor with saline and inoculating the suspension on agar; any other apparently abnormal organs were also examined.

Recovery of organisms from surface lesions

TABLE 2 Guineapigs.

Organism	Dose range in viable units	Mean number of cells per viable unit	Number of lesions inoculated	Number of lesions from which organisms were recovered
Staphylococci				
PS 80	84-525	1.7 - 2.2	36	32
Farrow	161-266	1.7 - 1.9	17	14
Warren	353-392	1.7 - 1.8	18	16
Micrococci				
Harrod	71-93	2.3 - 2.6	12	1
NCTC 7944	114-129	1.6	12	3
Escherichia coli				
E 56	187-239	1.05	11	5
Streptococci				
NCTC 8194	20-121	7.2 - 8.6	18	0

TABLE 3 Mice.

Staphylococci				
PS 80	35-430	1.5 - 2.2	10	7
Micrococci				
Harrod	161	2.2	5	4
NCTC 7944	41-52	1.7 - 1.9	8	0

RESULTS

Six guineapigs each had 6 lesions on which serial 10-fold dilutions of staphylococcus PS 80 were inoculated. The inoculum ranged from about 2×10^5 viable units to about 2 viable units. When the lesions were examined a grey exudate was generally found to be present which was composed of blood cells, bacteria and debris, but no other tissue response was ever seen. No difference could be detected in the response to the various inocula and the organisms were generally recovered from all lesions except those receiving the smallest inoculum; when the same procedure was carried out using sterile saline as the inoculum, lesions of the same appearance developed.

It has been reported that repeated infection with staphylococci may lead to the development of hypersensitivity but when the test was repeated 4 times on all 6 guineapigs at intervals of 6 weeks, no change in response was seen.

In experiments with organisms other than staphylococcus PS 80, the lesions were inoculated with between 100 and 400 viable units (Table 2). Recovery of staphylococci from the lesions was good, but that of the coliform organisms was erratic; in 5 samples where the coliforms were recovered, there was a heavy growth indicating that multiplication had taken place despite the presence of leucocytes in the pus. Micrococci were

recovered in only a few instances but no streptococci were ever isolated from the lesions.

Similar experiments were carried out on mice (Table 3). There was always less exudate from the mouse lesions but the results were basically similar to those obtained using guineapigs.

Examination of exudates at times other than 24 hours

The above results refer to the appearance of the lesions at 24 hours.

Twenty-four lesions were made on guineapigs and inoculated with 2 x 10^2 viable units of PS 80. Twelve of the lesions were uncovered and examined immediately and staphylococci were recovered from each in small numbers (mean of 5·5 viable units from each lesion on the agar plate). The scanty exudate at this time consisted almost entirely of erythrocytes.

The other twelve lesions were examined after a period of 4·5 hours at which time considerable multiplication of the organisms had taken place; the exudate was still about 64% erythrocytes.

Exudate from lesions inoculated with sterile saline showed a very similar picture, at 4 hours there were 86% erythrocytes and at 24 hours only about 7% erythrocytes.

If the lesions were left for more than 24 hours before examination, the exudate began to dry up and the

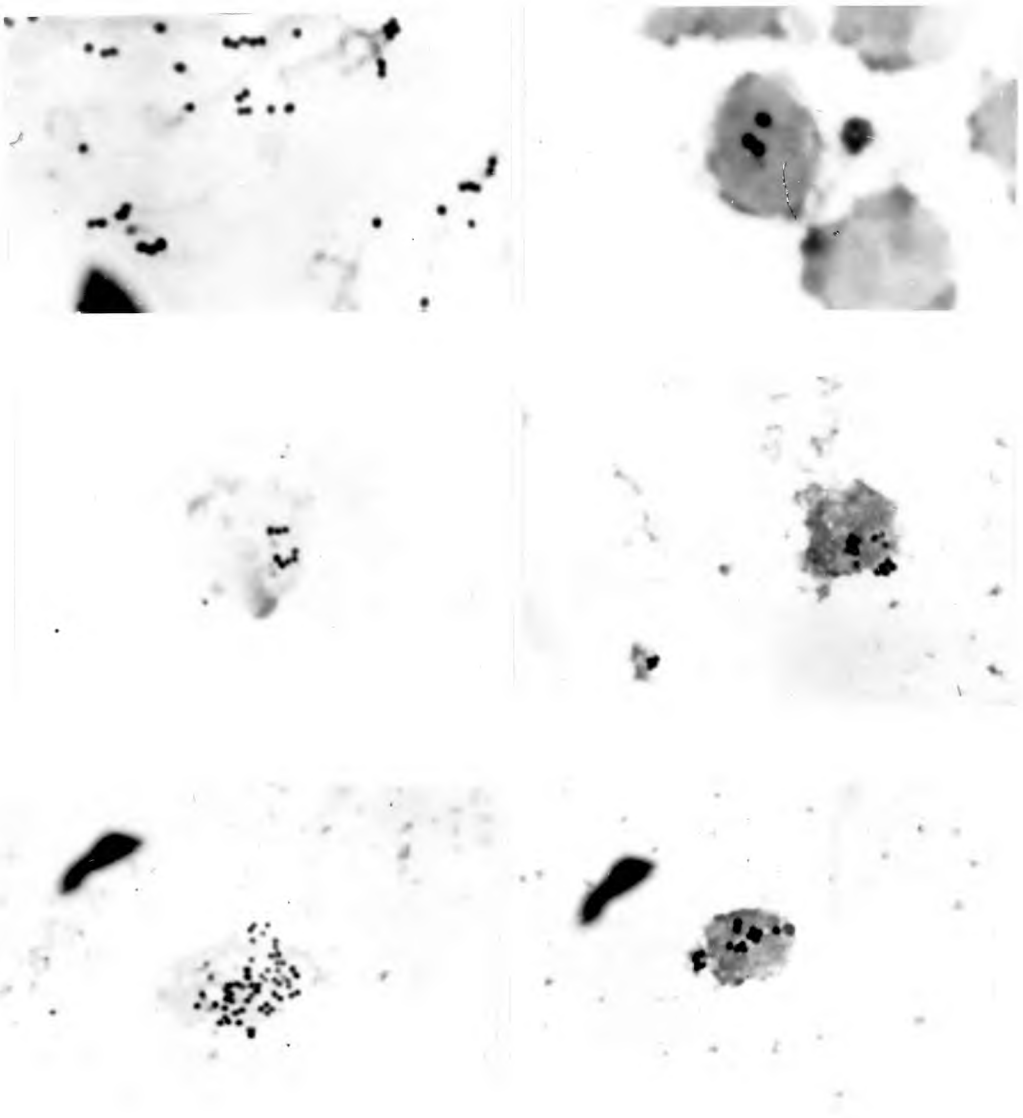


Figure 1 Leucocytes and cocci in exudates from surface lesions.

organisms were difficult to recover. This may have been due to mechanical trapping in the scab.

Examination of the exudate for organisms

Films of all exudates were stained by Gram's method. There were in all 167 lesions inoculated with PS 80 and from which this organism was recovered by cultural methods; Gram positive cocci were seen in films of 79 (47%) of these.

In many exudates the cocci appeared to be unassociated with the leucocytes and counts of cocci in each unit (clump size) gave a distribution very similar to that of the broth culture with which the lesion had been inoculated (Figure 1). The clump size was of the order of 2 cocci per clump. In some of the films however cocci were seen which were closely associated with the leucocytes. Examples were found of every stage in an apparent process of ingestion of the cocci by a leucocyte, multiplication of the cocci, death of the leucocyte and the formation of a 'colony' of cocci within the leucocyte (Figure 1).

Discussion

The results presented here bear a very close resemblance to those described by Foster and Hutt in their experiments on humans. A seropurulent exudate was found on all the lesions and no differences could be detected following

the inoculation of staphylococci, micrococci or saline. The behaviour of the experimental animals necessitated the use of more adhesive tape than in the experiments conducted by Foster and Hutt and reaction to this may have masked the erythema which these authors noticed when staphylococci were inoculated. The finding that most of the organisms were extracellular was also reported by Foster and Hutt.

Conclusion

These experiments demonstrate the ability of organisms to multiply on skin lesions and to survive in a cellular exudate. However, no difference in the appearance of the lesions could be detected when various strains of micro-organisms were used or when the lesions were inoculated with saline, nor were there any marked differences in the survival of the staphylococci in the exudates. This method could, therefore, not be used as a measure of pathogenicity.

THE PRODUCTION OF SUBCUTANEOUS LESIONS

The technique used by Foster and Hutt had proved to be unsatisfactory for determining pathogenicity and it appeared pointless to search for differences in virulence by this method.

The use of contaminated sutures stitched under the skin of the animal seemed to be worth investigation. The role

of the suture appeared to be to act as a mechanical depot for the staphylococci or as a mechanical irritation, or both. The use of sutures has two disadvantages however; one is that many of the sutures may be bitten or scratched out by the animal unless the area is covered or the ends of the sutures sealed to the skin; the second is that difficulty is sometimes experienced in assessing the dose of bacteria inserted on the suture, especially if, for example, the suture is cut off at the skin surface.

Cotton dust had proved a satisfactory substrate on which to study the survival of staphylococci on drying (Noble, 1961; M.Sc. thesis), since a suspension of the dust in a fluid such as saline could be pipetted as though there were no solid phase present. It seemed possible that cotton dust might prove as satisfactory a depot for the staphylococci as the cotton sutures and, if it were introduced under the skin, it might be difficult for the animal to remove, thus eliminating the need for a dressing over the lesion site. It was found that if the cotton dust were pushed into the lumen of a transfusion needle and the needle introduced under the skin of a mouse, the cotton could be expelled by means of a wire plunger. The cotton dust compacted in the needle to a plug which remained intact in the tissue but broke up in broth or saline to give a suspension. Staphylococci

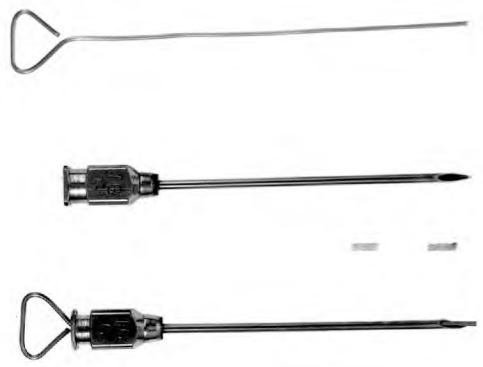


Figure 2 Needles and plungers used to inoculate cotton dust. Two cotton plugs are shown (normal size).

introduced under the skin of mice in this way gave rise to septic lesions and so this method was adopted and explored.

Materials and methods

The needles used were No. 16 gauge transfusion needles fitted with a wire plunger to expel the cotton dust (Figure 2). The plunger was marked so that it could be withdrawn a given distance (0.5 cm.) up the barrel of the needle; the needle was then filled by packing loose cotton dust into the space in the lumen of the needle between the point and the plunger. In this way about 2·5 mgm. of cotton dust was used for each plug.

The cotton was C4 white cotton flock which is pure cellulose and corresponds to the cotton in bleached cotton fabric.

The needle can be charged either with contaminated cotton dust on which bacteria have been dried or with clean cotton dust. When clean dust was used, the inoculum was picked up on a standard loop and touched against the cotton in the needle. Capillary forces rapidly drew the fluid culture into the cotton plug. The number of viable cocci on the cotton plug was determined either by suspending the plug in saline and inoculating the suspension on agar or by inoculating a standard loopful of a suitable suspension of staphylococci directly on agar.

Male and female mice of the Wright-Fleming Institute strain weighing 20-25 grams and 9 to 12 weeks old were used in

the tests. The mice were housed in groups of up to 24, in cages 200 square inches in area and were fed 'Diet 18' and water without restriction throughout the tests. The back of each mouse was clipped and depilated with a barium chloride powder 1 to 2 hours before challenge. When the mice became dry again after the depilation they were inoculated with the cotton plug; the skin of the back was pinched up between the fingers, the needle inserted at the base of the fold and the plug expelled.

No difference in reaction was detected between the sexes, but within any one test mice of one sex only were used. Records were kept of the sex of pairs of mice used in a survey of virulence in which 232 staphylococcal cultures were selected at random and inoculated subcutaneously. The results showed that the male mice gave a mean score of 8.96 marks and the females 8.41 marks. This difference is not significant ($10\% > P > 5\%$, $t = 1.71$, $n_{male} = 104$ pairs, $n_{female} = 128$ pairs).

The results were read daily, although a reaction was generally present at 24 hours.

Method of scoring lesion severity

The lesions were scored 0, 1, 2 or 3 for no reaction, slight, moderate or severe reaction. 'No reaction' was defined as complete absence of pus visible at the skin surface and no

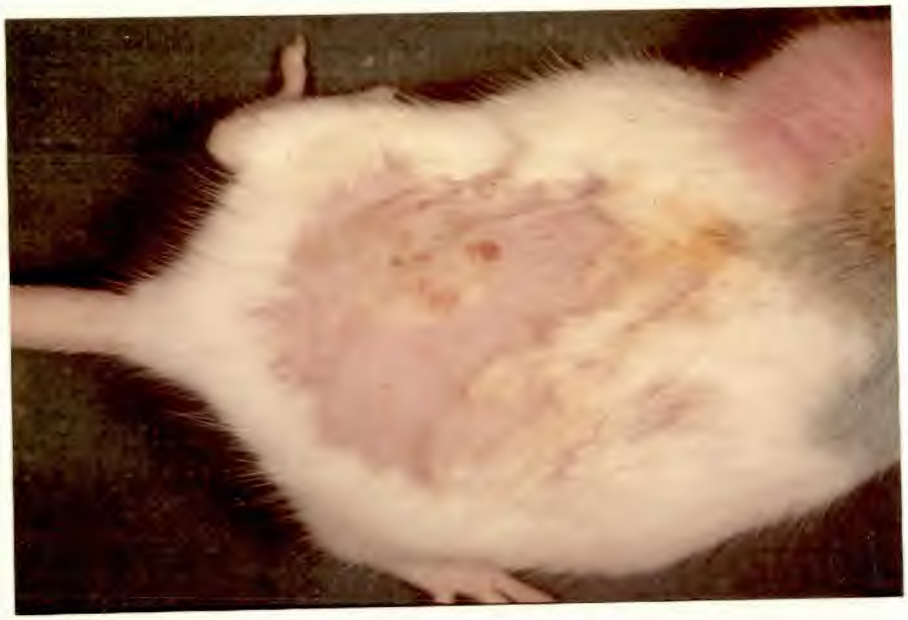


Figure 3 Severe lesion showing white zone

Figure 4 Slight lesion

erythema except in the skin immediately above the cotton plug. A slight reaction was defined as minimal pus immediately above the plug (this usually showed as a 'head') and erythema in the skin around the plug. A moderate reaction was one in which the pus was accompanied by a white zone or area of necrosis not more than ½ cm. in diameter and a severe reaction as one in which the diameter of this zone was greater than ½ cm. The white zone became necrotic after 2 to 4 days.

Most lesions tended to be circular or oval, but occasional difficulty was encountered in assessing the score of asymmetric lesions. An example of a severe and a slight reaction are shown in Figures 3 and 4. In the text the results are usually quoted as the sum of scores for the first two days following challenge; thus a mouse which showed a moderate reaction at day 1 and a severe reaction at day 2 would score 2 + 3 = 5 marks. The results are usually quoted as the sum for 6 or 12 mice.

A series of investigations was undertaken to determine the general characteristics of the lesions produced by the subcutaneous inoculation of cotton dust containing staphylococci.

Characteristics of the lesions:

The effect of inoculating sterile cotton dust

Twelve mice were inoculated with sterile cotton dust. One showed a mild erythematous reaction at 2 days. Nine showed a mild reaction at 4 days and all showed a mild reaction at 7 days; none ever showed evidence of the white zone. After 15 days the mice were killed and the cotton and any pus present excised. All of the mice had retained the cotton and 3 of the twelve had a little pus around the plug, the other 9 showed no macroscopic reaction other than thickening of the connective tissue in which the plugs were situated. One of the 3 with pus yielded coliform organisms on culture but the other 11 were sterile.

As will be seen from the following passages, the reaction to the introduction of even small numbers of some micro-organisms was very marked so the reaction to the cotton alone has been discounted in the following experiments.

The effect of the presence of cotton dust on the severity of lesions

Broth cultures of staphylococci were inoculated subcutaneously with and without cotton dust. Cultures without cotton dust were injected with a number 20 gauge needle, 0·05 ml. being introduced under the skin. The lesions produced in the absence of cotton dust were, dose for dose, much less severe than those produced in the presence of cotton, except at the

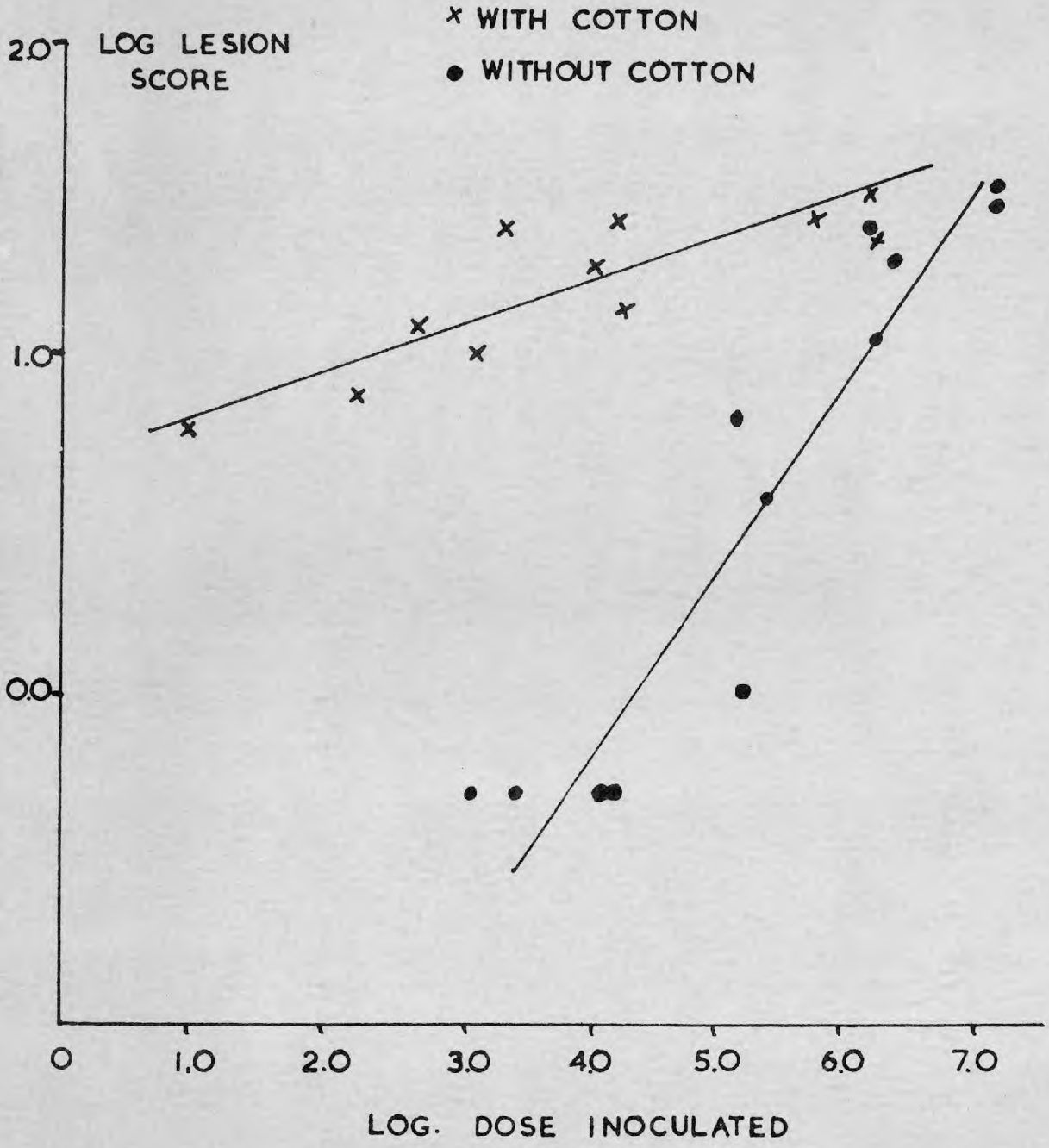


Figure 5 Comparison of lesion scores obtained when staphylococcus PS 80 was injected with and without cotton dust.

highest dose levels. The experimental results are given in Appendix 1 Table 1, and the results for staphylococcus PS 80 are given in Figure 5. The regression slope of 'log.lesion score' on 'log.dose' was calculated, and comparison of the regression lines confirmed, that they were significantly different (e.g. PS 80, $P < 0{\cdot}1\%$, $t = 6{\cdot}5$, $df = 21$). It may thus be concluded that the presence of the cotton dust exerted a marked influence on the lesions formed by the subcutaneous introduction of staphylococci.

Although there was almost no reaction to the introduction of micrococci alone (Appendix 1 Table 2), lesions were produced by micrococci in cotton dust. The lesions were of a different nature from those produced by staphylococci however; only rarely did pus appear at the surface of the lesion within 2 to 4 days as was the case with staphylococci and no white zone or necrosis was seen. The lesions remained unbroken for as much as 14 days and gradually increased in size; figure 6 illustrates such a lesion at 10 days. Moreover, almost all the lesions were identical except those produced by the lowest doses which tended to be small. When the strict criteria used in assessing the staphylococcal lesions were applied to the micrococcus lesions, the result had always to be classed as 'no reaction' or 'slight reaction' (scoring 0 or 1 mark). When excised, or finally ruptured

Figure 6 'Micrococcus' lesion 10 days after inoculation

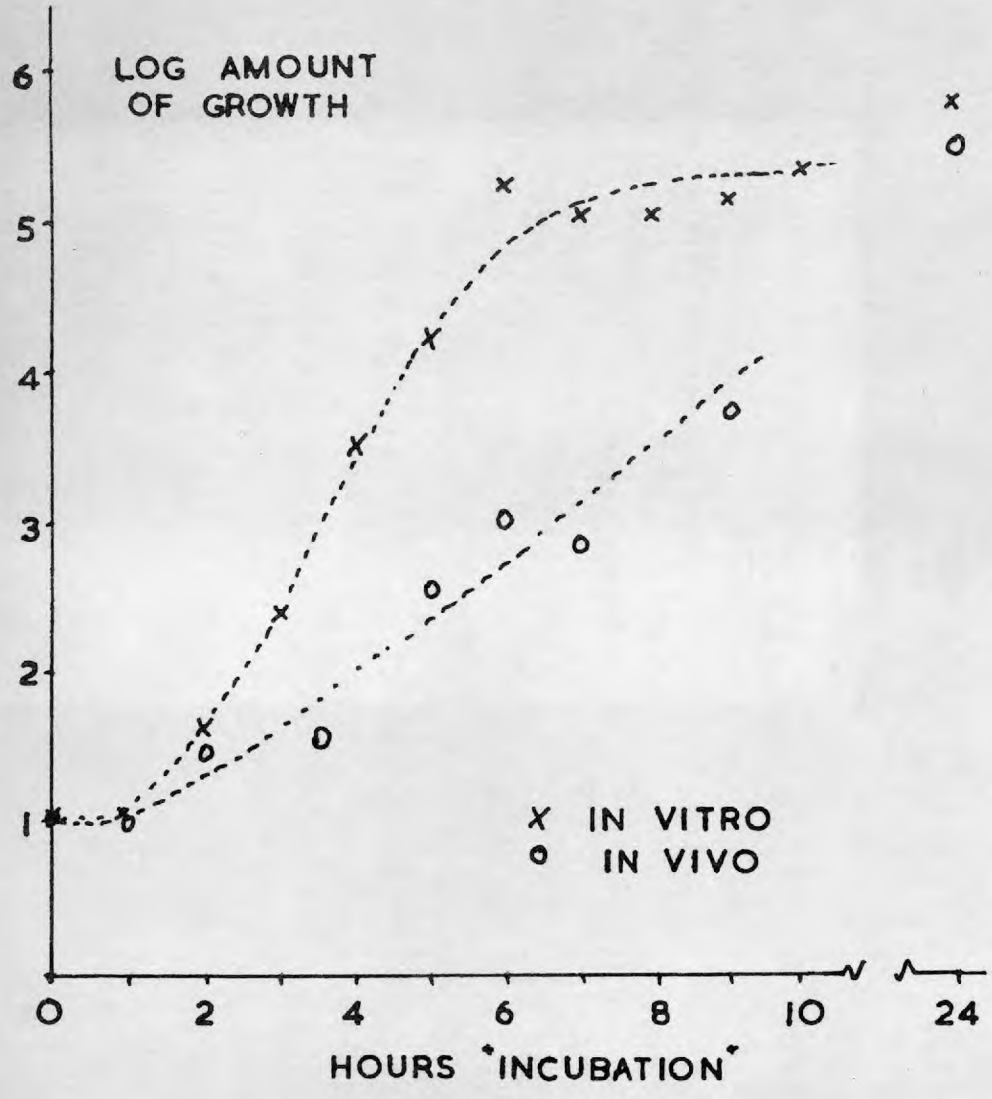


Figure 7 Comparison of the growth rates of staphylococcus PS 80 in vivo and in vitro.

spontaneously, these large micrococcus lesions were full of pus which consisted largely of cellular debris from which the micrococcus could sometimes be recovered by culture. The lesions appeared to be the result of the accumulation of debris resulting from the growth of organisms and from the infiltration of leucocytes into the lesion site.

Growth of staphylococci in the lesions

Cotton plugs containing about 100 viable units of staphylococcus PS 80 were inoculated under the skin of a large group of mice. At intervals after inoculation, pairs of mice selected haphazardly were killed and the cotton and any pus present excised, homogenised in an M.S.E. blendor with 3 ml. of saline and dilutions of the saline suspension inoculated onto agar. The results are shown in Figure 7 and Appendix 1 Table 3. The 0 hour counts were made after killing the animal directly the plug had been inserted; the low counts for the first few hours probably reflect the difficulty of recovering organisms from the cotton. It can be seen that a very rapid increase in the numbers of organisms recovered from the lesions occurred after the first 4 - 5 hours.

Similar experiments with the Type 52 staphylococcus also showed an increase in the viable count after 4 - 5 hours.

Young (1954) reported that staphylococci injected into the cheek-pouch of hamsters rapidly became dispersed over a large area, and it seemed very likely that bacteria injected under the skin of the mouse would also be dispersed in the same manner. It was found that 0·05 ml. volumes of Indian ink injected subcutaneously in the mice spread from an initial area of about 0·5 cm. diameter to about 2-3 cm. diameter in about 2 hours. By contrast, when cotton plugs soaked in Indian ink were injected under the skin, very little ink spread into the surrounding tissue, although some remained in the track made by the transfusion needle. It is tempting to suppose that the bacteria also remained in or around the plug and did not become dispersed through the tissue. In view of the wide dispersal of material injected without cotton dust it did not seem worth trying to follow the growth rate of staphylococci injected under the skin by bacteriological methods. Other methods, such as the use of radioactively labelled bacteria could however be used to follow dispersal of the bacteria (see for example Fisher & Robson, 1963).

Invasion of the plug by leucocytes

Cotton plugs inoculated with about 2×10^6 viable units of staphylococcus PS 80 were introduced under the skin of mice and pairs of mice selected haphazardly were killed at hourly intervals.

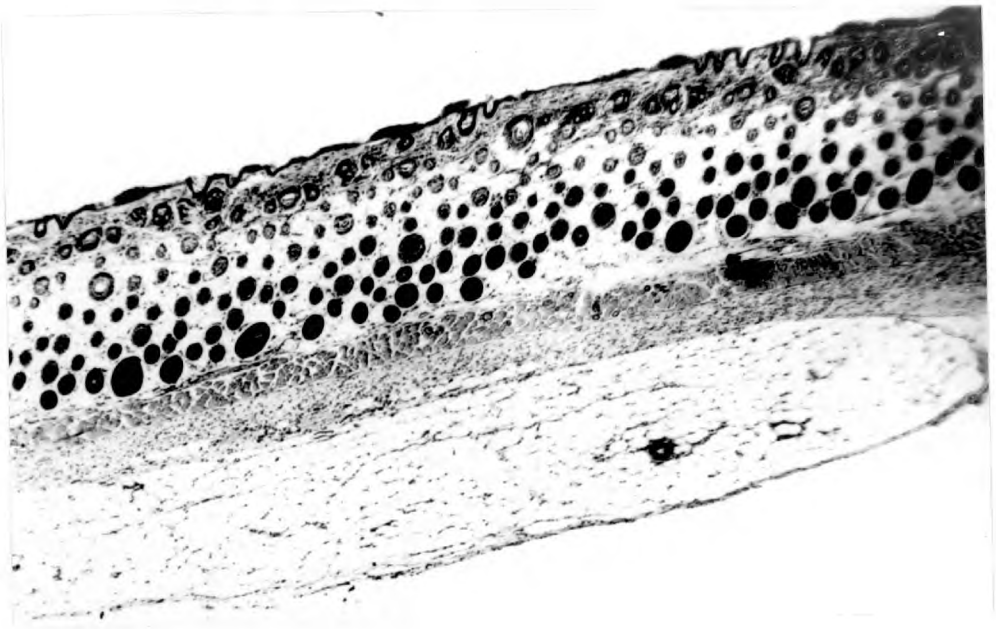
The plugs were dissected out, emulsified in saline and a film of the suspension stained with Leishman's stain.

Only cotton fibres and cocci were seen for the first 2 hours, but at 3 hours a few leucocytes were seen on the film and the numbers increased thereafter. At 4 hours a few leucocytes were seen which appeared to have ingested the cocci but at 5 and 6 hours a number of the leucocytes had the appearance of being 'overfull' of cocci. For example, 6½ hours after infection, 26/102 leucocytes appeared to have ingested cocci and 5 of these appeared 'overfull'. This could be interpreted in 2 ways. Either these leucocytes were especially efficient at ingesting cocci or the cocci were multiplying inside the leucocytes. The rather ragged appearance of the leucocytes suggests that the latter is the most likely explanation.

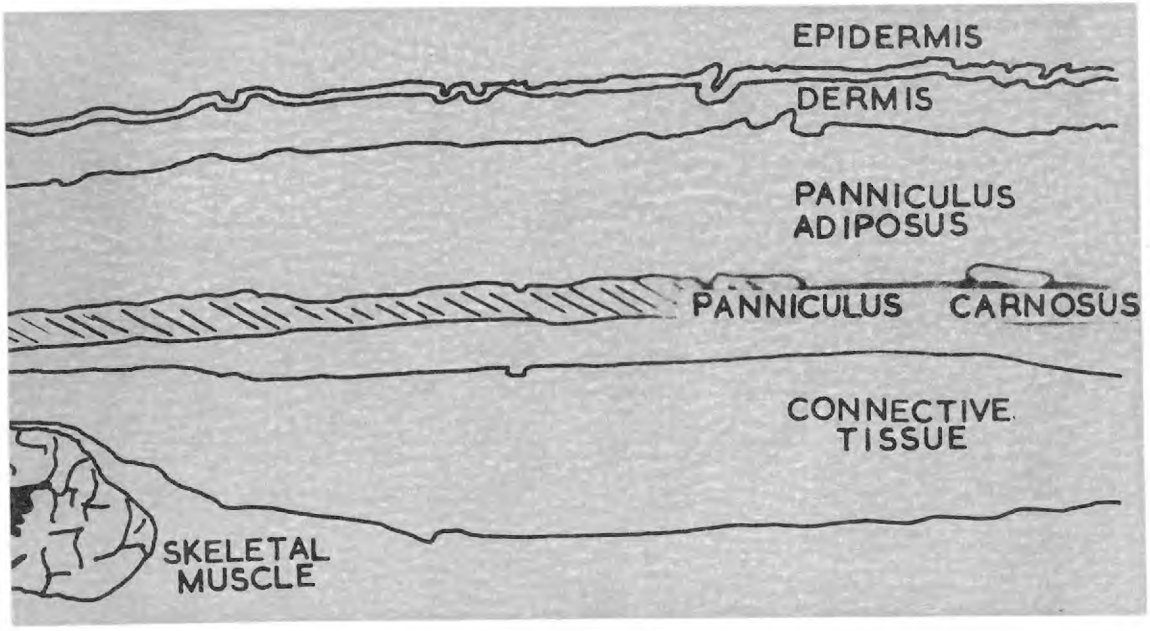
To judge from the appearance of the blood vessels of the subcutaneous tissues, the infected plug makes it presence felt in about 2 hours. No signs of a reaction could be seen on the outside of the skin until about 6 hours after an infected plug had been inserted.

Histology of the lesions

The histology of the lesions is shown in the diagrams and photographs. A section of normal tissue is shown in Figure 8 A and B. In the mouse there is, beneath the epidermis and dermis, a fatty layer and then a muscular layer, the

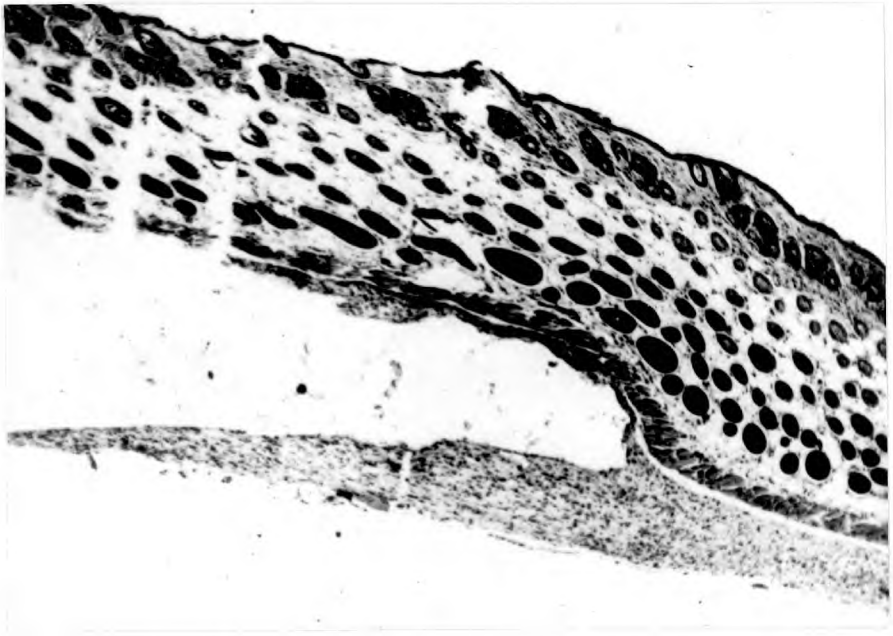


A

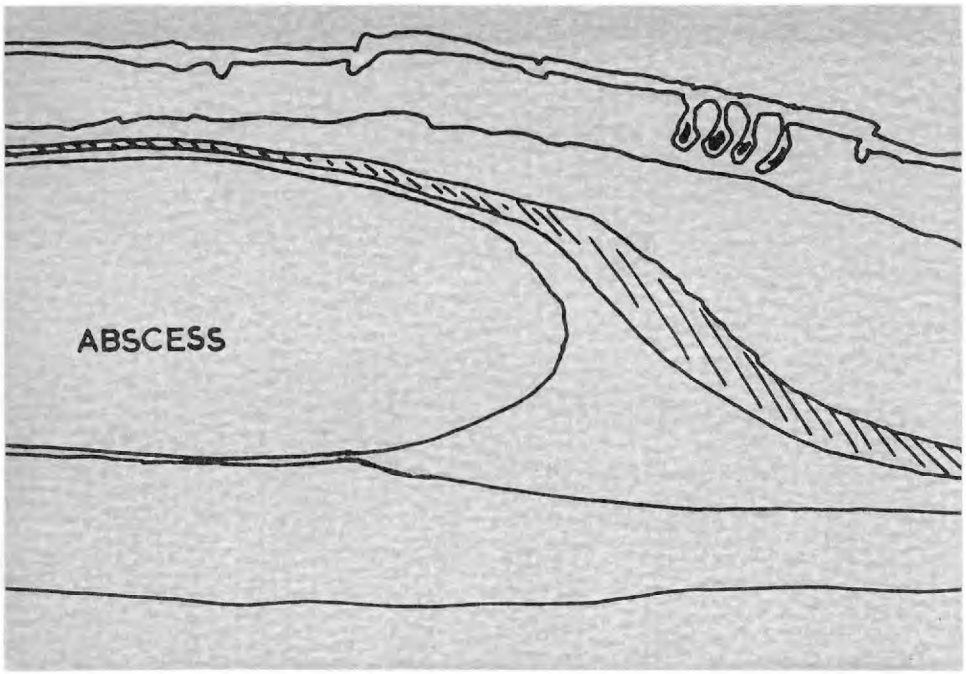


B

Figure 8 Histology of subcutaneous lesions.
Normal skin.

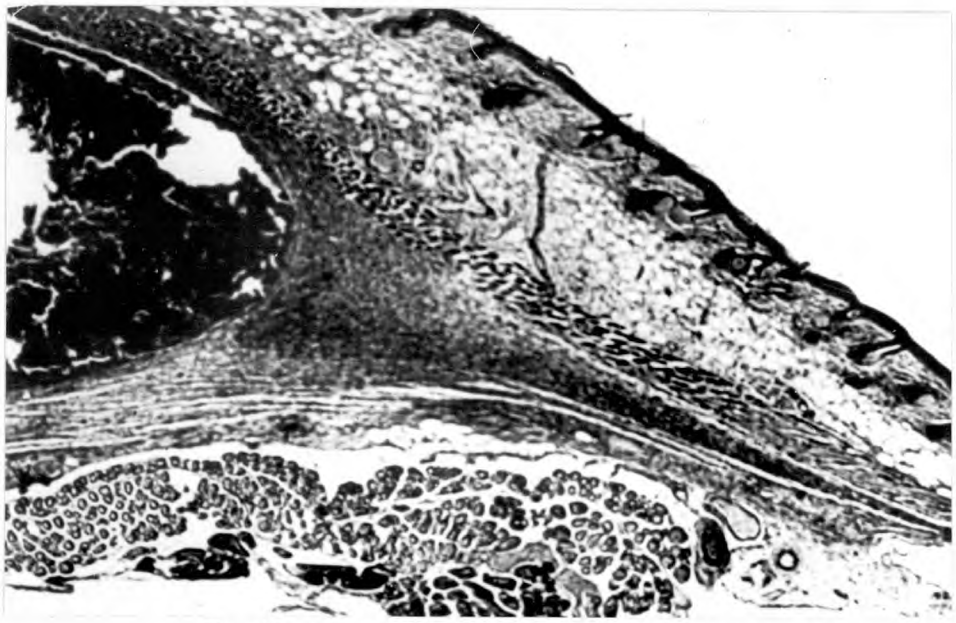


C Sterile cotton plug in tissue

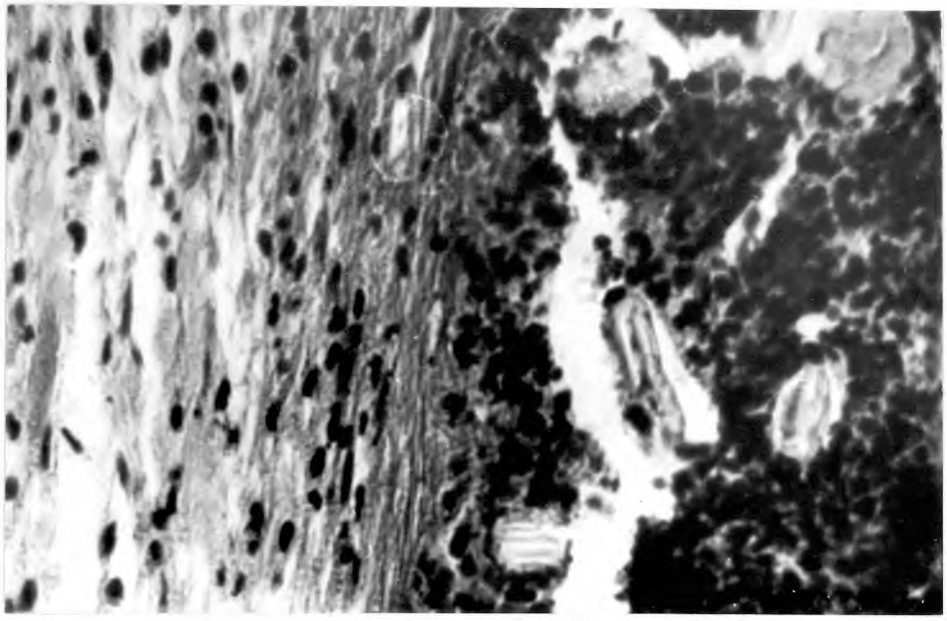


D Site of abscess

Figure 8 Histology of subcutaneous lesions.



E Abscess showing deformation of tissue



F Edge of abscess, high power view.

Figure 8 Histology of subcutaneous lesions.

panniculus carnosus. Between the panniculus carnosus and the musculature of the body wall there is a layer of very loose connective tissue, and it is in this tissue that the cotton plug is deposited. Figure 8 C shows a sterile cotton plug in position and illustrates how the tissue is deformed by the presence of the plug (the mouse was killed before any reaction to the plug had taken place).

After 24 hours the lesion has developed into a typical abscess (Figures 8 D and E). The cotton plug has become walled off and infiltrated with leucocytes, with a resultant increase in size. The walling off and infiltration is shown in more detail in Figure 8 F in which the cotton fibres are clearly seen.

Dose response curve for different strains

Four strains of Staphylococcus aureus (PS 80, Charlton, Wood 46, Type 52) were used as standards throughout much of this work (see Appendix 2) and the responses obtained in the mice to various doses of these 4 strains have been plotted in the form 'lesion score' against 'log.dose inoculated' (Figure 9), and also as 'log.lesion score' against 'log.dose inoculated' (Figure 10). On statistical grounds there is little to choose between the two transformations, the correlation coefficients being remarkably similar (Table 4).

The standard deviations of the score about the regression lines were smaller for the 'log.score/log.dose curves'

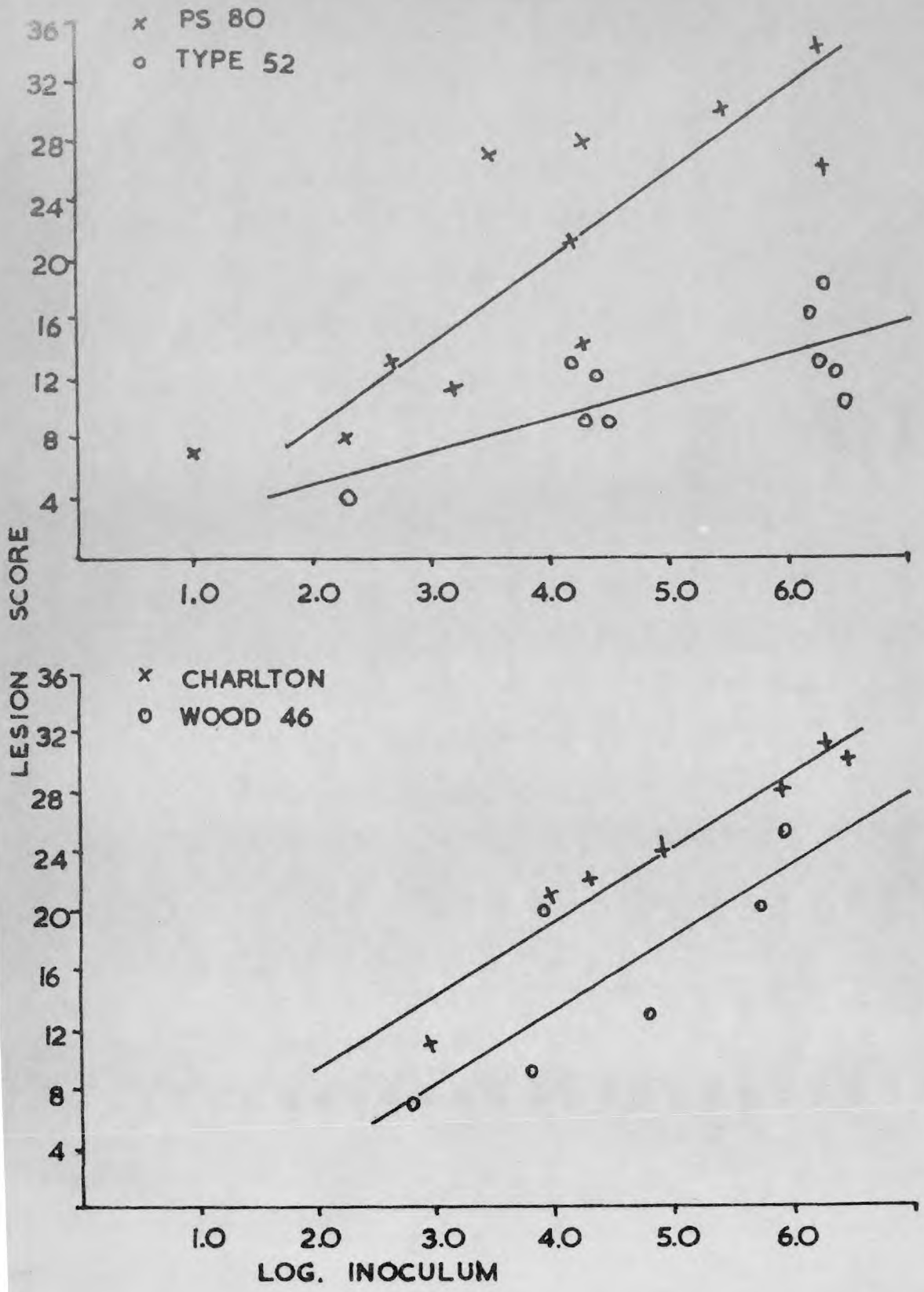


Figure 9 Comparison of regression curves

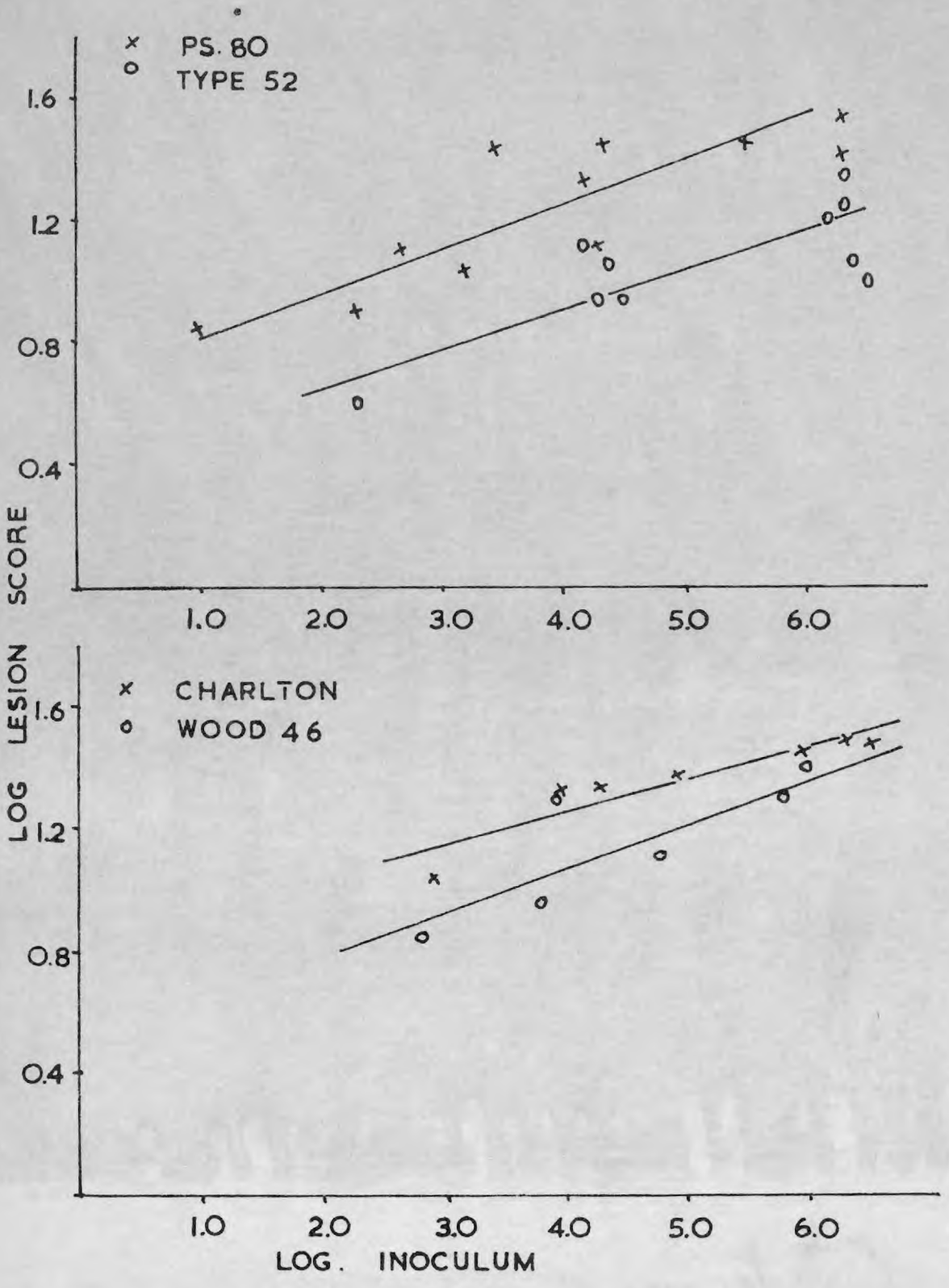


Figure 10 Comparison of regression curves

TABLE 4 Statistical data for dose/response curve

Strain	PS 80	Charlton	Wood 46	Type 52
degrees of freedom	9	5	4	8
score/log.dose curves				
correlation co-efficient	0·96	0·97	0·82	0·92
standard deviation*	14.2%	7.5%	28.6%	13.8%
Log.score/log.dose curves				
correlation co-efficient	0.89	0.92	0.83	0.78
standard deviation*	9.3%	4.9%	11.7%	13.1%

* The standard deviation of the score about the regression line expressed as a percentage of the mean score.

than for the other series. (In Table 4 the standard deviations are shown as a percentage of the mean score in order that they may be compared for the two transformations.) In addition, the regression lines of 'log.lesion score' were rather more parallel than those with the other transformation and were therefore employed. Analysis of covariance showed that, when allowance had been made for the difference in dose, the differences in the scores between strains were significantly different ($5\% > P > 1\%$, $F = 3{\cdot}5$, $n_1 = 3$, $n_2 = 29$).

The use of a grading system for the lesions (slight, moderate and severe) has advantages over a more simple scheme such as 'lesion/no lesion'. Figure 9 shows that, although there is some scatter, the points conform reasonably well to a straight line; if, however, only lesions with the white zone had been considered positive, the curves would have been even more sigmoid than those in Figure 9 (this can be seen from Table 5). Miles (1955) gives a full discussion of the advantages of using dose/response curves over using the ID_{50} or LD_{50} determined from a sigmoid curve.

Reproducibility of lesion score

Mice varied in degree of response to a given dose of bacteria (Table 5). This is only to be expected since not only will the mice themselves vary in resistance to infection, but

TABLE 5 Reproducibility of lesion scores in mice

Strain	Inoculum size	Score for the second day: Number of mice showing this score				total number of mice used
		0	1	2	3	
PS 80	10^6	0	0	2	10	12
	10^5	0	0	1	11	12
	10^4	0	0	1	11	12
	10^3	0	3	9	0	12
	10^2	0	10	2	0	12
	10^1	0	12	0	0	12
	10^6	0	0	2	4	6
	10^6	0	2	1	3	6
	10^4	0	2	3	1	6
	10^2	0	5	1	0	6
Charlton	10^6	0	0	2	4	6
	10^6	0	0	3	3	6
	10^5	0	1	4	1	6
	10^4	0	2	4	0	6
	10^4	0	3	3	0	6
	10^3	1	5	0	0	6
Type 52	10^6	0	6	6	0	12
	10^6	0	2	4	0	6
	10^6	0	2	4	0	6
	10^6	0	5	1	0	6
	10^4	0	3	3	0	6
	10^4	0	5	1	0	6
	10^4	0	6	0	0	6
	10^2	2	4	0	0	6

there will be at least a Poisson variation in the dose of bacteria injected.

Experiments showed that the variance of viable counts of bacteria on the cotton plugs was greater than expected for a Poisson distribution. For a Poisson distribution the variance is equal to the mean of the counts and under the most favourable conditions used in the laboratory (inoculation of 10 drops of a suitable dilution of staphylococci on to agar using the same dropping pipette) this was attained (mean of counts = 26·3, variance = 26·2, n = 18). When cotton plugs were inoculated with staphylococci and then suspended in saline and dilutions inoculated on to agar using a fresh dropping pipette for each culture, the variance was about 7 times the Poisson (mean = 126·1, variance = 928·5, n = 17). When the plugs were made with cotton on which staphylococci had been dried (for details see later) the variance was about 15 times the Poisson (mean = 111·1, variance = 1697, n = 18).

Greater precision in experiments can always be obtained by the use of large numbers of animals but the defects arising from the use of smaller numbers of animals can be offset by statistical methods of analysis and by pooling the results of a number of experiments. Although generally 6 animals were used to determine each piece of information, in the section of this

thesis dealing with a comparison of the virulence of a number of strains of staphylococci, only 2 mice were infected with each staphylococcus. In this survey a total of 259 pairs of mice were inoculated and in 120 of the pairs, both mice gave the same result; in 82 pairs the result differed by only one mark and in 53 pairs it differed by 2 marks. Only 4 of the pairs gave a result which differed by 3 marks. The maximum difference which could have been obtained, in which one mouse showed a severe reaction on both days whilst the other showed no reaction at all, would have been 6 marks. There were a number of staphylococci of each strain thus giving rather more precision to the estimated virulence of the strain than is implied in the small numbers of mice used for each isolate.

Life-history of the lesions

Most of the lesions produced by staphylococci were at a maximum within 1 to 3 days. Necrosis always followed the appearance of the white zone. When necrosis occurred the cotton plug was discharged and the lesion healed, usually within 10 days, and the fur grew again in about 3 to 4 weeks. Figure 11 shows a lesion at 14 days.

When a very small dose of staphylococci (10 viable units) or almost any dose of micrococci was introduced, the progress of the lesion was different. Little or no erythema and no necrosis

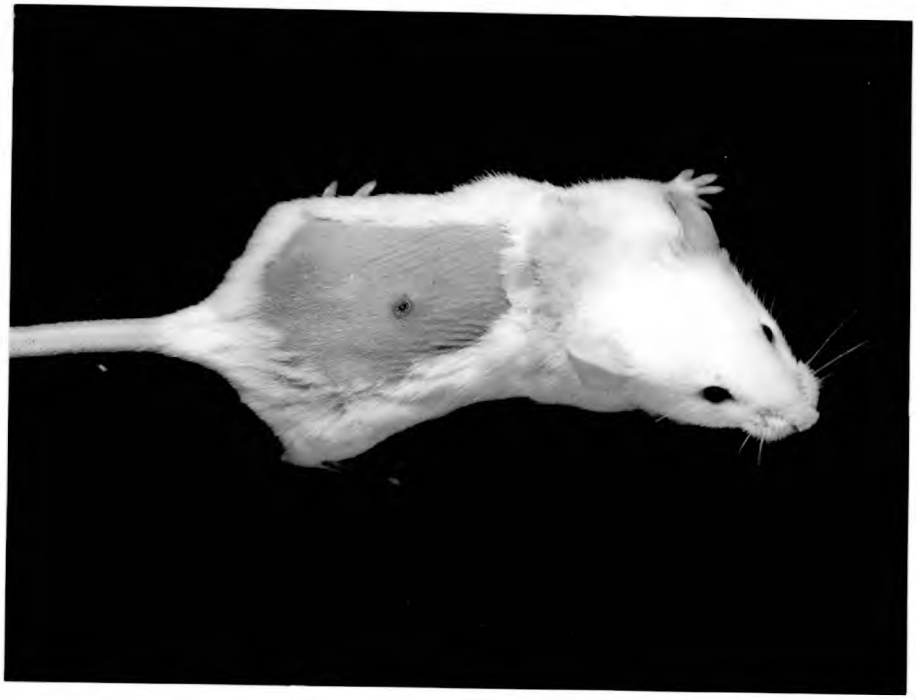


Figure 11 Lesion healing 14 days after challenge.

occurred and the lesion progressed for several days before finally breaking down and releasing large quantities of pus. Figure 6 shows such a micrococcus lesion 10 days after challenge.

None of the mice died from generalisation of these subcutaneous staphylococcal lesions. The staphylococci appeared to be confined to the lesion itself and did not spread through the animal's body. Examinations were made of 270 mice post mortem, 192 had been injected with staphylococcus PS 80, 48 with other staphylococci and 30 with micrococci. On no occasion was any micro-organism isolated from the kidneys and on only 3 occasions were staphylococci isolated from the heart blood. In view of the heavy growth from the heart blood and the lack of growth from the kidneys of these animals it seems possible that the bacteria on the heart swabs were contaminants from the lesion. However, Fisher and Robson (1963) reported that 0•2% of staphylococci injected subcutaneously find their way into the blood-stream very rapidly.

In contrast, mice infected intraperitoneally and intravenously with staphylococci or subcutaneously with Streptococcus pyogenes, died of generalised infections and the appropriate organisms were recovered from the liver, spleen, heart and kidneys of all those animals that died.

Summary of general investigations into lesion production

The subcutaneous inoculation of staphylococci in cotton dust produced a lesion that was in many ways like a human boil or abscess; the lesions which in appearance most closely resembled human boils were those produced by small doses of staphylococci. More severe lesions were produced in the presence of cotton dust than in its absence; the severity of the lesions was also dependent on the dose of cocci inoculated. The presence of the cotton alone did not result in the production of a significant lesion at the skin surface, although a method similar to this has been used to study the formation of granulation tissue in rats (e.g. Penn & Ashford, 1963).

The histological appearance of the lesions also resembled that of similar human infections. The site of the lesion was walled off by a thickening of the tissue and there was an extensive infiltration of the area with leucocytes. The staphylococci appeared to remain localised at the site of the lesion.

The fact that the response to a given dose of staphylococci could be reproduced provided that a group of animals was used, and the fact that the response to different staphylococci varied, suggested that this technique might provide a method by which differences in virulence between groups of staphylococci and

differences in the virulence of one strain treated in different ways might be investigated. Experiments of this nature are reported on in the following sections of this thesis.

THE USE OF THE MOUSE TEST TO DISTINGUISH BETWEEN STRAINS OF STAPHYLOCOCCI

In view of the different responses obtained when 4 strains of staphylococci were injected subcutaneously with cotton dust, it was decided to test a collection of strains obtained during studies on cross-infection to see whether any differences in mouse virulence could be correlated with observed differences in behaviour of the strains in the wards. The strains available were those from studies carried out at St. Bartholomew's Hospital reported by Williams et al., (1962) and Noble (1962). The data available for each strain included phage-type, antibiotic sensitivity, total number of patients infected nasally and the number who suffered sepsis, and counts of the organisms in the hospital environment. It was thus possible to classify most of the strains as 'epidemic' (causing more than 3 lesions) or 'non-epidemic' with a fair degree of accuracy. A few additional strains were available from further studies at St. Bartholomew's Hospital (I am grateful to Dr. M. Patricia Jevons for these strains) and from studies at St. Mary's Hospital.

AN 'INCIDENT' OF CROSS-INFECTION IN A HOSPITAL WARD

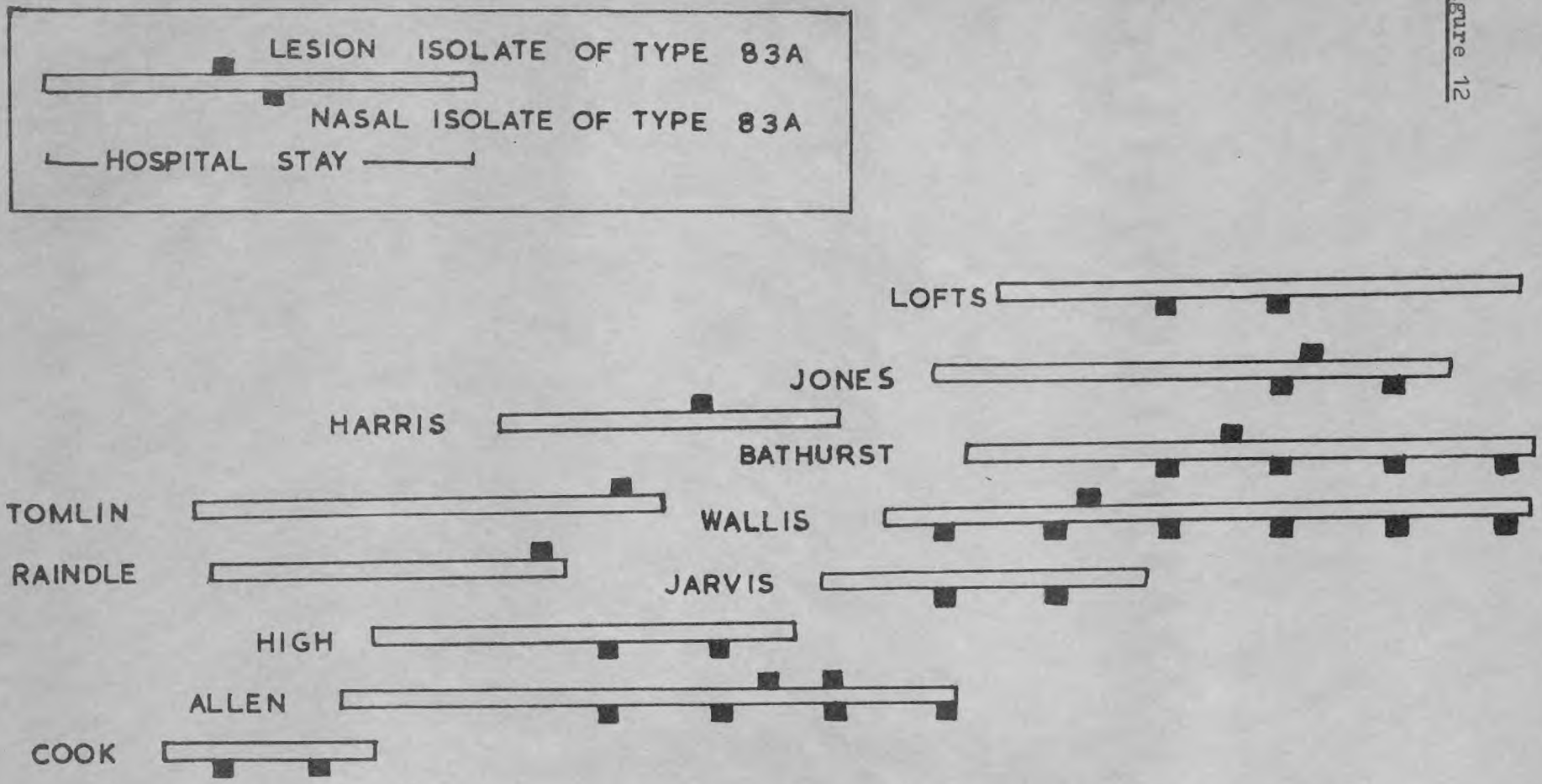


Figure 12

The term 'strain' is here used to mean a staphylococcus of distinct phage and antibiotic sensitivity pattern which was present in the ward over a definable period. Each strain used in the experiments had caused an 'incident' of cross-infection (Figure 12), in which a number of patients had become infected either as nasal carriers or with clinically infected lesions. Each patient from whom the strain was recovered was regarded as yielding an 'isolate' of the strain; not more than one isolate from each site on each patient was included in the experiments. Altogether 200 'isolates' belonging to 35 different 'strains' of 22 different 'phage-types' of staphylococci were examined.

In addition, a further 21 isolates from 8 strains which were present in the wards for at least one week without spreading to the other patients were examined; all of these were from nasal carriers and caused no sepsis.

It should be noted that some of the isolates tested, particularly those recovered early in the work at St. Bartholomew's Hospital, had been stored for many months at room temperature on nutrient agar in airtight bottles, without being subcultured. All the isolates had, after being recovered from the patients, been subcultured 3 or 4 times on artificial media.

Each of the isolates used in the tests for mouse virulence was freeze-dried under a code number, so that the

source of the strain was not apparent at the time of making any test. This was important in order to avoid bias in reading the results. This and the fact that the strains were selected at random for the tests had the effect of off-setting the differing ages of the cultures since primary isolation.

Preparation of the inoculum

One batch of nutrient broth was used for all the cultures. The inoculum was prepared by opening an ampoule of the freeze-dried culture directly into the broth, which was then incubated for 18 hours. A small wire loop, used throughout the experiments, was employed to charge the cotton plug with one drop of the culture and the plug was then inoculated under the skin of the mouse. Viable counts of the inoculum were carried out on serum agar containing phenolphthalein phosphate and the number of cells per viable unit (the clump-size) was calculated as described in Appendix 2.

RESULTS

Strains which spread in the wards:

Comparison of the severity of lesions

Two mice were inoculated with each isolate and the results scored at 24, 48 and 72 hours. As necrosis often made it difficult to assess the severity of the lesion after 72 hours, the mice were killed at this stage. No mice died during the

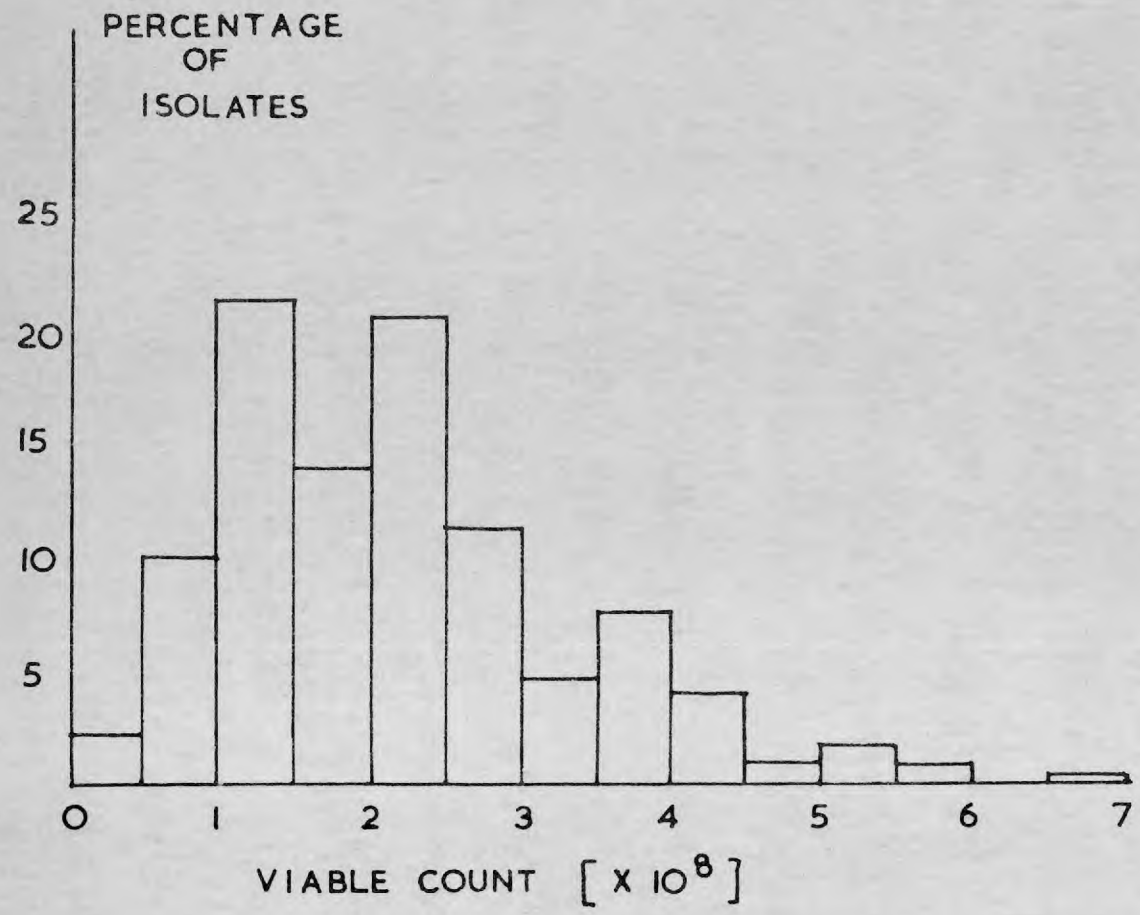


Figure 13 Range of viable counts per ml of culture, of the 259 isolates inoculated into mice

course of the experiment and, in view of earlier experience, no post mortem examinations were conducted.

The sums of scores for the 2 mice for the first two days were tabulated for each isolate of each strain (Appendix 1 Table 4). There was some variation in the scores but an analysis of variance showed that the differences between strains were greater than those within strains, and that the difference was significant at the 0.1% level (variance ratio = 2.61, $n_1 = 34$, $n_2 = 165$).

Relation of score to dose inoculated

As has already been demonstrated, there was a strong correlation between the size of inoculum as determined by the viable count, and the score obtained in the mouse test. Since the broth cultures were used without preliminary standardization there was a range of inoculum size from 1×10^7 to 7×10^8 cocci per ml., with the majority of the counts lying in the range 0.5 to 3×10^8 (Figure 13). The volume of broth culture inoculated on the cotton dust was about 0.01 ml. Analysis of variance showed that in this series of experiments also, the correlation between the viable count and the lesion score was unlikely to have occurred by chance ($P < 0.1\%$, $F = 13.0$, $n_1 = 1$, $n_2 = 198$).

The effect of variation in dose can be overcome statistically by the use of an analysis of covariance in which allowance is made for the variation in dose inoculated and the

TABLE 6 Adjusted mouse scores for each isolate

Rank number in order of mouse virulence	Adjusted scores for each isolate*	Mean Score	Rank order number in unadjusted series
1	11.1 11.7	11.40	2
2	10.3 10.8 11.7 12.6	11.35	3
3	9.7 10.0 11.7 12.0 12.2	11.12	4
4	9.7 10.5 11.9 12.1	11.05	1
5	8.0 11.6 12.0	10.53	5
6	10.2 10.5	10.35	7
7	8.4 8.7 9.8 12.7	9.90	11
8	7.0 7.5 8.3 8.4 9.4 9.5 9.5 9.5 9.6 9.8 11.1 11.1 12.4 12.4 12.6	9.87	12
9	6.7 7.7 9.5 9.5 9.8 9.9 9.9 11.8 11.9 11.9	9.86	8
10	6.4 10.0 10.5 10.6 11.6	9.82	9
11	4.7 8.1 9.3 11.7 12.3 12.6	9.78	16
12	7.4 9.1 10.2 12.3	9.75	13
13	7.9 8.3 9.6 9.9 11.2 11.5	9.74	6
14	6.2 7.4 9.6 9.8 10.7 10.8 11.0 12.0	9.68	14
15	5.9 7.6 12.0 12.5	9.50	17
16	7.3 9.3 9.9 11.1	9.40	10
17	7.9 7.9 8.3 9.0 10.9 11.8	9.30	15
18	7.0 8.0 9.7 11.8	9.12	18
19	4.4 9.7 9.9 10.3 10.7	9.00	20
20	4.4 5.9 8.4 10.0 12.0 13.0	8.95	19
21	3.7 5.7 7.8 8.5 8.7 9.3 9.5 10.9 12.5	8.51	23
22	4.4 4.6 5.6 6.1 9.1 10.0 10.1 10.1 11.3 12.2	8.35	22
23	5.0 5.3 7.1 8.1 8.1 10.3 10.6 11.1	8.20	21
24	4.6 4.8 6.0 6.1 6.2 6.4 8.5 8.5 9.4 10.3 10.3 10.4 11.0 12.2	8.19	26
25	4.2 9.9 10.1	8.06	24
26	5.2 5.3 7.0 7.4 8.1 8.6 11.0	7.51	25
27	4.5 6.7 6.8 7.6 7.6 7.7 8.4 8.5 9.7	7.50	29
28	5.2 6.9 6.9 10.1	7.27	27
29	4.7 4.8 4.9 7.5 8.2 9.7 10.1	7.12	28
30	6.3 6.4 7.2 8.0	6.97	31
31	3.5 6.6 7.9 9.6	6.90	30
32	4.0 5.4 6.3 7.4 8.4	6.42	32
33	5.6 7.0	6.30	33
34	4.2 6.1 7.1	5.80	34
35	2.9 4.7 6.5	4.70	35

* Each score is based on the scores of 2 mice for 2 days.

corrected values tested for significant differences. The analysis table is given in Appendix 1 Table 5 which shows that, when allowance had been made for the different inoculum size, there was still a significant difference between the strains ($1\% > P > 0.1\%$, variance ratio = 2.04, $n_1 = 34$, $n_2 = 164$). As a result of adjusting the scores for the effect of inoculum size, the variance attributed to the 'between strains' differences dropped from 12.15 (34 degrees of freedom) to 10.29 and this is seen to be reflected in a change in P (from $P < 0.1\%$ to $1\% > P > 0.1\%$). This presumably means that there was a tendency for most of the isolates in some strains to grow to a greater cell density in broth than did those of other strains; this point is examined later.

Before discussing the relation between mouse virulence and factors such as the resistance to antibiotics etc., it is desirable to correct the mouse scores obtained by direct observation for the effect of differing doses. This was done according to the following formula:

Adjusted score = original score - bx

where b is the regression co-efficient

and x is the deviation of the dose from the mean dose.

An example of this adjustment follows:-

Isolate number 49, mouse score Day 1: 3 + 2 (one mouse showed a severe and one a moderate reaction), Day 2: 3 + 3, total score = 11. Viable count of inoculum = 198, mean viable count for series = 221. (These numbers, the number of colonies growing on the agar from a standard dilution of the culture, rather than the actual dose inserted were used in the calculation of the regression of score on dose inoculated. For a viable count of 198 the dose inoculated was 99×10^5 viable units.)

Regression co-efficient of score on dose inoculated, calculated for the whole series, r = 0·005.

Unadjusted mouse score = 11

Deviation of dose from mean dose = 198 - 221 = -23

Adjusted score = 11 - (0.005)(-23)

= 11 - (-0·115)

= 11.1

The result of this adjustment is given in Table 6. As may be seen, this made little difference to the order in which the strains fell. Unless specifically qualified, the 'rank order number' given in the following tables refers to the rank order of mouse virulence for adjusted scores given on the left hand side of Table 6.

In order to test whether the changes in position in the table were significant or not the following method was used:

The rank correlation co-efficient was calculated as follows:-

$$r_s = 1 - \frac{6 \cdot Sd^2}{n(n^2 - 1)}$$

where

r_s is the rank correlation co-efficient

Sd^2 is the sum of squares of the deviations of one rank order from the other

n is the number of ranks

Like the correlation co-efficient, r_s can range from -1, (complete discordance) to +1 (complete concordance). The degree of significance can be found from tables of the correlation co-efficient.

In this example

$$Sd^2 = 174$$

$$r_s = 1 - \frac{6 \times 174}{35(35^2 - 1)} = 1 - 0.024 = 0.976$$

The concordance was significant at the 0.1% level, degrees of freedom 35 - 2 = 33.

Comparison of scores of isolates from different sources

It has often been suggested that staphylococci from, for example, a wound may be more virulent than those isolated from the nose. This point was examined here by comparing the scores obtained in the mouse test of isolates recovered from lesions and from the nose.

Isolates from wounds. There were 31 isolates from clinically infected surgical wounds and 61 nasal isolates of the same 14 strains. When the mean scores of these two groups were compared it was found that the wound isolates had a lower mean score than those from the nose and that, on the basis of 'Students' t test these scores were significantly different. ($5\% > P > 2\%$, $t = 2{\cdot}24$, wound isolates mean score = 8·34, $\sigma^2 = 6{\cdot}01$, $n = 31$; nasal isolates mean score = 9·45, $\sigma^2 = 4{\cdot}57$, $n = 61$).

Comparison of wound and nasal isolates from the same patients. Among the 31 isolates from wounds, there were 15 for which there was a paired isolate of the same strain recovered from the nose of the same patient within one week of the wound isolate. When these were compared it was found that the mean scores were not significantly different although the nasal isolates still gave a higher score ($P > 10\%$, $t = 1{\cdot}20$, wound isolates mean score 7·97, $\sigma^2 = 4{\cdot}64$, $n = 15$; nasal isolates mean score = 8·89, $\sigma^2 = 3{\cdot}64$, $n = 15$).

Isolates from urine. A similar analysis was carried out on isolates from infected urine specimens. It was found that the mean score for the 8 isolates from urine was lower than that of the 27 nasal isolates from the same 5 strains and that the difference was statistically significant ($2\% > P > 1\%$, $t = 2.69$,

urine isolates mean score = 6·15, σ^2 = 4.42, n = 8;
nasal isolates mean score = 8·68, σ^2 = 5.69, n = 27).
There were only 2 urine isolates for which there were paired nasal isolates and no significant difference could be shown between the mean scores of these.

Hence there is no evidence that staphylococci isolated from septic lesions were more virulent for mice than thos isolated from nasal sources when tested by this method.
It might indeed be argued that staphylococci isolated from urine and perhaps adapted to this environment were less virulent than staphylococci of the same strain isolated from the nose.

A comparison of virulence based only on nasal isolates

In view of the differences found between isolates from different sources it might be argued that it would be fairer to construct a virulence table based only on nasal isolates. Table 7 shows such a table. Whilst the mean scores of some of the isolates have changed quite markedly, there has been remarkably little effect on the order in the table as a whole (rank correlation co-efficient = 0·864, df = 33 concordance significant at the 0.1% level). In view of this lack of difference, virulence tables quoted hereafter are based on isolates from all sources.

TABLE 7 A comparison of rank orders of mouse virulence based on isolates from all sources or on nasal isolates only.

Rank order number on nasal isolates only	Rank order number on full table	Mean score based on nasal isolates only	Mean score based on full table
1	2	11.45	11.35
2	1	11.39	11.40
3	20	11.14	8.95
4	3	11.12	11.12
5	4	11.04	11.05
6	5	10.53	10.53
7	14	10.52	9.68
8	6	10.37	10.35
9	19	10.21	9.00
10	8	10.13	9.87
11	11	10.10	9.78
12	9	10.09	9.86
13	25	10.01	8.06
14	18	9.81	9.12
15	10	9.80	9.82
16	12	9.75	9.75
17	13	9.74	9.74
18	15	9.50	9.50
19	16	9.39	9.40
20	17	9.30	9.30
21	22	9.24	8.35
22	7	8.98	9.90
23	21	8.60	8.51
24	29	8.23	7.12
25	23	8.21	8.20
26	27	7.68	7.50
27	24	7.55	8.19
28	26	7.49	7.51
29	28	7.26	7.27
30	30	6.62	6.97
31	31	6.61	6.90
32	32	6.40	6.42
33	33	6.33	6.30
34	34	5.79	5.80
35	35	4.68	4.70

TABLE 8 A comparison of rank order number from results at 24 and 48 hours after infection.

Rank order number based on results at 24 hours	Rank order number based on results at 48 hours
1	1
2	4
3	2
4	3
5	6
6	5
7	7
8	8
9	9
10	10
11	12
12	11
13	13
14	15
15	16
16	19
17	14
18	18
19	21
20	20
21	17
22	22
23	24
24	23
25	25
26	26
27	29
28	27
29	28
30	34
31	30
32	31
33	33
34	32
35	35

Differences in virulence detected at 24 hours

All the analyses given above were based on the assessment of the severity of the mouse lesion over the first 48 hours. This period was chosen because it offered the maximum range of scores.

It might be argued that some of the more virulent strains could produce a lesion within 24 hours but that the effect might be masked by other, less virulent, strains 'catching up' at 48 hours. Accordingly Table 8 shows the strains placed in order of virulence judged by their mean unadjusted scores at 24 hours compared with the score at 48 hours. There is little difference in the overall order of the strains (rank correlation co-efficient = 0.99, df = 33, concordance significant at the 0.1% level). All the following tables are therefore based on the mouse score at 48 hours.

The relation of clump size to virulence

In the preceding sections, the number of bacteria inoculated into the mouse was determined solely on the basis of the viable count (the number of viable colony-forming units of cocci) and no attempt was made to utilize the data on clump size the number of viable cocci in each colony-forming clump). As is shown in Figure 14, the clump sizes of the broth cultures were remarkably alike and appeared to conform to a normal distribution.

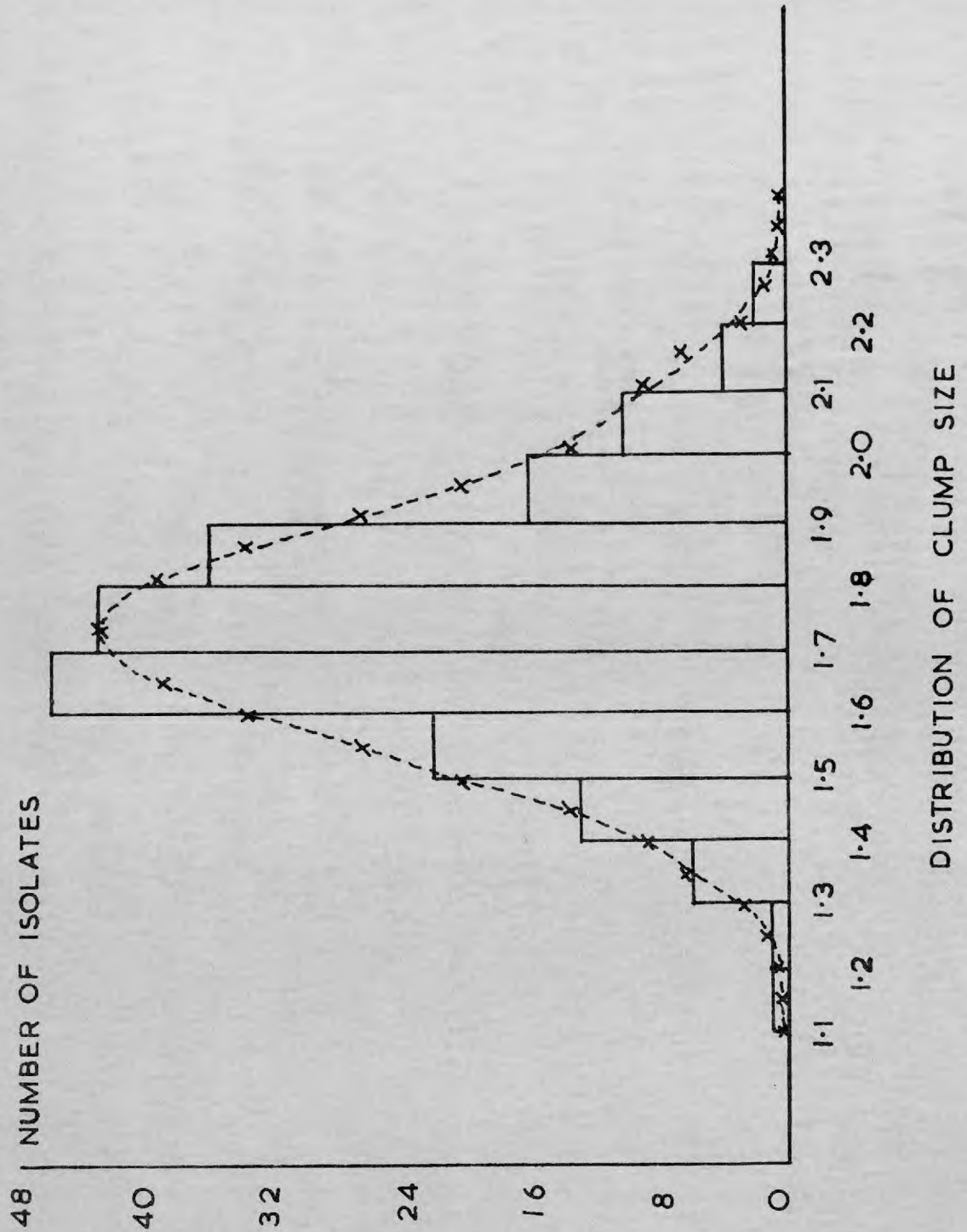


Figure 14 Distribution of clump size and the normal distribution curve appropriate to this data

An analysis of variance carried out on the correlation between the clump size and the adjusted mouse score (in which, of course, allowance has been made for the differences in viable count) showed that there was no significant relation of clump size to virulence ($P > 20\%$, $F = 0.62$, $n_1 = 1$, $n_2 = 198$).

Summary

In agreement with the previous observations, significant differences were detected between the reactions of mice to subcutaneous injections of a number of strains of staphylococci on cotton dust. The severity of the reaction was dependent on the dose of cocci inoculated but even when allowances had been made for the effect of inoculum size, there were still significant differences between the strains. Staphylococci isolated from septic lesions and from urine specimens tended to give less severe lesions in the mice than did those isolated from the noses of patients. No significant changes in the rank order of virulence was seen however when only isolates from the nose were considered. The 'virulence' did not appear to depend on the number of cocci in each viable unit, although the differences in clump size were small.

TABLE 9 Observed epidemicity of strains

Rank order number	Source in index patient	Number of other patients infected				If strain regarded as 'epidemic'
		N only	L only	N + L	Total	
1	N + L	4	0	3	7	+
2	N	15	3	1	19	+
3	N + L	9	0	1	10	0
4	N + L	3	0	1	4	0
5	not known	-	-	-	-	+
6	not known	-	-	-	-	NK
7	N + L	4	3	2	9	+
8	L	37	2	10	49	+
9	N + L	17	2	4	23	+
10	N	11	0	0	11	0
11	N + L	15	0	2	17	+
12	N	3	0	0	3	0
13	L	3	1	2	6	+
14	L	5	3	2	10	+
15	N	3	0	0	3	0
16	N + L	4	0	2	6	+
17	not known	-	-	-	-	+
18	N + L	4	0	2	6	+
19	N	5	3	4	12	+
20	N + L	6	1	4	11	+
21	N + L	12	0	4	16	+
22	N	15	1	2	18	+
23	not known	-	-	-	-	+
24	N + L	14	1	13	28	+
25	N + L	11	0	2	13	+
26	N	6	0	1	7	0
27	N	7	0	5	12	+
28	N + L	10	0	1	11	0
29	N	2	1	1	4	0
30	N	10	0	2	12	0
31	N + L	3	2	1	6	+
32	N	4	0	0	4	0
33	N	5	0	0	5	0
34	N + L	6	0	1	7	0
35	N	4	0	0	4	0

N = nose L = lesion

Strains 5, 17 and 23 were obtained from studies at St. Mary's Hospital where only lesions were investigated. Strain 6 was obtained at the end of the investigations at St. Bartholomew's Hospital and was not observed for more than 1 week.

The relation of mouse virulence to other characteristics of the staphylococci:

Observed epidemicity of the strains

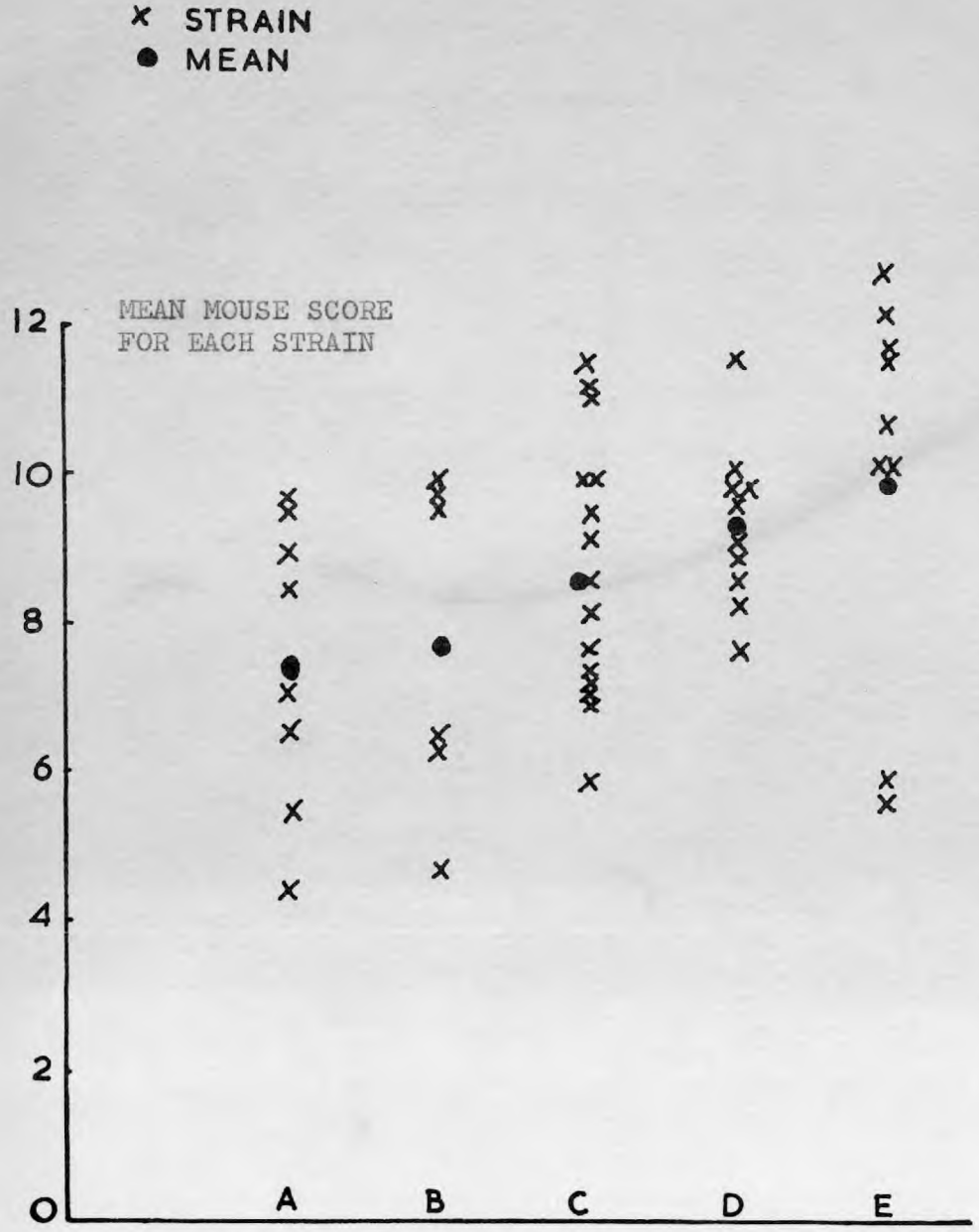
Table 9 presents the epidemic history of the strains as observed in the wards, the strains being listed in order of mouse virulence. On the basis of the definition of epidemicity given by Williams and Jevons (1961), (the isolation of the staphylococcus from 3 or more septic lesions), it can be seen that there was a tendency for the strains which were epidemic for man to be virulent for mice. The significance of this relationship may be tested by the Mann-Whitney U statistic (Siegel, 1956) which is calculated as follows:-

From Table 9 calculate the number of times that a non-epidemic strain precedes an epidemic one in rank order number. Thus strain 3, which is non-epidemic, precedes 19 epidemic strains, and so does strain 4. Strain 10 precedes 15 epidemic strains and strain 12 precedes 14 epidemic strains, etc. These are then added together to derive U

$$U = (19+19+15+14+12+2+1+1+1) = 84$$

$$z = \frac{\left| U - \frac{1}{2}mn \right| - \frac{1}{2}}{\sqrt{\left(\frac{1}{2}mn(m+n+1)\right)}}$$

where m = number of non-epidemic strains = 13
n = number of epidemic strains = 21
$| \; |$ indicates numerical value ignoring the sign



A Strains not spreading in the wards, mean score = 7.2
B Strains which spread but caused no lesions, mean score = 7.74
C Strains which spread and caused 1-3 lesions, mean score = 8.63
D Strains which spread and caused 4 or more lesions, mean score = 9.28
E Strains from animal sources, mean score = 9.96

Figure 15 Comparison of virulence of isolates with different 'epidemic' abilities

$$z = \frac{\left| 84 - 136.5 \right| - \frac{1}{2}}{\sqrt{(136.5 \times 35)}}$$

$$= 0.75$$

From tables of Fisher (1948) we see that this result is significant between the 1% and the 0.1% levels. Hence we may conclude that those strains which were epidemic for man had a significant tendency to be virulent for mice on subcutaneous inoculation in cotton dust.

Some caution must be used in assessing these results however, since some of the strains were recovered from wards in which patients harbouring tetracycline resistant staphylococci were isolated from the other patients in ventilated cubicles or in side wards (Williams et al., 1962) and thus the full epidemic potential of some of these strains may not have been realised. In addition, some of the strains (no. 1, 24, 25, 28, 30) came from incidents in which staphylococci were widely distributed about the wards by individual patients 'dispensers'. This dispersal is now thought to be a function of the patient rather than the staphylococcus and the apparent epidemicity of these strains may, therefore, be exaggerated; it is interesting that with one exception they were among the least virulent strains in the mouse test. Figure 15 shows that the mean scores in the mouse test were highest among those strains that caused the most sepsis.

TABLE 10 Virulence of strains of staphylococci which did not spread.

Mean score	Phage type	Antibiotic resistance*	Adjusted score in mouse test			
9.65	187	-	7.3	12.0		
9.50	3B/3C/55/71	P	7.2	11.8		
8.97	52A/79	P	6.0	10.1	10.8	
8.35	79/6/53/77	-	7.7	9.0		
7.1	81	-	6.1	8.1		
6.5	52	-	3.2	6.1	6.7	10.2
5.4	NT	-	4.8	6.1		
4.4	52A	P	2.2	3.2	4.2	8.0

TABLE 11 Virulence of staphylococci from animals.

Mean score	Phage type	Antibiotic resistance*	Source
12.7	29/52/52a/80/3A/3B/3C/55/47/53	-	NCTC 7485 bovine
12.1	42E/77/42D	-	cow
11.7	NT	-	dog
11.5	NT	T	pig
10.5	6/7/42E/47/53/54/77/42D/81+	-	hamster
9.9	29/52/52A/79/80/7/47/53/54/75/83A/81	P	sheep
9.9	29/52/52A/79	-	chicken
5.9	52A/79	P	gerbil
5.5	77/42D	-	cow

* P = resistance to penicillin T = resistance to tetracycline

Strains which did not spread in the wards

A number of tetracycline sensitive strains which did not spread about the wards were investigated in the same manner as the 'spreading' strains. In an attempt to get material which could be investigated in the same way as the other series, repeat nasal isolates of the same strain from the same patient were examined. All the isolates were from the weekly swabbing of patients and each strain was present in the wards for a period of at least one week and so, in theory, had the opportunity to spread. The mouse scores, corrected for differences in dose in the same way as for the spreading strains, are shown in Table 10, together with the phage and antibiotic sensitivity patterns. The differences between the strains were not significantly greater than those within strains ($P > 20\%$, $F = 1.44$, $n_1 = 7$, $n_2 = 13$).

Figure 15 shows that the range of mean scores was the same as that of the lower 2/3 of the table for spreading strains; the mean score for the group was 7.2 marks. It is curious that the type 187 should again be near the top of the table, for this type is not generally regarded as 'virulent' for man.

Strains from animal sources

It has been suggested that staphylococci from animal sources are more 'virulent' for animals than strains from

TABLE 12 Phage-type and antibiotic sensitivity in relation to virulence for mice.

Rank order number	Phage type+	Epidemic* type	Antibiotic resistance pattern
1	6/47/53/75	*	-
2	80	*	PT
3	53/75		PTS
4	187		-
5	83A	*	PT
6	52/80	*	C
7	52/55		PTSE
8	80/81	*	PT
9	53/77		PTS
10	7/47/53/75/77	*	PTS
11	29/52		-
12	55/71		-
13	55/71		P
14	80/81	*	PT
15	6/7/47/53/54/75	*	-
16	6/7/47/53/54/75	*	P
17	80/81	*	PT
18	6/7/47/53/54+	*	-
19	83A	*	PTS
20	83A	*	PTS
21	52/80/81	*	PT
22	75/77		PSE
23	29/52/80		P
24	53/77		PTS
25	79/6/53/77		P
26	52A/79		P
27	52/52A/80	*	PT
28	6/53/75		T
29	52/80	*	PT
30	79		P
31	53		PTS
32	52A/79		P
33	52/52A/79/80/7/42E		-
34	inhib 52A		P
35	3B/3C/55/71		-
non-spreading strains:			
1	187		-
2	3B/3C/55/71		P
3	52A/79		P
4	79/6/53/77		-
5	81		-
6	52		-
7	NT		-
8	52A		P

+ Phage-types underlined indicate lysis at 1000 x RTD

* Types causing more than 4% of hospital sepsis 1954-1960

/ø P=resistant to penicillin, T=resistant to tetracycline
S=resistant to streptomycin, E=resistant to erythromycin
C=resistant to chloramphenicol, -=sensitive to all antibiotics

human sources (e.g. D. D. Smith, 1963). A collection of strains isolated from animals was kindly sent to me by Dr. H. Williams-Smith of the Animal Health Trust. These were tested by the mouse test and the scores and other details given in Table 11. In general they gave consistently higher scores than many of the human strains (Figure 15), with a mean of 9·96 marks, thus supporting Smith's findings.

COMPARISON OF VIRULENCE WITH IN VITRO CHARACTERS

As is apparent from the literature discussed in the first part of this thesis, there are a considerable number of characters which have been investigated with a view to their possible relationship to the virulence of staphylococci. A number of these characters have been investigated here and it is proposed to deal with them separately in the first instance, pooling the results for the separate isolates of each strain.

Antibiotic resistance

The possibility of an association of virulence for mice with resistance to tetracycline or streptomycin was investigated using the Mann-Whitney U statistic described previously. No evidence for any such association was found however (see Table 12). (Tetracycline, $P > 5\%$, $z = 0.42$; streptomycin, $P > 5\%$, $z = 0.25$.)

We may note that whilst none of the strains which failed to spread in the wards were resistant to tetracycline, 13 of the 16 strains which spread to more than 10 patients were resistant to penicillin and tetracycline or streptomycin, and two of the other three were spread by dispersers. The fact that no relation with streptomycin resistance could be found, rules out the possibility that any relationship of virulence with multiple antibiotic resistance exists, since with one exception all the streptomycin resistant strains were also resistant to penicillin and tetracycline.

TABLE 13 Comparison of resistance to mercury salts and tetracycline.

Rank order number	Resistance		Rank order number	Resistance	
	mercury	tetracycline		mercury	tetracycline
Spreading strains			Non-spreading strains		
1	-	-	1	-	-
2	R	R	2	-	-
3	R	R	3	-	-
4	-	-	4	-	-
5	R	R	5	-	-
6	-	-	6	-	-
7	R	R	7	-	-
8	R	R	8	-	-
9	R	R			
10	R	R			
11	-	-			
12	-	-			
13	-	-			
14	R	R			
15	-	-			
16	-	-			
17	R	R			
18	-	-			
19	R	R			
20	R	R			
21	R	R			
22	R*	-			
23	S/R	-			
24	R	R			
25	S/R	-			
26	S/R	-			
27	R	R			
28	-**	R			
29	R	R			
30	S/R	-			
31	R	R			
32	R	-			
33	-	-			
34	-	-			
35	-	-			

R = resistant - = sensitive S/R = equivocal results

* this strain is resistant to penicillin, streptomycin and erythromycin although sensitive to tetracycline.

** this strain is resistant to tetracycline only.

Mercury resistance

The phenyl-mercuric-nitrate disc method described by Green (1962) was used to determine the sensitivity of the strains to mercury (see Appendix 2). Table 13 shows that there was, in general, a very good correspondence between tetracycline resistance and mercury resistance; only 7 of the 43 strains did not correspond. Of these 7, two nevertheless fit in with general experience; one strain sensitive to tetracycline but resistant to mercury was also resistant to penicillin, streptomycin and erythromycin; and one resistant to tetracycline but sensitive to mercury was also sensitive to all other antibiotics.

Four of the 'spreading' strains however gave results which did not conform to the general pattern in that the sensitivity recorded appeared to depend on the density of the inoculum and in one other strain the resistance to mercury and tetracycline did not correspond. All these strains were resistant to penicillin but sensitive to tetracycline and other antibiotics. The phage-types of these strains were 79/6/53/77, 79, 29/52/80, 52A/79, 52A/79. It is curious that 3 of these strains (79/6/53/77, 79 and 52A/79) were spread about the ward by dispersers. Such dispersal is thought to be a result of the colonisation of the skin of the patient (Noble & Davies, in preparation) and it may be asked whether this partial resistance to mercury is a reflection of

'partial' virulence in that the strains are able to colonise healthy skin but not to cause disease. It is noteworthy that another type 52A/79 resistant to penicillin and type 79/6/53/77 sensitive to all antibiotics, both non-spreading strains, were uniformly sensitive to mercury.

Phage-type

The relation of phage-type to virulence is more difficult to analyse. Williams and Jevons (1961) found that 6 basic phage-types of staphylococci each accounted for more than 4% of the hospital sepsis in 1954-60 (types 80/81), 52/52A/80 and 52/80, 6/7/47/53/54/75+, 47/53/75/77, 83A).

If these types can be regarded as the 'epidemic' phage-types for man, we see (Table 12) that the relation between virulence for mice and 'epidemic' phage-type is significant ($1\% > P > 0.1\%$, Mann-Whitney U, $z = 0.8$, $n_1 = 16$, $n_2 = 19$). We may note however that this holds true only for staphylococci from human sources. The phage-types of the staphylococci from animals (Table 11) are unlike those associated with disease in man, yet the animal strains were in general more 'virulent' than those from humans.

Another way in which the relationship between phage-type and virulence might be explored is to test the variability in the scores given by strains of the same phage-type irrespective

of the other features. Table 14 shows material analysed in this manner; in this rather limited series, however, the differences within the types were as large as those between the types ($P > 20\%$, $F = 1{\cdot}53$, $n_1 = 9$, $n_2 = 16$).

Coagulase

All the strains were coagulase positive for human plasma by the slide and the tube test. It has been suggested that the virulence of a staphylococcus for an animal depends on its ability to coagulate the animal's plasma. Accordingly, all the isolates were tested for coagulase using pooled mouse plasma; with one exception (the isolate from the dog) all were positive by the slide coagulase test. By the tube test, 11 of the 136 tested were positive but these 11 were scattered throughout the entire range of isolates. As noted by Selbie and Simon (1952) the clots produced were not like those obtained using human plasma; they were far looser and broke up very easily. It seems possible that some positive results may have been missed because of the frailty of the clots. Seven of the 11 positives were obtained only after incubation at bench temperature overnight. Addition of normal rabbit serum to mouse plasma enabled most of the staphylococci to produce a clot but this was still very fragile.

Haemolysins

The haemolysins were investigated using rabbit and sheep red cells which had been washed 3 times with saline and

TABLE 14 Analysis of virulence according to phage-type.

Phage-type	Mean mouse scores for the strains of this type			
80/81	9.30	9.68	9.86	11.35
52/52A/80/81	7.12	7.50	8.51	
52A/79	7.51	8.88		
3B/3C/55/71	4.70	9.46		
53/77	8.19	9.87		
6/47/53/75	7.27	11.40		
6/7/47/53/54/75/77	9.12	9.40	9.50	9.82
83A	8.95	9.08	10.53	
187	9.64	11.05		
79/6/53/77	8.06	8.36		

re-suspended at the original cell density in saline.

The strains were 'streaked' on agar containing 5% of the rabbit red cell suspension at right angles to filter-paper strip soaked in staphylococcus antitoxin (200 units per ml.) and the plates incubated at 37°C in air overnight; it is recognised that this is not the optimum method for the production of alpha-haemolysin but the interest lay in finding differences between strains. The use of the antitoxin enables the alpha and delta lysins to be distinguished. The results were recorded as moderate or minimal lysis or absence of lysis. Only one strain, Wood 46, produced abundant alpha lysin using this technique although all strains produced more alpha lysin on further incubation of the plates; the early results showed the biggest differences between the strains. The results are shown in Table 15; the majority of the 'virulent' strains produced moderate amounts of alpha lysin, whilst the less virulent strains produced minimal amounts of alpha lysin (these results are based on the mean for the various isolates of each strain; there was good agreement within the strains). The delta lysin showed a very similar pattern. The associations between moderate production of alpha and of delta lysin with virulence were significant at the 0.1% level (alpha lysin $z = 1.19$, $n_1 = 15$, $n_2 = 20$; delta lysin $z = 0.91$, $n_1 = 13$, $n_2 = 22$).

TABLE 15 Production of haemolysins and diffusible precipitins.

Rank order number	Haemolysins			Number of antigen/antibody diffusion lines
	alpha	beta	delta	
1	+		+	5.0
2	+		+	4.3
3	+		+	3.8
4	+	+	+	2.5
5	+		+	4.0
6	min		none	2.0
7	+		+	2.0
8	+	+	min	2.7
9	+	+	+	3.8
10	+		+	4.6
11	min		min	2.6
12	+		+	4.3
13	+		+	2.2
14	min		min	1.9
15	min		+	2.5
16	+		+	3.8
17	+		+	2.2
18	+		+	3.3
19	+		+	4.4
20	+		+	4.8
21	+		min	2.9
22	min		+	3.4
23	min		+	4.4
24	+	+	+	4.1
25	min		min	3.0
26	min		+	3.1
27	min		none	1.9
28	+		none	1.7
29	min		min	1.7
30	min		+	3.0
31	none		min	2.0
32	min		min	2.8
33	min		none	2.0
34	none		+	3.3
35	+		min	2.3
non-spreading strains:				
1	+		+	4.0
2	min		+	2.0
3	min		+	4.3
4	min		+	4.0
5	min		+	3.5
6	min		+	1.7
7	none		+	4.0
8	min		min	2.2

+ = moderate production of lysin min = minimum production

Isolates of some strains were tested for quantitative production of alpha lysin by the tube method described by Selbie and Simon (1952), (see Appendix 2). The results were consistent for each strain and tended to confirm the rather more crude plate technique (Table 16). It was clear that the severity of the lesion in mice was not correlated solely with the production of alpha haemolysin in vitro however, since the strain which was least virulent for mice (rank order number 35) produced as much alpha lysin in the tube test as did the most virulent for mice (no. 1).

Addition of 10 units of staphylococcus antitoxin to the tubes prevented all haemolysis except at the 1/4 dilution.

The sheep blood agar plates were inoculated from a broth culture and were incubated at 37°C for 48 hours in 30% CO_2. After examination the plates were kept at 4°C for a further 18 hours to demonstrate the 'hot/cold' lysis of the beta haemolysin. Four strains consistently produced beta lysin (Table 15) and in addition a few isolates of other strains showed some 'hot/cold' lysis; these have not been included here but the data is utilized in other analyses. Three of the four strains are among the most virulent and the fourth is of the same phage-type and antibiotic sensitivity pattern as one of the three; the two epidemics due to this type occurred in different wards following the transfer

TABLE 16 Quantitative determination of alpha haemolysin.

Rank order number	Number of isolates tested	Dilutions showing haemolysis						Result on plate test
		1/4	1/16	1/64	1/256	1/1024	1/4096	
1	2	+	+	+	±			+
2	3	+	+	+	±			+
3	4	+	+	+	±			+
5	1	+	+	+	+	±		+
6	2	+	+	±				min
7	2	+	+	+	+	±		+
8	5	+	+	±				+
10	3	+	+	+	+	±		+
11	3	+	+	±				min
12	2	+	+	+	±			+
23	4	+	+	+	±			min
24	4	+	+	+	±			+
32	3	+	±					min
33	2	+	±					min
34	2	+	±					none
35	2	+	+	+	±			+
PS 80	1	+	+	+	±			
Wood 46	1	+	+	+	+	+	±	
Smith	1	+	+	+	±			

+ indicates complete haemolysis

± indicates 50% haemolysis

of one patient. In common with Levy (1952), this work showed that beta lysin and fibrinolysin were not found to be mutually exclusive.

Antigen/antibody diffusion lines

The antigen/antibody plate diffusion technique described by Elek and Levy (1950) was followed exactly using refined staphylococcus antitoxin issued by Burroughs-Wellcome & Co., at a strength of 1080 units per ml. (20,000 units in 18.5 ml.). The lines were counted and are given separately for each isolate in Appendix 1 Table 6. Although a difference in the mean number of lines per strain existed, the differences were not associated with mouse virulence (rank correlation co-efficient = 0·32, $10\% > P > 5\%$, n = 33), (see Table 15).

It is noteworthy that fewer lines were detected with these strains than with those described by Elek and Levy. There are two basic reasons why this might be so: (i) the strains had been kept in storage for a long period and may therefore have lost the ability to produce some of the antigens; (ii) the antitoxin or growth conditions may have been different in some way to those used by Elek and Levy. These authors reported the presence of 10 lines when Wood 46 was tested against the antitoxin, but only 8 lines were detected in this series. Dr. H. Proom of the Wellcome Research Laboratories (personal communication) is of

TABLE 17 Pigmentation on glycerol monoacetate agar.

	Rank order number	Pigment
Spreading strains:	1	orange
	2	orange
	3	yellow
	4	cream
	5	cream
	6	orange
	7	yellow
	8	orange
	9	orange/cream
	10	cream
	11	orange
	12	orange
	13	orange
	14	cream
	15	orange
	16	orange
	17	orange
	18	orange
	19	cream
	20	cream
	21	orange
	22	yellow
	23	orange
	24	orange
	25	orange
	26	orange
	27	cream
	28	orange
	29	orange
	30	orange/cream
	31	yellow
	32	orange
	33	orange
	34	orange
	35	orange
non-spreading strains:		
	1	white
	2	orange
	3	orange
	4	orange
	5	orange
	6	orange/cream
	7	white
	8	orange

the opinion that different batches of antitoxin might give different numbers of lines, although the method of preparing the refined globulins has not changed in the last ten years. Some differences in antibody content were recorded by Gladstone and van Heyningen (1957) and were seen subsequently in this series of experiments when some batches of antitoxin were found to be extremely weak in the anti 'S' component of leucocidin.

Pigmentation

The glycerol monoacetate medium described by Willis and Turner (1962), (Appendix 2), was used to investigate pigmentation. Although the isolates of each strain tended to conform to a specific colour pattern (Table 17), no correlation between yellow pigment and mouse virulence was found ($P > 5\%$, $z = 0{\cdot}18$, $n_1 = 4$, $n_2 = 31$); nor was there any obvious relation of pigment to epidemicity for humans. Sompolinsky (1962) has pointed out that the normal 'aureus' pigment of staphylococci becomes poorer after storage of the staphylococci on artificial media, and so perhaps it is unfair to apply the glycerol monoacetate pigmentation test to strains which had been isolated from the patient up to 5 years previously. All the staphylococci used here possessed the normal 'aureus' pigment although this was slow to develop in some isolates even on blood agar.

TABLE 18 Egg-yolk reactions

Position in mouse virulence table	Egg-yolk* broth	Egg-yolk* agar	Antibiotic+ resistance pattern	Phage$^{\phi}$ group
1	+	+	-	III
2	-	-	PT	I
3	-	-	PTS	III
4	+	+	-	M
5	-	+	PT	III
6	+	+	C	I
7	-	-	PTSE	M
8	-	-	PT	I
9	-	-	PTS	III
10	+	+	PTS	III
11	-	+	-	I
12	+	+	-	II
13	+	+	P	II
14	+	+	PT	I
15	+	+	-	III
16	+	+	P	III
17	+	+	PT	I
18	+	+	-	III
19	+	+	PTS	III
20	+	+	PTS	III
21	+	+	PT	I
22	-	-	PSE	III
23	+	+	P	I
24	-	-	PTS	III
25	+	+	P	M
26	+	+	P	I
27	+	+	PT	I
28	+	+	T	III
29	+	+	PT	I
30	+	+	P	I
31	-	-	PTS	III
32	+	+	P	I
33	+	+	-	M
34	+	+	P	M
35	+	+	-	II
nasal strains:				
1	+	+	-	M
2	+	+	-	II
3	+	+	P	I
4	ND	ND	-	M
5	+	+	-	I
6	+	+	-	I
7	ND	ND	-	NT
8	+	+	P	I

* + = egg-yolk positive - = egg-yolk negative ND = not done

+ P = penicillin, T = tetracycline, S = streptomycin, E = erythromycin

ϕ = phage-groups I, II, III, M = mixed groups, NT = not typable

TABLE 19 Relation of antibiotic resistance to egg-yolk test.

Egg-yolk reaction on agar	Strain resistant to n antibiotics				
	0	1	2	3	4
positive	13	11	6	3	0
negative	0	0	2	5	1

The egg-yolk test

The egg-yolk reaction of all isolates was investigated by both the broth method (Gillespie & Alder, 1952) and the agar plate method (Willis & Turner, 1962; see Appendix 2). Table 18 shows that the strains which were most virulent for mice tended to be negative by both tests (the association between the negative reaction and virulence was statistically significant (P = 1%, $z = 0.6$, $n_1 = 8$, $n_2 = 27$). As may be seen from the table, there was no relation with phage group but all the negative strains were resistant to 2 or more antibiotics although the converse was not true, (Table 19).

It was suggested by Rosendal (1963) that the virulent egg-yolk negative strains produce an enzyme which splits the lipase and thus renders the strains egg-yolk negative. The isolates which were egg-yolk negative in this series were accordingly mixed with a known egg-yolk positive strain (PS 80) and incubated in egg-yolk broth. After 18 hours' incubation all the cultures gave a positive reaction. This would appear to suggest either that the negative strains are truly negative or that the lipase destroying enzyme, if produced, had no effect on the lipase of another staphylococcus.

Gelatine

The ability of staphylococci to liquify gelatine was studied by inoculating 10 ml. amounts of nutrient gelatine

TABLE 20 *In vitro* characters in relation to virulence.

Position in virulence table	Reaction with							
	Gelatine	Haemoglobinase	Litmus milk	Egg-yolk agar	Lactose	Urea	Fibrinolysin	V.P.
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	+	+	+	+	+	-	-	-
Non-Spreading strains								
1	+	+	-	+	-	-	+	-
2	+	-	-	+	+	+	-	+
3	+	+	-	+	+	-	-	+
4	ND							
5	-	+	+	+	+	-	-	-
6	+	-	-	+	+	-	-	+
7	ND							
8	-	+	-	+	-	-	-	+

with staphylococci and incubating at 37°C. The tubes were placed in cold water after periods of 24, 48 and 72 hours and those which failed to solidify were regarded as positive.

The agreement within strains was good; the results for each strain are shown in Table 20. There was a tendency for the strains which did not liquify gelatine to be virulent for mice and this association is significant at the 5% level ($z = 0.41$, $n_1 = 13$, $n_2 = 22$).

Haemoglobinase

Elek (1959) described a test for a haemoglobinase in which saponin laked horse red cells were incorporated in agar. The isolates were streaked on the medium and the presence of the haemoglobinase detected by decolourisation of the haemoglobin.

In these experiments, 5% of laked red cells were added to nutrient agar. The results are shown in Table 20; there was a good agreement between the isolates within the strains. Table 20 shows again that there was a tendency for the strains which were negative by this test to be virulent for mice. The association is significant at the 0.1% level ($z = 0.85$, $n_1 = 16$, $n_2 = 19$).

Litmus milk

The ability of the staphylococci to produce acid and to clot litmus milk was investigated. The agreement within the strains was good except for 3 strains which gave equivocal results;

these have been counted as positive. Table 20 shows a relationship between those strains which were virulent and those which were negative by the litmus milk test. The association is significant between the 1% and 0.1% levels ($z = 0.61$, $n_1 = 13$, $n_2 = 22$).

Correlation between tests

There was a very good, though by no means perfect, agreement between the three tests, gelatinase, haemoglobinase and litmus milk. It seems reasonable to suppose that, if these were independent tests, there would be an equal number of each possible combination (++-, +-+, +++ etc). This was not so, and using the χ^2 test, the discrepancy was seen to be significant at the 1% level ($\chi^2 = 27.3$, df = 7). Thus there was a significant tendency for strains to possess or lack enzymes affecting all 3 of these materials. These differences were closely linked to phage group, for example 12 of the 14 phage group III strains were negative for 2 or more of the 3 enzymes, compared with 3 of the 17 phage group I strains.

Relation of cell density in broth to virulence

One measure of the biochemical ability of an isolate might be the cell density to which it grew in nutrient broth and, as the isolates had been grown in the same batch of broth for a standard length of time, it seemed that this might be investigated.

It will be remembered that there was a strong correlation between the dose of bacteria inoculated and the mouse score. When uncorrected for differences in the dose inoculated, the variance attributable to the 'between strains' differences was greater than after correction for dose. This suggested that, in some strains, all or most of the isolates had grown to a greater number of viable units per ml. than had the isolates of other strains, implying greater synthetic biochemical activity among certain strains.

Analysis of variance showed that this was so; the differences between strains were significantly greater than those within strains ($P < 0.1\%$, $F = 2.73$, $n_1 = 34$, $n_2 = 165$.).

There was however no tendency for the strains which grew well in broth to be those which were virulent for mice. The rank correlation co-efficient between mouse virulence (on adjusted dose levels) and the growth in broth was -0.02 ($P > 10\%$, $df = 33$).

Using the data on clump-size and viable count, it is possible to calculate how many cocci were present per ml. of broth. When this was done, however, it was found that the differences between strains were not significantly larger than those within strains ($P \geq 20\%$, $F = 0.28$, $n_1 = 34$, $n_2 = 165$). Hence it would appear that the cell count reached in broth is not related to virulence for mice.

Ammonium molybdate test

One hundred isolates selected at random from the total of 259 were tested by the ammonium molybdate test described by Myers (1959a). All were strongly positive and no inferences could be drawn with regard to virulence on the basis of this test.

Other characters investigated

A considerable number of other characters were investigated, not because it was thought that they might have any direct bearing on 'virulence' but because they might demonstrate those strains which had the greatest biochemical activity. Many of the 'sugars' tested gave the same results for all isolates. Thus sorbitol, raffinose, glycerol, rhamnose, inositol, aesculin, arabinose, xylose and inulin were not fermented whilst sucrose, maltose, fructose, glucose and trehalose were fermented by all isolates. Lactose was not fermented by some strains but there was no significant relation with virulence ($P > 5\%$, $z = 0.37$, $n_1 = 4$, $n_2 = 31$). Salicin was fermented by a few isolates but showed no obvious pattern even in relation to strain. All isolates were phosphatase positive on the phenol phthalein medium of Barber and Kuper (1951).

There was no relation between virulence and the ability to split urea ($P > 5\%$, $z = 0.21$, $n_1 = 15$, $n_2 = 19$), the

TABLE 21 Assay of leucocidin

Rank order number	Reciprocal of direct reading highest dilution	Antigen/Antibody lines		
		F	S	Others
1	32	-	+	1
2	4	+	-	-
3	16	±	+	-
4	8	±	-	-
5	8	+	-	1
6	ND			
7	8	-	-	-
8	4, 8, 8	-	-	-
9	8	-	-	-
10	8, 32	-	+	2
11	4	-	-	-
12	16	-	-	1
13	8	-	-	2
14	8	+	-	2
15	16	-	-	1
16	4	-	-	1
17	8, 16	-	-	-
18	8	-	-	1
19	4	-	+	1
20	8			
21	4	±	-	-
22	32, 64	+	-	-
23	32	-	-	1
24	8	-	-	1
25	ND			
26	16	-	-	1
27	16			
28	ND			
29	16	-	-	1
30	4	-	-	1
31	8	-	-	1
32	8	-	-	1
33	4, 8	-	-	1
34	8	+	-	1
35	32	-	-	-
PS 80	16	-	-	-
Wood 46	8	-	+	-
Charlton	16	+	-	3
V 8	64	+	+	-

ability to produce fibrinolysin ($P > 5\%$, $z = 0.36$, $n_1 = 13$, $n_2 = 22$), or to give a positive Voges-Proskauer reaction ($P > 5\%$, $z = 0.37$, $n_1 = 10$, $n_2 = 25$) when the pooled results for each strain were considered.

Assay of leucocidins

Owing to the rather more complex nature of the techniques involved it did not prove possible to test all the isolates for leucocidin. One isolate from each strain was chosen on a 'stratified random sample' basis and these and four other staphylococci tested.

Two methods of assay were used based on the work of Gladstone and Woodin (e.g. Gladstone & van Heyningen, 1957; Woodin, 1961). I am indebted to Dr. Gladstone for helpful discussion regarding the assay of leucocidin and for gifts of the standard strain 'V 8' and of the F and S components of leucocidin.

For both assays the staphylococci were grown in the medium described by Gladstone and van Heyningen (1957). All glassware was kept scrupulously clean and stored in alcohol until required.

Method 1. The cultures were grown overnight with constant shaking and the bacterial cells removed by centifugation and discarded. The culture supernatant was diluted in 2-fold steps in 20% rabbit serum saline to prevent the delta lysin from

interfering in the tests. Plaques of leucocytes, obtained by allowing blood from a finger prick to clot on a coverslip, were inverted over the culture dilutions contained in cavity slides. After half an hour's incubation at 37°C the plaques were examined; death of the leucocytes was seen to follow the pattern described by Gladstone and van Heyningen (1957). The cells rounded up and potocytosis occurred; the highest dilution at which this happened was noted. Assessments made in this way were necessarily rather subjective. The results are shown in Table 21. Using the Mann-Whitney U statistic no relation of mouse virulence to leucocidin production could be demonstrated ($P > 5\%$); nor was there any relation to the epidemicity of the staphylococci for humans.

In considering the leucocidal activity of a staphylococcus, it suffices to carry out a direct determination of activity as described above. Woodin has shown however that leucocidin is composed of 2 components 'F' and 'S', and that both of these must be present before leucocidal activity is shown; whichever of the components is present in the smallest concentration determines the level of activity. An investigation of the 'F' and 'S' components was conducted as follows.

Method 2. The method of Woodin (1961) was adapted. The cocci were removed from 100 ml. of culture by centrifugation

and the supernatant dialysed against solid ammonium sulphate for 24 hours. This had the effect of reducing the volume to about 30 ml. and of precipitating the protein. It was found difficult to separate the protein by centrifugation and so the reduced volume of material was dialysed against water for 24 hours to remove the ammonium sulphate and then reduced to dryness at a temperature of 37°C. The deposit was resuspended in 0.2 ml. of saline at a pH of 7. This material was used as the antigen in Ouchterlony plates against commercial staphylococcus antitoxin (Burroughs-Wellcome) and lines identified by comparison with the standard F and S antigens supplied by Dr. Gladstone.

The results, however, were very difficult to interpret (Table 21). Even under the conditions of the experiment given above, some of the strains produced one or more extra antigens which could not be identified with 'F' or 'S'. The standard commercial antitoxin may contain at least 10 precipitating antibodies and so it is difficult to distinguish between the various precipitation lines. In addition, the 'F' component given to me by Dr. Gladstone was known to contain D.N.A. ase which precipitated as a broad band next to the F line and so obscured the results. Finally some of the batches of antitoxin gave no lines against the 'S' component at all.

Attempts to separate the components of leucocidin by paper chromatography and electrophoresis on both paper and

cellulose acetate were not successful. Clearly further work is needed before these techniques for the separation of leucocidins can be applied to large collections of staphylococci.

Since many human sera contain antibodies to leucocidins, it seems probable that leucocidin production *in vivo* is very different from that *in vitro*. Gladstone and Glencross (1960) found that both the F and S components of leucocidin were produced more abundantly in intraperitoneal sacs in rabbits than in similar menstrua *in vitro*.

Investigation of capsular material

To investigate the possibility that some of the staphylococci might possess a hyaluronic acid capsule, a method based on that of Clausen and Rosenkast (1962) was used. The strains in the stratified random sample of staphylococci were inoculated onto agar plates, one plate per strain, and incubated overnight at 37°C. The growth was scraped off and suspended in 10 ml. of phosphate buffer (9 parts $\frac{M}{15}$ KH_2PO_4 and 1 part $\frac{M}{15}$ Na_2HPO_4) for 14 days. The staphylococci were removed from the buffer by centrifugation and the supernatant evaporated to dryness at 37°C. The deposit was resuspended in 0.2 ml. of distilled water and 2 microlitres of the solution placed on 10 x 5 cm. strips of cellulose acetate, 2 unknowns and 1 standard on each strip. The standard solution was composed of hyaluronic acid and

chondroitin sulphate. Electrophoresis at 120 volts for 1½ hours was carried out at a temperature of 4°C in phosphate buffer of pH 7.2. After electrophoresis the strips were dried and stained with Toluidine Blue (0.1% in water). This stain is more specific than the mucicarmine used by Clausen and Rosenkast.

RESULTS

Seven of the 35 strains gave a result which might be interpreted as hyaluronic acid. No great weight can be placed on these results, however, for the 'spots' were very diffuse and, unlike the standard, tended to spread very widely. In most of the strains, a diffuse spot appeared about half way between the hyaluronic acid and the chondroitin sulphate of the standard. Nine of the strains showed a 'spot' which had run ahead of the standard; no interpretation of the spot can be given at present. No relation between any of the apparent mucopolysaccharides and virulence for man or mouse was found.

Comparison of this work with that of Clausen and Rosenkast (1962) is made difficult by their use of a dye which appears to be less satisfactory than Toluidine Blue and also by the fact that they compared unknowns with standards on separate strips of cellulose acetate. Examination of the movement of the standards on different strips in the same run in the present series of experiments showed that movement on different pieces of

cellulose acetate was not identical.

This work was a joint experiment with Mr. B. C. Pratt (also of the Bacteriology Department, The Wright-Fleming Institute) and is being continued in an attempt to determine the nature of the substances found. We are indebted to Dr. Helen Muir (St. Mary's Hospital) for advice in regard to staining and technique.

Sensitivity to antibiotics produced by viridans streptococci

Myers (1959b) drew attention to the susceptibility of staphylococci to 'antibiotics' produced by viridans streptococci. It seemed possible that the resistance or susceptibility to such antibiotics (which might of course be present in the mouth or nose) might influence the communicability of staphylococci.

Accordingly, 9 strains of viridans streptococci isolated from the upper respiratory tract of patients were tested for antibiotic production. Three of the 9 strains produced 'antibiotics'; the antibiotic of one strain appeared to be diffusing into the agar. One of the 3 strains of streptococci inhibited only a few of the staphylococci; the other two, having a similar but not identical spectrum, inhibited a number of the staphylococci.

A total of 208 isolates of staphylococci was then tested against these 3 streptococci (see Appendix 2 for methods). The results were consistent for each isolate but not for each

TABLE 22 Analyses based on isolates.
A comparison of virulence and lysin production.

Mouse score	Total isolates	% producing		
		alpha lysin	beta lysin	alpha + delta + fibrinolysin
11 - 12	48	85	35	44
9 - 10	49	67	16	14
7 - 8	39	44	10	15
5 - 6	24	50	8	8
1 - 4	11	9	0	0

strain (Appendix 1 Table 7). Some of the isolates in almost every strain were sensitive and some resistant. No correlation could be found between susceptibility to streptococcal antibiotics and the observed epidemicity for man to source, phage-type or conventional antibiotic resistance, or to mouse virulence.

Analyses based on isolates

The previous analyses of virulence factors were based on the mean response for each strain and the individual variations of each isolate have been ignored.

There are, however, grounds for regarding each isolate as a separate entity since within a strain the isolates were obtained from different patients over a period of as long as 2 months and each isolate would have undergone considerable multiplication within its host.

To facilitate analysis on these lines, the results for each isolate were recorded on Copeland-Chatterson 'edge-punch' cards. Analyses could thus be carried out in a variety of ways with ease.

RESULTS

Full tables of results are given in Appendix 1 and simplified tables are given in the text.

Relations of the lysins to virulence for mice

The isolates which gave the highest scores in mice more frequently produced alpha haemolysin *in vitro* than did other isolates. This was to be expected in view of the fact that the white or necrotic zone appeared to be produced by alpha lysin (this is described more fully later). The more 'virulent' isolates also more frequently produced beta lysin, fibrinolysin and combinations of these than did the other isolates; delta lysin was more evenly spread amongst the isolates (text Table 22, Appendix 1 Tables 8, 9, 10).

Other enzyme factors

The negative correlations of virulence with egg-yolk factor, gelatinase, haemoglobinase and litmus-milk enzymes observed on the basis of strain became less pronounced when results for isolates were analysed (Appendix 1 Tables 12, 13). These enzymes appeared to be more nearly evenly distributed than formerly; only urease was markedly less frequent amongst the isolates giving the highest mouse scores. The reason for this may lie in the fact that the production of certain enzymes was almost confined to some phage groups, for example haemoglobinase was far more frequent among the phage group I strains than amongst the phage group III strains (Table 23, Appendix 1, Table 14).

TABLE 23 Analyses based on isolates.

A comparison within phage groups.

Phage group	Total isolates	% producing			
		alpha lysin	alpha + delta lysin	haemoglo-binase	litmus milk
I	84	48	30	69	55
II	12	12	67	17	33
III	61	79	72	3	8
Mixed	14	50	43	50	21

TABLE 24 A comparison of pigmentation.

Pigment on glycerol monoacetate	Total isolates	% producing				
		alpha lysin	egg-yolk factor	haemoglo-binase	urea	litmus milk
yellow	16	63	6	0	6	0
orange	109	61	75	49	40	43
other	46	59	74	32	30	24

As observed by Willis, Jacobs and Goodburn (1964), the strains which produced a yellow pigment on glycerol monoacetate agar were physiologically distinct from strains producing other pigments on this medium (Table 24, Appendix 1 Table 15). Yellow strains in this series did not produce beta lysin.

Production of antigen-antibody precipitation lines in agar is, of course, partly a reflection of lysin production (Appendix 1 Tables 16, 17, 18). It is curious that, although commercial antitoxin contains little or no anti-delta haemolysin, there was a relation between the production of delta lysin and the number of lines observed. Delta lysin production is presumably closely linked to some other factor. There was a slight tendency for strains which were 'virulent' to produce more precipitation lines than those which were 'avirulent' (Appendix 1 Table 17).

The relation of virulence factors to disease in humans

The previous analyses are based on the observed virulence of the staphylococci for mice. No comparable data regarding the 'virulence' of these staphylococci for humans was available but it seemed permissible to divide the staphylococci into three groups according to their 'epidemicity'.

The three groups were defined as follows:-

TABLE 25 Analysis of enzyme in relation to human disease.

	Epidemic strains	Non-epidemic strains	non-spreading strains
Total isolates	90	66	16
	% of isolates producing enzymes		
Alpha lysin	70	56	25
Beta lysin	24	12	6
Delta lysin	64	74	56
Fibrinolysin	23	53	19
Gelatinase	59	74	75
Haemoglobinase	32	45	63
Egg-yolk	53	85	81
Urea	31	39	69
Litmus milk	29	42	25
Voges-Proskauer	68	54	50
Lactose	92	85	63
Number of antigen/antibody precipitation lines:			
5 or more	27	11	25
3 - 4	37	54	25
1 - 2	36	35	50
Phage group:			
I	44	48	75
II	4	9	12
III	49	27	0
misc.	2	15	12

Non-spreading staphylococci - isolates from strains which failed to spread to other patients.

Spreading, non-epidemic staphylococci - isolates of strains which had spread about the wards, but which had caused less than 3 septic lesions.

Epidemic staphylococci - isolates of strains which had spread and caused 3 or more septic lesions.

The production of various factors by isolates of these 3 groups is shown in Table 25. Alpha and beta haemolysins were more frequently produced by the epidemic strains than by the others, but the production of delta lysin and fibrinolysin have no apparent relation to epidemicity. Of the other characters investigated, only a positive Voges-Proskauer reaction and lactose fermentation were more frequent among the isolates from 'epidemic' strains. These differences are probably attributable to the differences in the distribution of phage groups. When staphylococci of one phage group only were considered there were no real differences between the 'epidemic' and 'non-epidemic' groups except in alpha and beta haemolysin production (e.g. Appendix 1 Table 18).

Discussion

In general, the results of the tests for diffusible products of the staphylococcus were very similar to those obtained

TABLE 26 Comparison of results of 'enzyme' production.

'Enzyme'	Percentage of strains positive		References
	Noble	Others	
Alpha lysin	100*	82-100	Elek, 1959.
Beta lysin	10	11	Elek, 1959.
Delta lysin	90*	97	Elek, 1959.
Leucocidin	78	50	Gladstone and van Heyningen, 1957.
Fibrinolysin	34	81	Elek, 1959.
Egg-yolk lipase	82	89	Shah, Russell and Wilson, 1963.
Gelatinase	64*	58*	Levy, 1952.
Haemoglobinase	49	28	Elek, 1959.
Litmus milk	34	75	Elek, 1959.
Lactose	85	95	Chapman and Stiles, 1940.
Urease	41	20-70	Elek, 1959.
Voges-Proskauer	71	64	Cowan, 1962.
Sucrose, maltose, fructose, glucose, trehalose	100	general agreement	Chapman and Stiles, 1940.
Sorbitol, raffinose, glycerol, rhamnose, inositol, aesculin, arabinose, xylose, inulin	0	general agreement	Chapman and Stiles, 1940.
Mercury sensitivity	linked to anti-biotic resistance	general agreement	Akinlade, 1962. Jessen et al., 1963.

* Not tested by the most efficient technique available.

by other workers (Table 26).

The main conclusions which can be drawn from these studies would appear to be that the staphylococci which were 'epidemic' for man were also able, in general to produce skin lesions in mice, and that to some extent both these abilities were dependent on the production of alpha lysin and other similar toxins. Most of the characters investigated did not appear to be associated with virulence in man or mouse and it was not possible to show that the 'virulent' strains had a total biochemical activity greater than that of the other strains. It must be emphasised, however, that almost all the tests used were qualitative rather than quantitative in nature.

As is demonstrated later, the results of the mouse virulence test were related to the production of alpha haemolysin *in vivo* but this did not exactly parallel the *in vitro* production. There must then be some other factors which enable the staphylococcus to produce the conditions *in vivo* in which it can elaborate alpha haemolysin. If, for one moment, it be assumed that the correct factors have been chosen for investigation, then an analysis of all isolates which produced alpha lysin *in vitro* should indicate the accessory factors which enable some isolates to be more virulent for mice than others.

TABLE 27 Analyses based on isolates.

Relation of virulence to specific lysin production.

	Mouse score		Significance of difference assessed on the χ^2 test
	11 + 12	1 - 10	
Number of isolates	41	63	
	% of isolates producing the enzyme		
Beta haemolysin	37	19	< 1% *
Delta haemolysin	80	79	> 90%
Fibrinolysin	59	40	2% *
Gelatinase	68	56	> 10%
Haemoglobinase	37	27	> 10%
Egg-yolk	59	59	> 90%
Urea	24	44	< 1% *
Litmus milk	34	40	> 10%
Voges-Proskauer	59	62	> 10%
Lactose	83	87	> 50%

All of these isolates produced alpha haemolysin.

* Result is significantly different to the expected.

Table 27 shows that although beta lysin, gelatinase, fibrinolysin and haemoglobinase were all more common among the most virulent isolates, only beta lysin and fibrinolysin were significantly more common. Urease was significantly less common in the more virulent isolates.

If, for the sake of argument, we consider those enzymes for which a 'use' can be found, i.e. beta and delta lysin, the 3 protein splitting enzymes (fibrinolysin, gelatinase and haemoglobinase) and the egg-yolk lipase, we find that 46% of the most virulent staphylococci possess 4 or more of these 6 enzymes compared to only 20% of the less virulent strains.

The conclusion that beta lysin and fibrinolysin appear to play a supporting role to alpha lysin in the production of lesions is in accord with the work of, for example, Selbie and Simon (1952), Lack and Wailling (1954) and Howard (1954).

There are then some grounds for believing that the most virulent staphylococci are those which have the greatest biochemical activity. It is essential to bear in mind, however, that the production of enzymes <u>in vivo</u> may not be the same as that <u>in vitro</u> and what is needed for investigations of this sort is a means of assessing which enzymes have been produced in the mouse.

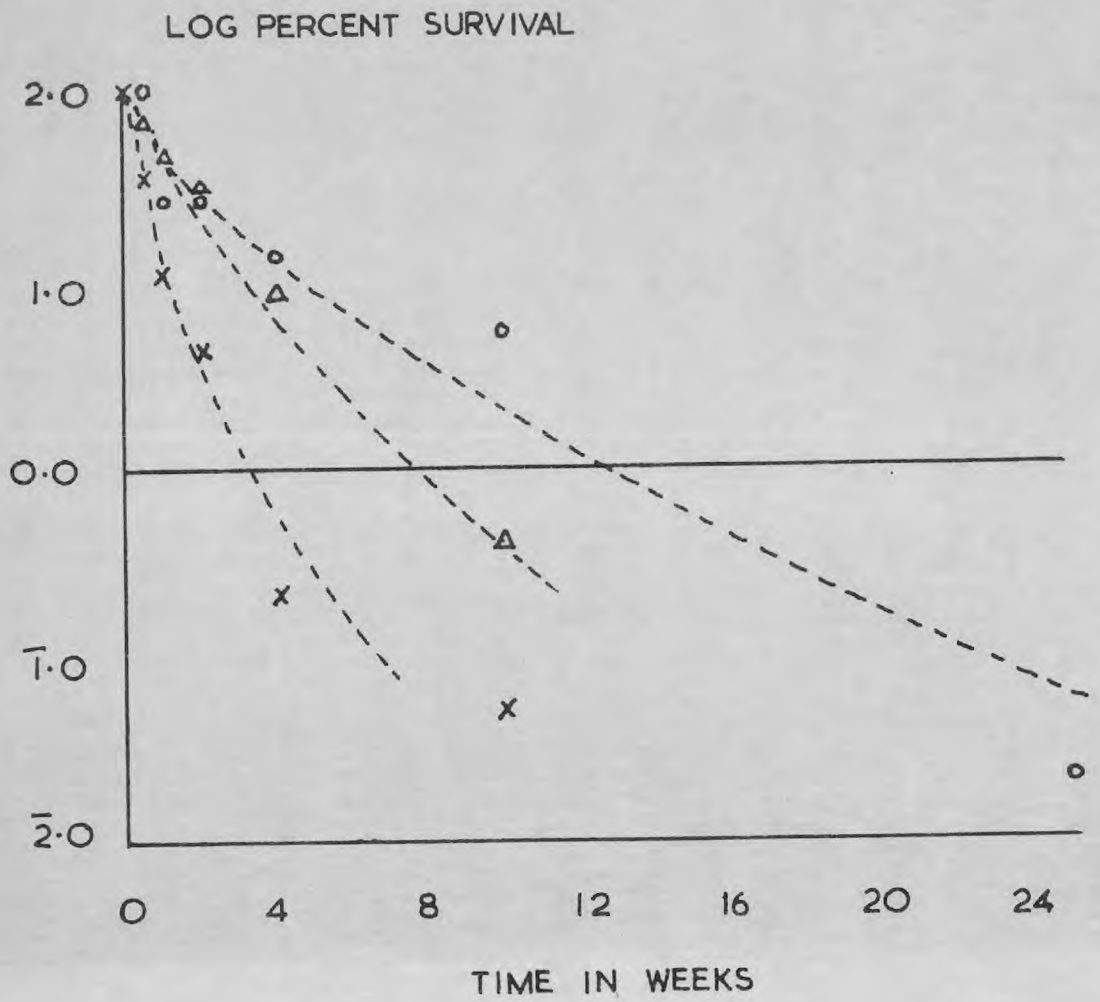


Figure 16 Determination of time which elapsed before the viable count fell to 1% of the inoculum. Three examples of die-away curves are shown.

THE SURVIVAL OF STAPHYLOCOCCI ON DRYING AND THE RELATION TO VIRULENCE

It might be supposed that, if disease is caused by bacteria that survive in the environment, then the strains which are most easily able to spread through wards will be those which are (a) virulent and antibiotic resistant and (b) those which survive drying well. Survival on drying may be considered under two headings; quantitative survival, the percentage of the original inoculum which can be recovered as viable cocci after dessication and qualitative survival, consideration of whether the bacteria which do survive dessication are as capable of causing disease as cocci of the same strain which have not been dried.

Quantitative survival

The survival of various strains of staphylococci was studied by putting 1/50 ml. of broth culture on to 0.1 gm. of cotton dust in bottles. The bottles were labelled and left in the dark without lids but covered with blotting paper. Immediately after inoculation and at intervals of 3 days, 1, 2, 4, 10 and 25 weeks one bottle of each culture was resuspended in 5% broth saline, diluted where necessary and inoculated on to serum agar. Colonies were counted after 18-20 hours incubation.

The percentage survival in relation to the count at 0 hours was calculated and plotted as 'log.percent.survival' against

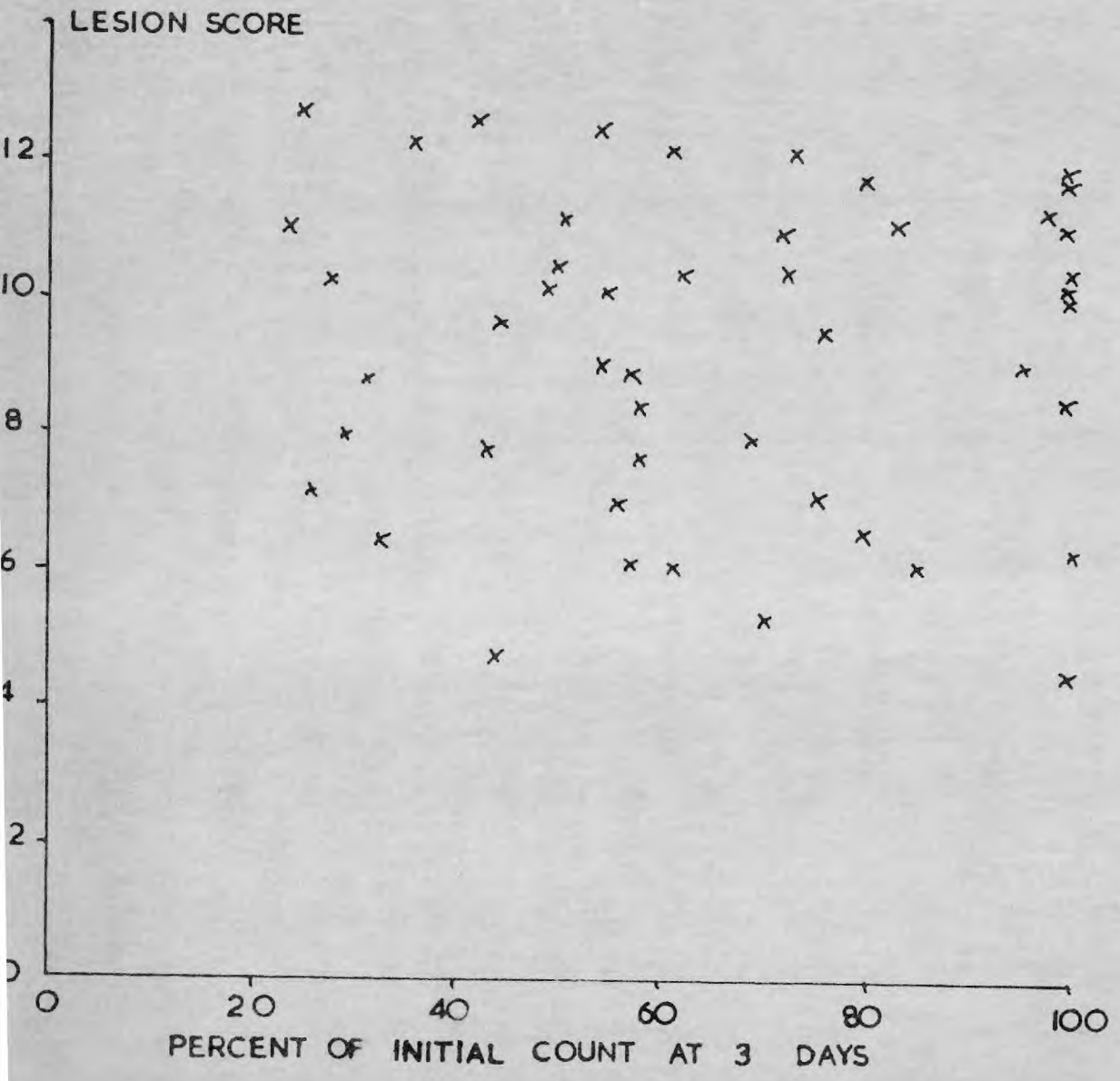


Figure 17 Relation of viable count at 3 days to lesion score.

'time'. From this graph the number of weeks which elapsed before the count fell to 1% of the inoculum was calculated (see Figure 16).

The percentage survivals at 3 days and the time which elapsed to 1% survival are given in Appendix 1 Table 19, where the strains are shown in order of mouse virulence. The results were plotted against the adjusted mouse scores for each individual culture (see for example Figure 17). There was no significant correlation between the mouse lesion score and the two measures of survival (lesion score/survival at 3 days correlation co-efficient = -0·012, df = 45, $P > 10\%$; lesion score/time to 1% survival correlation co-efficient = -0·245, df = 45, $10\% > P > 5\%$).

The staphylococci were divided into 2 groups according to whether or not the isolate was from a strain regarded as 'epidemic' or not (Williams & Jevons, 1961). Although the epidemic strains appeared to survive better than the non-epidemic strains, the differences were not statistically significant (survival at 3 days epidemic mean = 68·1%, non-epidemic mean = 56·8%, t = 1.36, df = 45, $P > 10\%$; time to 1% survival, epidemic mean = 8.1 weeks, non-epidemic mean = 7.6 weeks, t = 1.45, df = 45, $P > 10\%$).

There is thus no significant evidence that staphylococci which were epidemic for humans or virulent for mice survived

TABLE 28 Survival of staphylococci and micrococci on dessication.

Organism	% survival at 3 days	Weeks to 1% survival	Virulence score* in mouse test
Staphylococci			
NCTC 7485	55	6.0	36
PS 80	100	7.0	33
Wood 46	74	2.5	22.5
Smith	50	13.5	16
PS 42E	76	10	15
Type 52	100	18	13.5
PS 83A	19.5	4.0	8
Micrococci			
Harrod	100	8.5	18
NCTC 7944	100	8.5	16

* The score is based on the pooled results, where available, of the effect of injecting 10^6 cocci into 6 mice; maximum score = 36.

drying in the environment any better than other strains under the conditions of the test used.

Survival of miscellaneous strains

In Table 28 are presented the results of a few additional strains whose survival was measured in the same way as recorded above. It can be seen that all survived fairly well except strains Wood 46 and PS 83A. Again, no relationship between mouse virulence and survival can be found. In agreement with the results of Rountree (1963a and b), Wood 46 survived dessication badly. Type 52 was investigated previously by Noble (1961, M.Sc. Thesis) and when dried on talc dust was found to drop to 48% of the original inoculum on drying and to drop to 1% of the inoculum in 6-7 weeks. Staphylococci in pus and sputum were found to take about 6-7 weeks to drop to 1% of the inoculum when dried on cotton dust or on wool blanket.

Qualitative survival

The virulence of dried staphylococci was investigated as follows:- 5 ml. of broth culture of the staphylococcus was poured on to 2 grams of cotton dust and allowed to dry at ambient temperature and humidity. The dust was then ground in an M.S.E. blendor and sieved to homogenize it, packed into needles and inoculated into mice. The dose was calculated by putting the dust from a needle in to 10 ml. of broth saline, diluting where

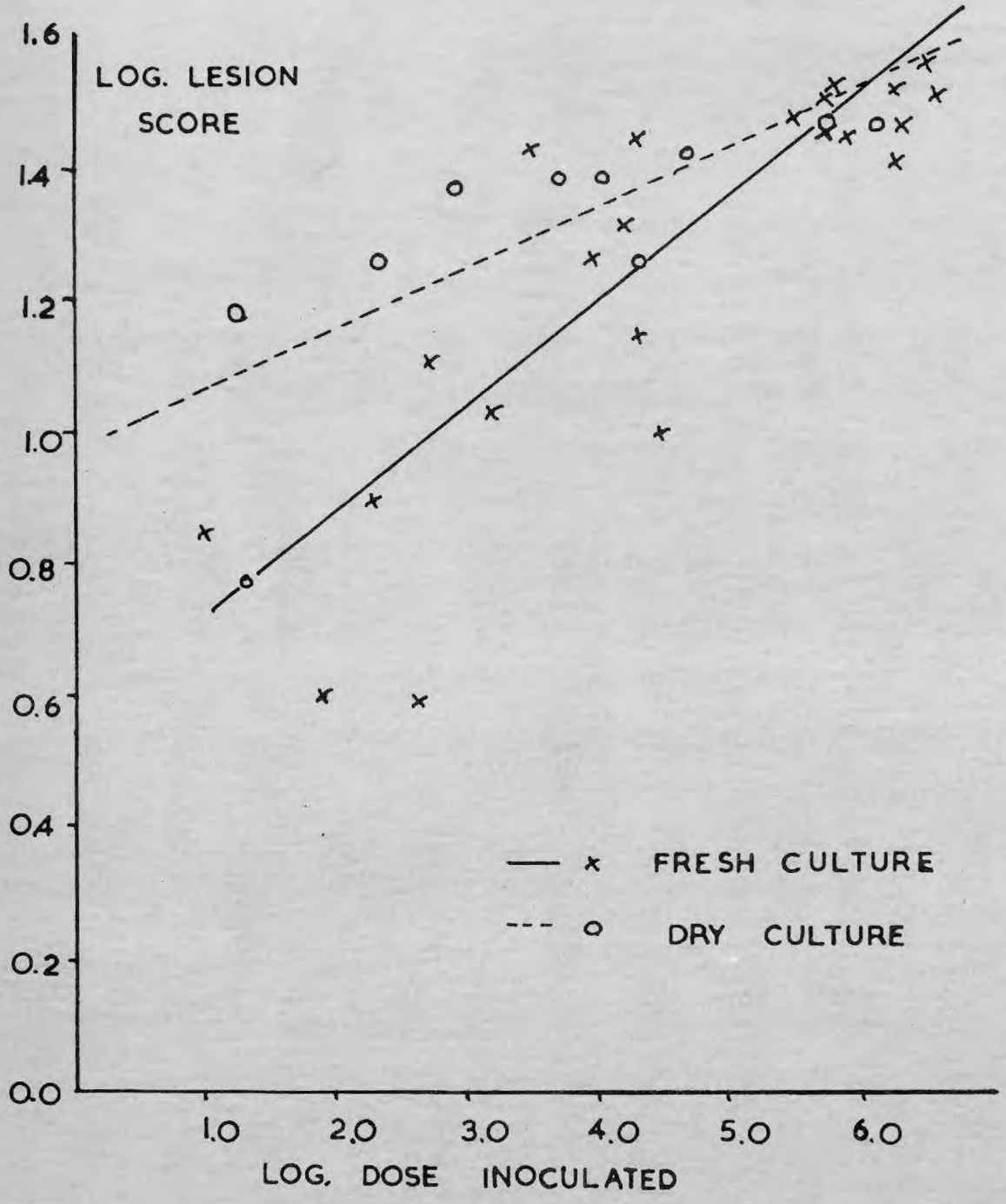


Figure 18 Dose/response curves for fresh and dried cultures of staphylococcus PS 80.

TABLE 29 Comparison of the regression lines of virulence against dose for fresh and dried cocci.

Strain of staphylococcus	slope of line		t	df	Probability = P		
	fresh	dry					
PS 80	0.155	0.093	1.8	32	10%	P	5%
Charlton	0.267	0.237	1.05	20	P	10%	
Wood 46	0.248	0.220	1.06	21	P	10%	
Type 52	0.204	0.046	0.69	20	P	10%	

necessary and inoculating onto agar. Because of the variability, at least 6 needles were used in the determination of the dose inoculated and 6 or 12 mice inoculated with the staphylococci.

Four strains of staphylococci were used in these investigations, PS 80, Charlton, Type 52 and Wood 46. The virulence of several preparations of dried cocci of each strain was measured after different times in the dried state. The regression lines of 'log.lesion score' against 'log. dose' were calculated and compared with the regression lines for fresh cultures. More data was available for 'fresh' cultures than previously because control counts for other experiments were included.

No significant differences were found between the regression lines for fresh and dried cocci (Table 29 and Figure 18) and from this it may be concluded that no differences in virulence were produced by drying. The full table of results is given in Appendix 1 Table 20. It is curious that the regression of score on dose is always greater for the fresh than the dry cocci but this may be a reflection of undetected differences in the clump size of the inocula.

Investigations into lag period

As noted in the discussion on the effects of dessication, other workers who resuspended the dried staphylococci and inoculated

TABLE 30 The effect of antibiotics on infection with fresh and dried staphylococci.

Strain	Dose inoculated	Score *									
		Control	5 mgm. of antibiotic given at x hours								
			0	3	4	5	6	7	8	9	10	11
PS 80+ Fresh	5×10^5	29	1	2			28					
	1×10^6	30	4	3	6	31	28					
	5×10^5	32		3	8	14	29					
	3×10^6	24		5	11	30	24	33	36	36	33	30
Dry	5×10^5	29		2	9	15	20					
	1×10^3	24		0	2	6	9					
	5×10^6	24		9	15	28	36	33	30	30	36	34
Wood 46+ Fresh	6×10^5	15		7	6	2	6					
	5×10^5	10		2	5	4	5					
	2×10^6	13	0	12	5	7	7					
Dry	3×10^5	18		4	3	6	24					
	1×10^7	26	30	29	26	21	31					
Charlton$^{\phi}$ Fresh	2×10^6	36	1	4	8	20	28					
	3×10^6	30	5	6	5	18	23					
Dry	1×10^7	36	5	16	33	30	36					
	3×10^6	36	7	12	20	32	32					

* Score is based on the results for 6 mice, maximum score = 36

+ Mice treated with streptomycin.

ϕ Mice treated with neomycin.

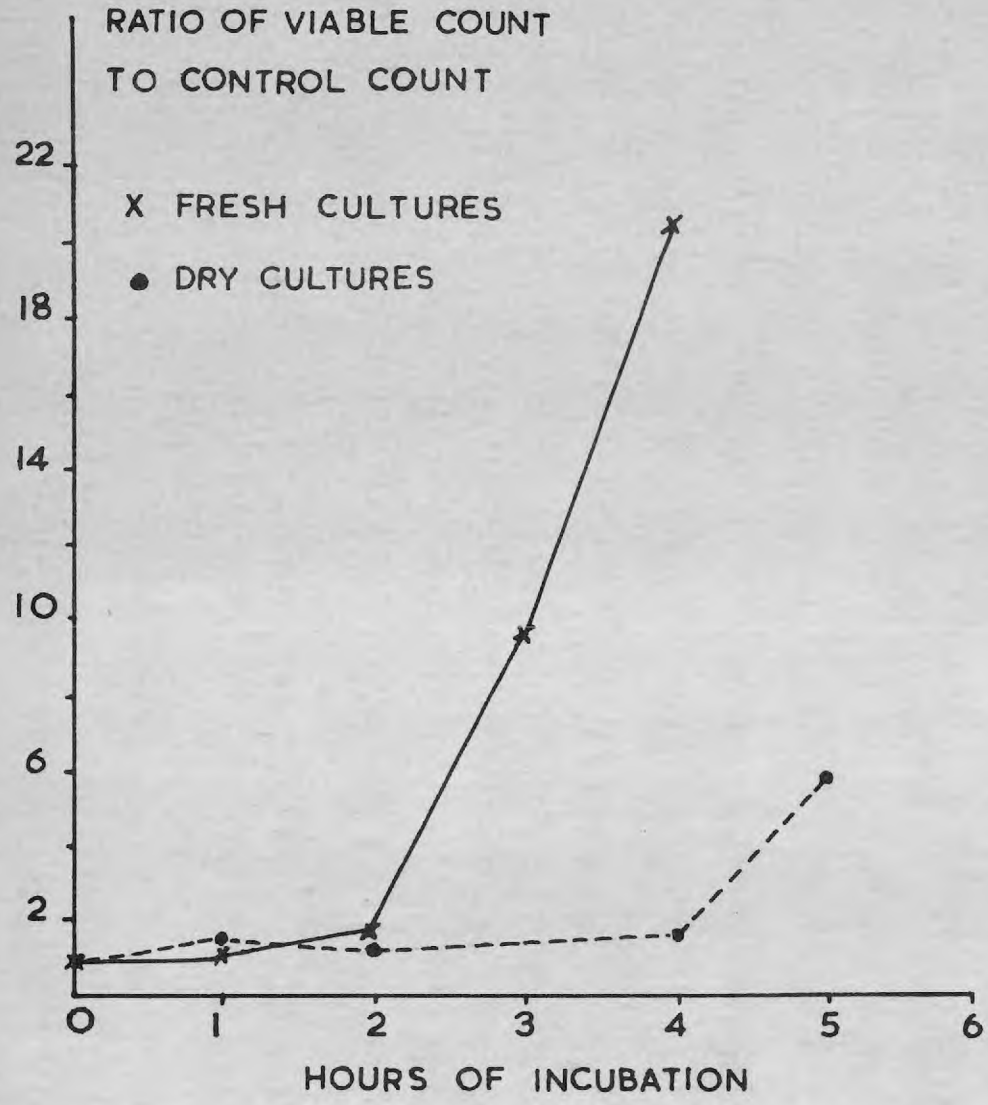
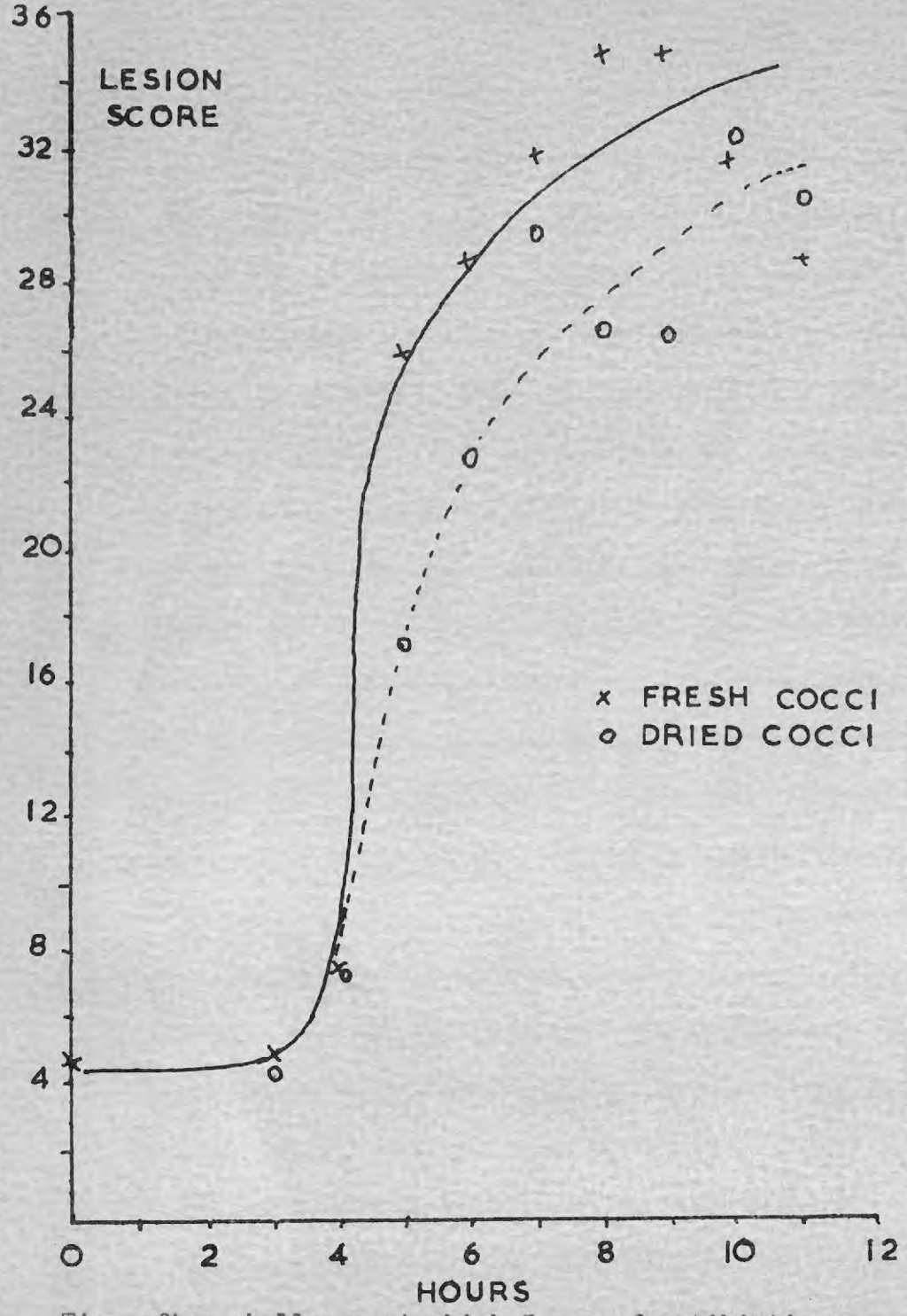


Figure 19 Lag phase in vitro of fresh and dried cultures of staphylococcus PS 80.

them into mice without a potentiator such as sutures or cotton dust, found a difference in virulence between the fresh and dry cocci. It seemed as if the ability of a suture to potentiate infection might have the effect of enabling the staphylococci to remain virulent. Maltman, Orr and Hinton (1960) found that dried cocci had a longer lag phase than fresh cocci when inoculated into broth. This was confirmed in the present series of experiments, when fresh and dry cocci were inoculated into broth and the viable count determined at hourly intervals (Figure 19). The discrepancy between published results on the virulence of dried staphylococci might be explained if, when injected alone, the dried cocci, having a longer lag period, were exposed to destruction by the body for longer. The potentiating effect of a suture might enable the staphylococcus to overcome this.

It has been shown that antibiotics given shortly after infection will prevent sepsis. If the dried cocci had a longer lag period <u>in vivo</u> as well as <u>in vitro</u> then the effective period for antibiotic therapy should be longer for dried cocci than for fresh cocci. Accordingly, mice were infected with fresh and dried cocci and a single dose of antibiotic given at intervals after infection. The results are given in Table 30 and are shown graphically for strain PS 80 in Figure 20.

Table 30 shows that the effect of one dose of antibiotic was dependent not only on the time after infection at which it



Time after challenge at which 5 mgm of antibiotic was given.

Figure 20 Susceptibility of 'fresh' and 'dried' staphylococci (PS 80) to antibiotics in vivo.

was given, but also on the number of bacteria with which the mouse was challenged. The effect of the antibiotic was markedly reduced in the presence of large doses of bacteria. To obtain more comparable results, the lesion scores were adjusted for the effect of dose in the same way as the lesion scores used previously in the comparison of virulence.

Adjusted score = original score - (regression slope)
(deviation of the dose from the mean dose)

The adjusted results are shown in Appendix 1 Table 21. The pooled results for staphylococcus PS 80 are shown in Figure 20. There is a suggestion that the dried cocci were susceptible to the effect of the antibiotic for a longer period than were the fresh cocci. If the dried cocci simply had a longer lag phase than the fresh cocci, the theoretical curves would be of the 'hysteresis' type shown in the diagram (Figure 20A), but these were not

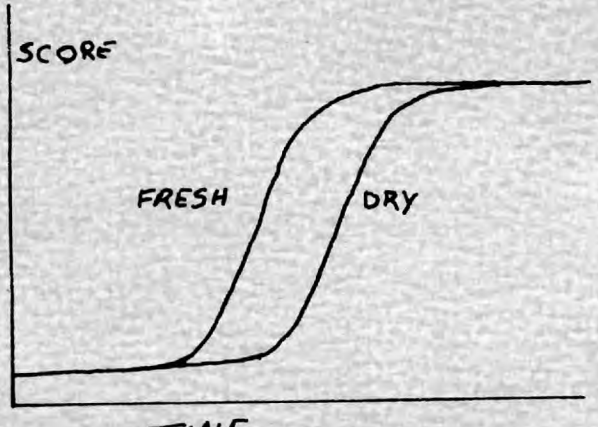


Figure 20A 'Hysteresis' curve.

obtained in practice. The results for Wood 46 and Charlton do not suggest a longer lag phase for the dried cocci and hence the evidence remains rather unsatisfactory.

Discussion

It must be remembered that all the bacteria used in these studies have been kept on artificial media for some years since isolation from the patient and some caution must, therefore, be used in extrapolating these results to considerations of cross-infection in hospitals. Further, these staphylococci have been dried from broth culture on cotton dust whereas it seems likely that most of the staphylococci in the hospital environment are dried from their natural growth medium on particles of desquamated human skin (Davies & Noble, 1962, 1963).

The experiments quoted above show that although the epidemic strains of staphylococci appeared to survive dessication better than the non-epidemic strains, the difference was not statistically significant. Exact comparison with the results of Rountree (1963b) is made difficult by Dr. Rountree's use of K values, for the value of K depends to a considerable extent on the portion of the curve over which the data is considered.

Dried cocci appear to be as capable of causing infection in mice as fresh cocci when introduced subcutaneously on a suitable

potentiator and there is a very slight suggestion that the dried cocci have a longer lag phase than the fresh cocci *in vivo* as well as *in vitro*. It is disappointing not to have shown a more marked difference in the lag period between the fresh and dried cocci but differences in the dose of live cocci inoculated and the presence of large numbers of dead organisms in the dried preparations may have influenced the result.

The dried cultures were all prepared at least one week before inoculation into the animals; some of them had been dried for many weeks before use. As may be seen from the tables, between 2 and 4 weeks elapsed before the count had fallen to less than 10% of the original inoculum and there is thus the possibility that the dried bacteria had a larger clump-size than did the fresh cultures. Examination of the dust on which staphylococci had been dried showed that, in addition to the usual groups of 1, 2, 3 and 4 cocci there were a number of large aggregates of perhaps 10 to 20 cocci. It is, of course, difficult to determine how many of the cocci in the clumps were viable. Attempts have been made to do this using ionising radiation (Lidwell, Noble & Dolphin, 1959), but further work has proved more difficult to interpret (Kingston & Noble, unpublished). The death rate of irradiated organisms depends on the nature of the menstruum in which the organisms are irradiated, the degree of exygenation, the speed

at which the dose of radiation is delivered and the presence of inducible phages in the cell (see for example Alper & Gillies, 1960).

In the present series of experiments, it was found that an increase of 1·7-fold in the viable count of the dried cocci could be obtained by blending suspensions of the dust in the M.S.E. blendor. But this increase could also be obtained with fresh broth cultures and it is therefore difficult to determine whether the clump size of the dried material was any different from that of the fresh growths. It seems likely that any such difference would be small however. It can be seen from Figure 18 that, because of the variability in the results, some leeway in the dose inoculated could be tolerated without markedly affecting the significance of the results.

Summary

There were no statistically significant differences in the survival rate on drying between epidemic and non-epidemic staphylococci.

When injected on cotton dust, no difference was found in the virulence of fresh and dried staphylococci of 4 strains. It seems possible, although by no means certain, that the lag period of dried staphylococci is longer both *in vitro* and *in vivo* than that of fresh staphylococci of the same strain.

TABLE 31 The effect of washing the inoculum on the score obtained after subcutaneous inoculation.

Strain	Control dose	Score	Washed dose	Score	Expected score
PS 80	1×10^6	32	1×10^6	32	31.5
	8×10^5	28	1.4×10^6	33	32.5
	8×10^3	18	1.4×10^4	15	19.5
	8×10	4	1.4×10^2	5	5.5
	1×10^2	13	1×10^2	12.5	9.5
Charlton	1.5×10^6	28	1.2×10^6	20	27
	1.5×10^4	10	1.2×10^4	11	18
	1.5×10^2	3	1.2×10^2	2	2.5
Smith	1.5×10^6	23	5×10^5	20	ND
	1.5×10^4	16	5×10^3	13	ND
Type 52	1×10^6	16	1×10^6	15	16
	1×10^4	13	1×10^4	13	13
Wood 46	9×10^5	13	1.5×10^6	11	14.5
	9×10^3	6	1.5×10^4	2	6.5
	9×10	1	1.5×10^2	1	1.5

The expected numbers were calculated on the basis of the slope of the regression line of lesion score against log. dose for each strain. They represent the score expected if the washing had no effect on the score.

No regression lines of dose against score were available for the Smith strain.

FURTHER INVESTIGATION INTO THE PRODUCTION OF LESIONS

A number of variations in technique were made in order to try to determine some of the factors involved in lesion production.

The effect of washing the inoculum

The majority of the tests reported in this thesis have been conducted using 18 hour broth cultures as the inoculum. It seemed desirable to determine whether the lesion score depended to any extent on the presence of preformed toxin in the broth culture. Accordingly inocula were prepared in which the cocci were washed twice in broth-saline and resuspended in broth-saline before inoculation into the mice. Table 31 shows that the effect of washing was marked for one strain only (Charlton). The control results were determined using unwashed staphylococci of the same broth culture and the 'expected' results were derived from these using the regression lines of 'lesion score' against 'log.dose' and they represent the result expected if the washing had had no effect. Overall the expected results do not differ significantly from the observed values (χ^2 = 11·12, df = 12, $0.9 > P > 0.5$).

It seems reasonable therefore to suppose that the severity of the lesions did not depend on the presence of preformed toxin. This result might have been anticipated in view of the fact that staphylococci remained 'virulent' even

TABLE 32 The effect of adrenaline on infection

Strain	Dose		Score	
	Adrenaline	Bacteria	Control	Adrenaline
PS 80	2 µgm	$1 \times 10_2^6$	32	33
		1×10^2	13	14
	25 µgm	$4 \times 10_2^6$	34	33
		4×10^2	1	4
Type 52	25 µgm	$2 \times 10_2^6$	11	9
		2×10^2	4	1

when diluted in saline; since the amount of original broth culture present when 10^4 cocci were introduced was about 0.0001 ml.

The effect of adrenaline on lesion severity

It has been shown that adrenaline injected into the skin of rabbits within 2 hours of an ordinary injection of bacteria enhances the production of lesions (Evans, Miles & Niven, 1948).

In the present series of experiments, adrenaline in doses of 2 micrograms and 25 micrograms failed to enhance the lesions produced by the introduction of staphylococci in cotton dust (Table 32). The adrenaline was given at the same time as the challenge. The mice were kept under observation for 4 hours after the injection of adrenaline, but no effects were seen.

As shown earlier, the cotton itself has a strong potentiating affect on the production of lesions and it may be that the adrenaline simply failed to potentiate infection further.

Modification of the Reticulo-Endothelial system

Staphylococci produced essentially a closed lesion when introduced subcutaneously whilst streptococci were found to invade the whole tissue of the mouse. Streptococcus pyogenes (NCTC 8194) introduced on the cotton plugs gave rise to lesions which were not surrounded by the white zone but which subsequently showed a considerable amount of necrosis. Unlike the staphylococci, a

number of the streptococci gave rise to generalised infections from which the mouse died, the streptococci being recovered from the tissues post mortem. The death rate was dependent on the dose of streptococci introduced. It is platitudinous to state that the streptococci were invasive whilst the staphylococci were not; what is unknown is why the staphylococci were not invasive.

It might be that the reticulo-endothelial system (RES) is able to cope with small numbers of staphylococci which 'get loose' in the tissue, but not with small numbers of streptococci. This suggested that, if the RES were interfered with, it might be possible to produce a generalised infection from a subcutaneous infection with staphylococci.

Accordingly, mice were injected intravenously with glycerol trioleate, which appears to stimulate the RES, or with ethyl stearate which depresses the action of the RES (Cooper & Stuart, 1962). Treated and control mice were challenged with the same dose of staphylococci subcutaneously on cotton dust and the results scored at 24 and 48 hours. The results are shown in Table 33. Only one mouse died (12 days after challenge following intravenous ethyl stearate) and only Proteus was recovered from kidneys and liver. All the mice were killed and examined 14 days after challenge. One mouse infected with staphylococcus

TABLE 33 The effect of modification of the RES

Strain	Dose	Scores in mice		
		Control	Treated with glycerol trioleate	Treated with ethyl stearate
Wood 46	9×10^5	13	12	16*
	9 x 10	1	0	0
Charlton	1.5×10^6	28	32	33ø
	1.5×10^2	3	7	6
PS 80	8×10^5	28	35	32
	8×10^2	4	14	7

* One mouse died 12 days after challenge, only Proteus was recovered from the viscera.

ø One mouse had this organism in the liver on post mortem examination.

TABLE 34 The effect of administering antibiotics

Strain	Dose	Time antibiotic given	Scores in mice	
			control	treated
Type 52	10^6	1 hr.	10	4
	10^4		9	4
Smith	10^6		8	4
	10^4		8	3
PS 80	10^6	0 hr.	30	4
			29	1
			26	6
			36	10
	10^4		34	9
	10^6	1 hr.	26	12
	10^4		17	10

See also section on virulence of dried organisms.

'Charlton' and treated with ethyl stearate yielded this organism from the liver, none of the other organs yielded staphylococci.

As may be seen from the table, the lesion score was not markedly affected by interference with the RES and the evidence that the mice were more susceptible to generalised infection was very slight. No attempt was made to check that injection of the lipids in the recommended doses had any effect on this particular strain of mice, but the method has been well documented.

Penetration of material into the plug

It seemed desirable to discover whether particulate or dissolved matter could penetrate into the cotton plug when this was under the skin of the mouse. Pairs of mice inoculated with plugs bearing 10^6 viable units of staphylococcus Type 52 were injected intravenously at intervals from 0 to 4 hours after infection with 0.5 ml. of 0.65% Pontamine Sky Blue. One of each pair was then killed at ½ hour and one at 1 hour after injection of the dye. In all cases both the skin around the plug and the plug itself were stained a deep blue. In animals given Pontamine Sky Blue 24 hours after infection, the white zone became coloured only very slowly, taking about 3 hours to stain completely. The surrounding tissue was deeply stained within 10-15 minutes of injecting the dye.

The penetration of antibiotics was studied by giving the mice 5 mgm. of streptomycin intramuscularly in the thigh after infection and noting the score obtained in these and control mice. In many of the treated mice the 'lesion' consisted solely of a lump around the cotton plug with little or no erythema, thus the effect of the antibiotic was probably greater than might appear from Table 34. As shown in the section of this thesis dealing with the virulence of dried staphylococci, antibiotics were effective when injected up to 4-5 hours after infection; this is, of course, the time at which the bacteria begin to multiply.

There is thus no reason to suppose that any material present in the blood stream of the mouse will fail to penetrate the lesions, especially in the early phases of lesion production.

Simon (1963) reported that oral or intramuscular tetracycline reduced by 10- 100-fold the dose of tetracycline resistant staphylococci needed to cause skin infection in guineapigs. The mechanism of this phenomenon is not known. In this series of experiments, the mice were not given antibiotics to which the staphylococci were resistant and no such potentiation of infection was seen.

Immunity to staphylococcal infection

As has been demonstrated, material circulating in the blood stream of an infected animal appears in the tissue surrounding

TABLE 35 Effect of immunizing mice with vaccine and vaccoid prepared from PS 80.

Challenge dose	7×10^5	7×10^4	7×10^3	7×10^2
			score*	
Control	25	21	7	10
1 dose vaccine	26	14	12	5
2 dose vaccine	22	16	12	4
1 dose vaccoid	23	20	11	6
2 dose vaccoid	27	17	8	7

* Score based on 5 mice, maximum score 30.

the cotton plug and in the plug itself. It seemed therefore that by introducing various substances into the mouse, it might be possible to determine some of the factors which are responsible for the production of these lesions.

Immunization of mice

A joint experiment with Mr. W. D. Brighton of the Applied Immunology Department of the Wright-Fleming Institute was conducted to test whether the use of subcutaneous cotton plugs would be a satisfactory method for assessing the efficacy of vaccines.

One hundred female mice aged about 6 weeks were divided into 5 groups by means of random numbers, one group being kept as control. Of the remaining four groups, two were given one dose (0.5 ml.) of vaccine or vaccoid prepared from staphylococcus PS 80 intraperitoneally 4 weeks before challenge and the other two, one dose of vaccine or vaccoid 6 weeks before challenge and a further dose 2 weeks before challenge. Vaccoid is a preparation of vaccine (dead cells) and denatured toxin (toxoid); details of the methods of preparation are given in Appendix 2.

All mice were challenged on one day with serial 10-fold dilutions of staphylococcus PS 80, the challenge doses ranging from 7×10^2 to 7×10^5 cocci. The scores are given in Table 35 which shows that neither the vaccine nor the vaccoid had any

TABLE 36 Effect of repeated infection with the same staphylococcus

Strain	Dose	Score*			
		Control	1st re-infection	2nd	3rd
PS 80	2×10^6	26	9		
	1×10^6	36	19	12	
	8×10^5	28			5
	3×10^4	34	12	8	
	2×10^4	16	10		
	8×10^3	18			6
Charlton	4×10^4	30	14		
			10		
			9		
	4	1	3		
			3		
Wood 46	2×10^6	26	9		
	2×10^4	1	3		
Type 52	2×10^6	12	14	13	
	1×10^6	10	12		
	2×10^4	12	8	11	
	1×10^4	9	12		
Smith	1×10^6	8	9		
	1×10^4	8	5		

* Score based on 6 mice.

effect on the production of lesions, except perhaps at the very lowest challenge doses.

Similar experiments using standard staphylococcus vaccine prepared from a number of strains, and a vaccine prepared by Greenberg (see for example Greenberg & Cooper, 1960; Greenberg, Cooper & Healy, 1961) in which the mice were challenged with one standard dose of staphylococci (9×10^5 viable units of PS 80), also failed to show any effect of the vaccine.

Effect of repeated infections with staphylococci

Mice which had been infected with various doses of different staphylococci were rechallenged at about 4 week intervals with the same strain at the same dose levels as used initially. Table 36 shows that the response to re-infection with PS 80, Charlton and Wood 46 was markedly reduced, especially at the high dose levels. No change in response was seen at the lower dose levels. The control scores were those obtained in previously uninfected mice inoculated with the same challenge dose. No reduction of score was shown with the strains Type 52 and Smith but no potentiation of infection was seen either.

The results for mice which were re-infected with different staphylococci from those used to produce the initial infections are shown in Table 37. The reduction in lesion score was as marked as in those re-infected with the same strains.

TABLE 37 Effect of re-infection with different staphylococci

Final challenge strain	Initial challenge strain	Dose	Control	Score* Number of previous infections		
				1	2	3
PS 80	Wood 46	6×10^5	34	15	4	
					3	
		6×10^3	12	6	4	
	Charlton	6×10^5	21		9	13
						13
		6×10^3	11		4	5
						5
Charlton	PS 80	4×10^6	29	10	11	
					12	
		4×10^4	13	4	5	
					5	

* The score is based on 6 mice.

It was noticeable that the reaction most markedly reduced was the white zone or the necrosis which surrounded the lesions initially.

The effect of antitoxin and toxin

In an attempt to discover more about the nature of the lesions, graded doses of Burroughs-Wellcome staphylococcus refined globulins (antitoxin) were given intraperitoneally to mice challenged with 10^{5-6} viable units of staphylococcus PS 80 and Charlton. Doses as low as 25 units of antitoxin per mouse markedly reduced the lesion abolishing the white zone or necrosis completely. Some vestiges of the white zone were seen when 2.5 units of antitoxin were given. Serum from mice which had had 4 infections with PS 80, in doses of 0.25 ml. intraperitoneally, reduced the lesions as much as did 2.5 units of antitoxin.

The lesions formed in 'immune' mice were almost entirely without the white or necrotic zone seen on normal animals. Pus formed at the surface of the lesion (slight reaction, score = 1) in most animals, but in those receiving the very smallest doses of staphylococci, no reaction except slight erythema was seen for up to 5 days. Some of the lesions, including those receiving up to 10^{6} cocci as the inoculum, progressed slowly for 10-14 days forming large spherical lesions similar to those produced by micrococci (see for example Figure 6).

It seemed at this stage that passive immunization with a non-staphylococcal serum or gamma globulin would show whether the action of the staphylococcus antitoxin was specific. Accordingly mice infected with 10^6 viable units of PS 80 were treated with 800 units of Burroughs-Wellcome diphtheria antitoxin. This too was found to abolish the white zone, but the diphtheria antitoxin was subsequently shown to prevent haemolysis of rabbit red cells by cultures of Wood 46 known to contain alpha lysin (for details see Appendix 2). It was also shown to produce at least 4 antigen/antibody lines against Burroughs-Wellcome staphylococcus alpha lysin. It may thus be assumed that the diphtheria antitoxin contained staphylococcus antibodies as well.

However, it was found that 0.07 units of the Burroughs-Wellcome staphylococcus alpha toxin injected subcutaneously on clean cotton dust produced a white flare and necrosis at 24 hours in exactly the same way as did a 'virulent' staphylococcus. Larger doses produced severe necrosis within 24 hours although no reaction was visible to the naked eye up to 6 hours after injection. In animals which had experienced a previous infection with staphylococcus PS 80, a dose of 0.7 units of alpha lysin (10 times the previous dose) was needed to produce a white or necrotic zone. It thus seems highly probable that the white zone seen round the lesions produced by the inoculation of staphylococci

TABLE 38 The presence of anti-alpha lysin in mouse sera

	Dilution of Wood 46 culture					
	1/4	1/16	1/64	1/256	1/1024	1/4000
Control	+	+	+	+	±	-
Control + B-W staph antitoxin	±	-	-	-	-	-
1 Control + Greenberg mouse serum	+	+	+	-	-	-
2 Control + Standard mouse serum	+	+	+	+	-	-
3 Control + Vaccine mouse serum	+	+	+	+	-	-
4 Control + Vaccoid mouse serum	+	+	+	+	-	-
5 Control + re-infection mouse serum	+	+	-	-	-	-

1 Serum from mice immunized with Greenberg vaccine.

2 Serum from mice immunized with Standard vaccine.

3 Serum from mice immunized with PS 80 vaccine.

4 Serum from mice immunized with PS 80 vaccine and toxoid.

5 Serum from mice which had had 4 previous infections with staphylococcus PS 80.

was caused by alpha haemolysin; although it was found that the Burroughs-Wellcome alpha lysin produced 5 antigen/antibody precipitation lines against staphylococcus antitoxin and therefore some of the other materials present might have had some part in the production of necrosis.

To complete the picture, sera from the mice used in all four vaccine experiments and from mice which had had 4 previous infections with PS 80 were added to dilutions of a culture of Wood 46 containing rabbit red cells (for details see Appendix 2). Only the serum from mice which had had 4 previous infections had any significant amount of anti-alpha lysin as judged by the ability to prevent haemolysis of the red cells (Table 38).

One may then ask the question, does the mouse virulence test merely measure the presence of alpha haemolysin? The answer appears to be that it measures the ability of the strain to produce alpha lysin <u>in vivo</u>. As has already been demonstrated, the production of alpha lysin <u>in vitro</u> does not exactly parallel mouse virulence measured by this test for the strain which was least virulent for mice produced as much alpha lysin <u>in vitro</u> as did the most virulent strain. Thus whilst the ability to produce alpha lysin is of great importance in this test, the staphylococcus must possess the ability to produce it <u>in vivo</u> as well as <u>in vitro</u> and this appears to depend on other enzymes and toxins.

SUMMARY OF EXPERIMENTAL WORK

No differences were detected in the severity of lesions or in the survival of staphylococci in the cellular exudate of surface lesions made by scraping away the epithelium of guineapigs and mice.

A new method for the production of abscesses in mice was investigated. The technique, which was based on descriptions of the production of abscesses using contaminated sutures, consisted of inserting a cotton plug bearing staphylococci under the skin of mice. Lesions developed in 1-3 days and histologically resembled human abscesses except for the presence of a white or necrotic zone surrounding the site of the cotton plug.

Using this technique it was found that there were significant differences between the lesions produced by different strains of staphylococci; there was a good correlation between the severity of the mouse lesions and the infectivity of the staphylococci for man as judged by observations of cross-infection in hospital wards.

In the second major section of this thesis, an attempt was made to correlate the observed 'virulence' of the staphylococci for man and mouse with the <u>in vitro</u> production of a number of enzymes and toxins and with 'markers' such as sensitivity to phage. There was a good correlation between the severity of the

mouse lesions and the *in vitro* production of alpha haemolysin. The correlation was not perfect however and other enzymes, particularly beta lysin and fibrinolysin, appeared to play a supporting role in the production of lesions. The virulence of the staphylococcus appeared to depend to some extent on the sum of its toxic abilities.

The third part of the experimental work describes experiments designed to detect differences between the virulence or infectivity of fresh and dried staphylococci. No such differences could be detected as judged by the production of subcutaneous lesions by staphylococci in cotton dust. There appeared to be little or no difference in the clump size (that is the number of live cocci in each viable unit) between the fresh and dried cocci, although only negative evidence was obtained on this point. Experiments in which the mice were given antibiotics at intervals after infection suggested that the dried cocci might have a longer lag phase *in vivo* than the fresh cocci, but this was by no means certain.

Experiments described in the final section of the thesis were concerned with attempts to determine further what factors played a part in the production of lesions. Injection of alpha lysin produced a white or necrotic zone around a cotton plug in the same way as did a 'virulent' staphylococcus.

This suggested very strongly that the severity of the staphylococcus lesion was related to the ability of the staphylococcus to produce alpha lysin *in vivo* (as distinct from *in vitro*). It was found that a single infection with a staphylococcus was more effective at producing antibodies, or other substances which prevented necrosis, than were vaccines. The lesions produced in animals which were passively or actively 'protected' against staphylococcal infection, were devoid of the white or necrotic zone and more closely resembled staphylococcal lesions seen in humans than did the lesions in 'unprotected' animals. No evidence of hypersensitivity was seen in these animals.

DISCUSSION

The subcutaneous injection of staphylococci on plugs of cotton dust gave rise to lesions which were to some extent like natural infections in humans, especially where the animal had previously experienced a staphylococcal infection. Animals 'immunized' in this manner showed a low level of anti-alpha lysin in the blood and it is perhaps significant that almost all humans appear to have at least a low level of antibody to staphylococci.

The experiments cited appear to show that the white or necrotic zone surrounding a staphylococcal lesion was produced by alpha haemolysin, although it must be remembered that the

Burroughs-Wellcome alpha lysin contained at least 5 precipitating antigens. It is therefore difficult to be sure that the alpha lysin alone caused the necrosis. Correlation of the *in vitro* production of other enzymes suggested that the beta lysin and fibrinolysin were also important in the production of lesions. In order to investigate the 'virulence factors' in more detail, it is desirable to obtain pure antigens for only in this way can the process of lesion production be dissected further with any degree of accuracy. It seems probable that micro-histological techniques will be necessary involving, for example, demonstration of the action of specific enzymes on substrates *in vivo*.

Under certain conditions, the subcutaneous lesions produced by the implantation of staphylococci on cotton dust were very slow growing and caused little necrosis outside the lesion itself. It seems possible that such lesions would be ideal for studying transduction, lysogenisation and synergism *in vivo*. Professor R. E. O. Williams has used this technique in a study of the production of staphylococcal L forms. The method is currently being used in a study of the lethal effect of fresh and dried streptococci.

In a study of staphylococcal infections of man we may agree with Pope (An Essay on Man) that 'the proper study of mankind is man'. In a comparative survey of this nature however,

in which more than 3,000 lesions were studied, the problems involved in carrying out the experimental work in man become enormous. It was therefore necessary to find a technique which could be used in an experimental animal and which would appear to have some relevance to human infection. As was discussed earlier, it seemed more likely that the experimental results would be relevant for man if the lesions formed in man and animals were similar than if wound infection in man were compared with, say, intraperitoneal infection in the mouse.

The techniques which meet this requirement are those producing non-fatal surface infections in animals, for example, the use of contaminated sutures to produce stitch abscesses. Many workers have found however that a considerable number of the sutures were removed by the experimental animals and that when this took place, no infections resulted. The animals were found unable to remove subcutaneous cotton plugs, however, until after the lesion had developed. The new technique would therefore appear to be as satisfactory as might be expected on theoretical grounds and in practice it was found that the severity of the mouse lesions was statistically significantly correlated with the infectivity of the strain for man as observed in the hospital wards.

APPENDIX 1

TABLES OF RESULTS

APPENDIX 1, TABLE 1

Comparison of the results obtained from the subcutaneous introduction of staphylococci in mice with and without cotton dust.

Strain	Cotton+		No cotton	
	Inoculum	Result*	Inoculum	Result*
PS 80/81	2×10^6	34	1.5×10^7	36
	2×10^5	26	1.5×10^6	34
	3×10^4	30	2.5×10^6	21
	2×10^4	28	1.5×10^6	29
	2×10^4	14	1.5×10^6	12
	1.5×10^4	21	2.5×10^5	4
	3×10^3	27	1.5×10^5	7
	1.5×10^3	11	1.5×10^5	1
	5×10^2	13	2.5×10^4	0
	2×10^2	8	1.5×10^4	0
	1×10	7	1.5×10^4	0
			1.5×10^3	0
Charlton	3×10^6	30	3×10^6	36
	2×10^5	31	3×10^5	8
	9×10^5	28	3×10^4	0
	9×10^4	24	3×10^3	0
	2×10^4	22		
	9×10^3	21		
	9×10^2	11		
Wood 46	9×10^5	25	2×10^7	36
	6×10^5	20	2×10^6	13
	6×10^4	13	2×10^5	0
	9×10^3	20	2×10^4	0
	6×10^3	9		
	6×10^2	7		
Type 52	3×10^6	10	3×10^7	7
	2×10^6	18	3×10^6	2
	2×10^6	13	3×10^5	0
	1.5×10^4	16	3×10^4	0
	3×10^4	9		
	2×10^4	9		
	1.5×10^4	13		
	2×10^2	4		
Smith mucoid	5×10^6	24	2.5×10^7	35
	1.5×10^4	23	2.5×10^5	10
	9.5×10^4	16	2.5×10^5	0
	1.5×10^4	8	2.5×10^4	0
	9.5×10^2	8		

* Each result is the total of the scores for 6 mice; maximum score 36.

+The results for 'cotton' are derived from a number of experiments and not from a series.

APPENDIX 1, TABLE 2

Comparison of the results obtained from the subcutaneous introduction of micrococci in mice with and without cotton dust.

Strain	Cotton		No cotton	
	Inoculum	Result*	Inoculum	Result*
NCTC 7944	1×10^6	13	1×10^7	0
	1×10^6	18	1×10^6	0
	1×10^5	18	1×10^5	0
	1×10^4	18	1×10^4	0
	1×10^3	18		
Harrod	1×10^6	18	7×10^6	2
	8×10^5	18	7×10^5	0
	3×10^5	18	7×10^4	0
	1×10^5	18	7×10^3	0
	1×10^4	12		
	1×10^3	12		

* Each result is based on the scores for 6 mice; maximum score 36.

Note however that the response is almost uniform, irrespective of the dose. The lesions seen with micrococci differ from those resulting from staphylococci.

APPENDIX 1, TABLE 3

Growth rates of staphylococcus PS 80 in vitro and in vivo.

In vitro		In vivo		
Time	Computed count per ml.	Time	Computed count per plug duplicate counts*	
0 hours	2160	0 hours	0	45
1	2520	1	15	30
2	9300	2	0	135
3	5.2×10^4	3½	15	135
4	7×10^5			
5	3.5×10^6	5	120	1365
6	3.9×10^7	6	2854	4944
7	2.2×10^7	7	600	2840
8	2.3×10^7			
9	2.9×10^7	9	3900	1.5×10^3
10	4.8×10^7			
24	9×10^7	24	37500	1.5×10^6
		48	1.6×10^5	4.7×10^5
		72	3.5×10^6	4×10^6
		96	2×10^4	7×10^5

* The numbers given are the computed numbers of viable units per plug. The initial inoculum was 100 viable units.

APPENDIX 1, TABLE 4

Sums of scores for mice, unadjusted for the effect of dose.

Rank order number unadjusted	Sums of scores for 2 mice for 2 days	Mean score
1	12, 12, 12, 12	12.00
2	11, 12	11.50
3	10, 11, 12, 12	11.25
4	10, 10, 12, 12, 12	11.20
5	9, 11, 12	10.70
5	9, 10, 10, 11, 12, 12	10.70
7	10, 10	10.00
8	7, 8, 8, 10, 10, 10, 10, 12, 12, 12	9.90
9	6, 10, 10, 11, 12	9.80
9	8, 10, 10, 11	9.80
9	8, 8, 11, 12	9.80
12	7, 7, 8, 9, 9, 9, 9, 9, 10, 10, 11, 12, 12, 12, 12	9.70
13	7, 9, 10, 12	9.50
14	6, 7, 9, 10, 10, 10, 12, 12	9.50
14	8, 8, 8, 9, 11, 12	9.30
14	4, 8, 9, 11, 12, 12	9.30
17	5, 7, 12, 12	9.00
17	7, 8, 9, 12	9.00
17	5, 6, 8, 11, 12, 12	9.00
17	5, 10, 10, 10, 10	9.00
21	6, 6, 8, 8, 8, 11, 11, 12	8.75
22	4, 4, 5, 6, 10, 10, 10, 10, 10, 12, 12	8.45
23	4, 6, 8, 8, 8, 9, 9, 11, 12	8.30
23	5, 10, 10	8.30
25	5, 7, 8, 8, 8, 10, 11	8.10
26	4, 5, 6, 6, 6, 6, 8, 8, 9, 10, 10, 10, 11, 12	7.90
27	6, 6, 7, 10	7.25
28	4, 4, 5, 7, 9, 10, 10	7.00
29	4, 6, 6, 7, 7, 7, 8, 8, 9	6.90
30	4, 6, 8, 9	6.80
30	6, 6, 7, 8	6.80
32	4, 6, 6, 8, 9	6.60
33	6, 7	6.50
34	5, 6, 7	6.00
35	3, 5, 7	5.00

APPENDIX 1, TABLE 5

Analysis of covariance table*.

Sources of variation	Degrees of freedom	Sums of squares and products			Error of estimation		
		Sx^2	Sxy	Sy^2	Sum of squares	Degrees of freedom	Mean square
Total	199	2668017.5	13448.95	1182.4	1175.62	198	
Effect of regression	34	960678.5	5634.25	320.86			
Within strains error	165	1707339.0	7814.70	861.54	825.74	164	5.04
Residual test of adjusted means		significance of			349.88	34	10.29

Variance ratio $= \frac{10.29}{5.04} = 2.04$ $n_1 = 34$ $n_2 = 164$

$1\% > P > 0.1\%$

* This table shows that, after allowance is made for the effect of dose on lesion score, there are still significant differences between the scores given by various strains of staphylococci.

APPENDIX 1, TABLE 6

Antigen/antibody diffusion lines.

Rank order number	Number of lines for each isolate
1	4, 6
2	3, 4, 6
3	3, 4, 4, 4,
4	2, 2, 3, 3
5	4, 4
6	1, 3
7	1, 3
8	1, 1, 2, 2, 2, 2, 3, 3, 3, 3, 3, 3, 5, 5
9	1, 1, 3, 3, 4, 4, 5, 5, 5, 7
10	4, 4, 5, 5, 5
11	1, 2, 2, 3, 5
12	4, 4, 5
13	2, 2, 2, 3, 3
14	1, 1, 1, 1, 1, 2, 4, 4
15	2, 3
16	3, 3, 4, 5
17	1, 2, 2, 2, 4
18	1, 4, 5
19	1, 5, 5, 5, 6
20	4, 4, 4, 5, 7
21	1, 2, 2, 3, 3, 4, 4, 4
22	2, 3, 3, 3, 3, 3, 4, 4, 4, 5
23	3, 4, 4, 4, 4, 5, 5, 6
24	2, 2, 3, 3, 3, 5, 5, 5, 5, 5, 5, 5, 6
25	2, 3, 4
26	2, 2, 2, 4, 4, 5
27	1, 1, 1, 1, 2, 2, 2, 3, 3
28	1, 1, 3
29	1, 1, 1, 2, 2, 2, 3
30	2, 3, 3, 4
31	0, 1, 2, 5
32	1, 2, 3, 3, 4, 4
33	2, 2
34	3, 3, 4
35	0, 3, 4

Total lines = 568
total isolates = 182
mean number of lines = 3.13

Non-spreading strains:

1	3, 5
2	1, 3
3	3, 5, 5
4	2, 2
5	2, 5
6	0, 2, 2, 3
7	4, 4
8	1, 2, 2, 4

Total lines = 60
total isolates = 21
mean number of lines = 2.85

APPENDIX 1, TABLE 7

Susceptibility of staphylococci to antibiotics produced by viridans streptococci.

Spreading staphylococci in order of mouse virulence.

No. tested	Streptococci					
	Class			Calicao		
	S	S/R	R	S	S/R	R
GROUP A (most virulent)						
2	-	-	2	-	-	2
4	2	1	1	1	2	1
5	-	-	5	-	1	4
4	1	-	3	-	1	3
2	-	1	1	-	-	2
2	-	-	2	-	-	2
2	-	1	1	-	-	2
15	4	1	10	3	3	9
10	-	1	9	1	-	9
5	-	2	3	-	1	4
6	1	2	3	-	1	5
3	-	-	3	-	-	3
5	-	1	4	-	-	5
8	1	2	5	1	1	6
3	1	2	-	1	2	-
4	-	1	3	-	-	4
6	1	2	3	1	1	4
2	-	-	2	-	-	2
GROUP B						
5	-	-	5	-	-	5
5	-	1	4	-	2	3
8	2	3	3	1	2	5
9	1	1	7	-	3	6
7	-	3	4	-	-	7
11	4	5	2	3	4	4
1	1	-	-	1	-	-
6	1	2	3	1	2	3
9	2	4	3	2	2	5
3	2	-	1	1	-	2
7	-	1	6	-	-	7

APPENDIX 1, TABLE 7 (continued)

GROUP C						
No. tested	Streptococci					
	Claes			Calicao		
	S	S/R	R	S	S/R	R
4	-	1	3	-	-	4
4	-	2	2	-	-	4
5	-	2	3	-	2	3
2	-	-	2	-	-	2
3	-	1	2	-	-	3
3	-	1	2	-	-	3
Non spreaders						
2	-	-	2	-	1	1
4	-	-	4	1	-	3
4	-	1	3	-	2	2
3	-	-	3	-	-	3
2	-	1	1	-	1	1
2	1	1	-	1	-	1
2	-	-	2	-	-	2
2	1	1	-	-	-	2
6	3	-	3	-	1	5

APPENDIX 1, TABLE 8

Analyses based on isolates.

Relation between mouse score and lysin production.

All isolates.

Mouse score	11-12	9-10	7-8	5-6	1-4
Total isolates	48	49	39	24	11
	% producing factors				
alpha lysin	85	67	44	50	9
beta lysin	35	16	10	8	0
delta lysin	81	67	67	71	9
fibrinolysin	52	27	28	33	27
$\alpha + \delta$ lysin	69	55	36	38	9
$\alpha + \delta$ + fibrin	44	14	15	8	0
$\alpha + \delta + \beta$ + fib	19	2	5	13	0

TABLE 9

All isolates producing alpha lysin.

Total isolates	41	33	17	12	1
beta lysin	37	21	18	17	0
delta lysin	80	82	82	75	0
fibrinolysin	58	30	47	50	[100]
δ + fibrin	51	21	35	25	0
$\beta + \delta$ + fibrin	22	3	12	0	0

APPENDIX 1, TABLE 10

Analyses based on isolates.

Isolates producing little alpha lysin.

Mouse score	9-12	1-8
Total isolates	23	44
	% producing factors	
beta lysin	13	2
delta lysin	52	48
fibrinolysin	17	20
δ + fib	17	14
β+ δ+ fib	9	0

TABLE 11

Analyses based on epidemicity.

	Epidemic isolates	Non epidemic	Non spreading nasal isolates
Total isolates	89	66	16
	% producing factors		
alpha lysin	70	56	31
beta lysin	25	12	6
delta lysin	64	74	60
fibrinolysin	25	53	19
α + δ lysin	57	42	25
α + δ + fibrin	16	29	6.2
α+ β+δ+fib	6.7	7.6	6.2

APPENDIX 1, TABLE 12

Analyses based on isolates.

Relation between mouse score and enzyme production.

All isolates.

Mouse score	11-12	9-10	7-8	5-6	1-4
Total isolates	48	49	39	24	11
	% producing factors				
haemoglobinase	33	33	54	54	27
gelatinase	73	63	59	75	63
egg-yolk	62	61	79	79	63
urea	25	33	33	67	18
litmus milk	31	31	38	42	27
Voges-Proskauer	60	61	56	62	82
lactose fermentation	85	89	85	88	82

TABLE 13

Isolates producing alpha lysin

Mouse score	11-12	9-10	1-8
Total isolates	41	33	30
	% producing factors		
beta lysin	37	21	17
delta lysin	78	82	77
fibrinolysin	58	30	50
haemoglobinase	37	21	30
gelatinase	68	58	50
egg-yolk	58	61	57
urea	24	42	47
litmus milk	34	33	47
Voges-Proskauer	58	67	57
lactose	83	88	87

APPENDIX 1, TABLE 14

Analysis by phage group

	Phage group			
	I	II	III	mixed
Total isolates	84	12	61	14
	% producing factors			
alpha lysin	48	75	79	50
beta lysin	14	83	23	29
delta lysin	52	92	80	85
fibrinolysin	31	8	43	57
alpha + delta	30	67	72	43
$\alpha+\beta+\delta$ + fib	6	0	8	14
haemoglobinase	69	17	3	50
gelatinase	81	33	51	71
egg yolk	79	92	46	85
urea	17	58	49	43
litmus milk	55	33	8	21
Voges-Proskauer	56	42	44	43
lactose	92	67	90	57

APPENDIX 1, TABLE 15

Analyses based on isolates.

Comparison of pigment production.

Pigment on glycerol monoacetate	yellow	orange	other
Total number isolates	16	109	46
	% producing factors		
alpha lysin	63	61	59
beta lysin	0	18	22
delta lysin	75	69	61
fibrinolysin	19	36	41
haemoglobinase	0	49	32
gelatinase	38	72	63
egg-yolk	6	75	74
urea	6	40	30
litmus milk	0	43	24
Voges Proskauer	75	53	76
lactose	75	90	85

TABLE 16

A comparison of precipitation lines and lysin production.

		% producing factors			
Number of precipitation lines	Total isolates	alpha lysin	beta lysin	delta lysin	alpha + delta lysin
1-2	63	40	8	41	27
3-4	73	70	21	81	57
5-7+	35	80	29	89	68

APPENDIX 1, TABLE 17

Production of precipitation lines in relation to virulence.

Mouse score	11+12	9-10	7-8	5-6	1-4
Total isolates	48	49	39	24	11
No: lines	% producing lines				
1-2	23	29	46	42	91
3-4	54	45	31	50	9
5-7+	23	26	23	8	0

TABLE 18

Phage group I strains only.

Enzyme production.

	Epidemic	non-epidemic
Total isolates	40	32
	% producing enzymes	
alpha lysin	60	41
beta lysin	25	6
delta lysin	45	63
fibrinolysin	23	50
gelatinase	85	81
haemoglobinase	68	69
egg-yolk	73	88
urea	18	19
litmus milk	55	63
Voges-Proskauer	60	50
lactose	100	91

APPENDIX 1, TABLE 19

Relation of survival on drying to virulence.

Rank order number	% survival at 3 days	No. weeks to fall to 1% of inoculum
1	51, 80	6.5, 6.5
2	62, 100	5.5, 7.0
5	100	13.0
7	25	5.0
8	50, 57, 58, 97	4.5, 5.0, 6.5, 8.5
9	43, 100	7.0, 13.0
11	29, 76	4.5, 11.0
14	72, 100	7.5, 8.5
17	72	13.5
18	75	8.5
19	72, 100	9.0, 14.0
20	61, 100	7.5, 11.0
21	41, 66	6.0, 9.5
22	85, 100	11.0, 14.0
23	26, 28	4.5, 13.0
24	36, 50, 61, 83	2.5, 5.5, 7.0, 7.5
25	95, 100	6.5, 7.0
27	44, 100	7.0, 7.0
28	49, 70	3.5, 4.5
30	33, 69	5.0, 15.0
33	56, 58	8.0, 11.5
34	54, 57	5.5, 6.5
35	44, 80	7.5, 11.5

APPENDIX 1, TABLE 20

Comparison of virulence of fresh and dried staphylococci.

Strain	Fresh		Dry	
	Inoculum	Score*	Inoculum	Score*
PS 80	2×10^6	34	1.3×10^6	30.5
	2×10^6	26	4.5×10^4	27
	3×10^5	30	5×10^3	24.5
	2×10^4	28	2×10^2	18.5
	2×10^4	14	1×10^4	24.5
	1.5×10^4	21	17	15.5
	3×10^3	27	2×10^4	18
	1.5×10^3	11	$2 \times 10_5$	6.5
	5×10^2	13	5.5×10^5	29
	2×10^2	8	8×10^2	24
	10	7		
	2.9×10^6	37		
	2.9×10^4	10		
	8×10^5	28		
	8×10^3	18		
	$8 \times 10_5$	4		
	5.2×10^6	29		
	1.9×10^2	30		
	4.3×10^5	4		
	5.4×10^5	32		
	6×10^5	34		
	4.3×10^6	33		
Charlton	3×10^6	30	1×10^7	34
	2×10^6	31	25	2
	9×10^5	28	2.2×10^6	32
	9×10^4	24	9.8×10^5	30
	2×10^2	22	7×10^5	30
	9×10^3	21	1.5×10^5	19
	9×10^2	11		
	1.5×10^6	28		
	1.5×10^2	3		
	1×10^6	34		
	1.5×10^4	10		
	4.5×10^4	30		
	1×10^2	2		
	4.5	1		
Type 52	2×10^6	18	9×10^6	14
	2×10^6	13	2×10^3	11.5
	1.5×10^6	16	2×10^6	11
	2×10^4	9	3×10^5	12
	1.5×10^4	13	1×10^5	11
	2×10^2	4	1.7×10^4	6
	3×10^6	10		
	3×10^4	9		

* Score based on 6 mice. Maximum score = 36.

APPENDIX 1, TABLE 20 (continued)

Strain	Fresh		Dry	
	Inoculum	Score*	Inoculum	Score*
Type 52 (cont.)	2.5×10^6	12		
	2.5×10^4	12		
	6.5×10^5	9		
	6.5×10^1	1		
	2.2×10^6	9		
	2.2×10^2	1		
Wood 46	9×10^5	25	7×10^5	26
	6×10^5	20	8.5×10^5	22.5
	6×10^4	13	6×10^5	22
	9×10^3	20	1.5×10^5	11
	6×10^2	9	4.5×10^4	15
	6×10^5	7	7.5×10^3	8
	9×10^5	13	3.3×10^5	18
	9×10^3	6		
	$9 \times 10_5$	1		
	7×10^5	20		
	7×10^3	4		
	2.3×10^6	26		
	2.3×10^4	1		
	8×10^5	15		

APPENDIX 1, TABLE 21

The effect of antibiotics on infection with fresh and dried staphylococci; scores corrected for the effect of dose.

Strain	Score*									
	Control	5 mgm. of antibiotic given at x hours								
		0	3	4	5	6	7	8	9	10	11
PS 80 fresh	31.5	3.5	5.5			30.5					
	31.9	5.9	4.9	7.9	32.9	29.9					
	34.5		5.5	10.5	16.5	31.5					
	22.8		3.8	9.8	28.8	22.8	31.8	34.8	34.8	31.8	28.8
mean	30	4.7	4.9	9.4	25.8	28.6	31.8	34.8	34.8	31.8	28.8
PS 80 dry	31.5		4.5	11.5	17.5	22.5					
	27.2		3.2	5.2	9.2	12.2					
	21.5		5.5	11.5	24.5	33.5	29.5	26.5	26.5	32.5	30.5
mean	27.0		4.4	9.4	17.1	22.8	29.5	26.5	26.5	32.5	30.5
Wood 46 fresh	17.1		9.1	8.1	4.1	8.1					
	12.5		4.5	7.5	6.5	7.5					
	13.4	0.4	14.4	5.4	7.4	7.4					
mean	14.3	0.4	9.3	7.0	6.0	7.6					
Wood 46 dry	20.8		6.8	5.8	8.8	26.8					
	9.8	13.8	12.8	9.8	4.8	14.8					
mean	15.3	13.8	9.8	7.8	6.8	20.8					
Charlton fresh	35.9	0.9	3.9	7.9	19.9	27.9					
	29.4	4.4	5.4	4.4	17.4	22.4					
mean	32.6	2.6	4.6	6.2	18.6	24.2					
Charlton dry	18.3	0	0	15.3	12.3	18.3					
	34.5	5.5	10.5	18.5	30.5	30.5					
mean	26.4	2.2	5.2	17.6	21.4	24.4					

APPENDIX 2

MATERIALS AND METHODS

MEDIA

Broth: Oxoid nutrient broth.

Todd-Hewitt Broth: Meat extract broth with added Eupeptone, sodium bicarbonate, sodium chloride, sodium phosphate and glucose (see Mackie & McCartney, 1959, Handbook of Practical Bacteriology, Livingstone, 9th edition, page 189).

Diluent: Physiological saline containing 5% broth.

Serum agar: Oxoid Blood agar base containing 5% horse serum. To this was usually added 0.1% phenol phthalein phosphate (Barber & Kuper, 1951).

Blood agar: Oxoid Blood agar base containing 10% horse blood.

Egg-yolk broth: The egg-yolk broth was prepared according to the method described by Gillespie and Alder (1952). Five parts of hen egg-yolk was added to 100 parts of digest broth. Kieselguhr (2 gm./100 ml.) was added and the whole thoroughly stirred, allowed to stand for 30 minutes, and then filtered through filter paper pulp. Sucrose (1%) was added to the medium and the whole sterilized by filtration through a Seitz filter.

Egg-yolk agar: Made by adding 1 egg-yolk to 250 ml. of digest agar containing 1% Tween 80.

Glycerol monoacetate medium for pigmentation: Nutrient agar to which was added 1% glycerol monoacetate prior to autoclaving (Turner & Willis, 1962).

Fibrinolysin medium: The method of Selbie and Simon (1952) was followed. Human plasma was heated to 60°C for 30 minutes to precipitate the fibrin which was centrifuged down and then shaken in saline and incorporated in nutrient agar. Clearing round the colony was taken as an indication of the production of fibrinolysin.

Quantitative determination of alpha haemolysin: The method used was that of Selbie and Simon (1952). Digest broth cultures were incubated at 37°C for 48 hours in 30% CO_2. Serial four-fold dilutions of the broth culture were prepared in saline and 0.1 ml of a suspension of rabbit red cells which had been washed 3 times in saline were added to each 3 ml of saline diluant. The suspension was incubated at 37°C for one hour and the results read then and again after a further 18 hours at 4°C. The highest dilution showing at least 50% haemolysis was recorded.

BACTERIA USED IN THE STUDY

A number of strains were used as standards throughout much of the work and are described below. The strains from the epidemics in hospital wards are described as regards phage-type etc. in the relevant section of this thesis.

Staphylococcus aureus

PS 80: (NCTC 9789) The propagating strain for phage 80. This strain was originally isolated by Dr. P.M. Rountree in Sydney. Phage type 80/81. Resistant to penicillin only.

Wood 46: (NCTC 7121) This strain produces large quantities of alpha haemolysin. Phage type 47+. Sensitive to all antibiotics.

Charlton: Isolated from a severely septic lesion during an outbreak of cross-infection. Phage type 53/77. Resistant to penicillin, tetracycline and streptomycin.

Type 52: Isolated from a nasal swab during a survey of nasal carriage in recruits. The survival of this strain was studied previously (Noble, 1961; M.Sc. Thesis.) Phage type 52. Sensitive to all antibiotics.

Smith: The mucoid staphylococcus described by Koenig (1962). This strain is not phage typable on account of the mucoid capsule. Sensitive to all antibiotics.

Warren: Isolated from a pustule. Not phage typable. Resistant to penicillin.

Farrow: Isolated from a boil. Phage type 3C+. Sensitive to all antibiotics.

PS 83A: (NCTC 10039) The propagating strain for phage 83A. Phage type 6/7/53/83A. Sensitive to all antibiotics.

PS 42E: (NCTC 8357) The propagating strain for phage 42E. Phage type 42E. Sensitive to all antibiotics.

Micrococci

NCTC 7944: The strain used by the Committee on Formaldehyde Disinfection (1958). Sensitive to all antibiotics.

Harrod: Isolated from an infected urine specimen.

Resistant to penicillin, tetracycline, streptomycin, chloramphenicol and erythromycin.

WCN: Carried as a commensal in the mouth. Sensitive to all antibiotics.

Streptococcus pyogenes

NCTC 8194: Lancefield group A, type 19.

Escherichia coli

E 56: Antigenic type O.128, B.12, H.2 Isolated from a case of gastro-enteritis and kindly supplied by Dr. Joan Taylor.

All cultures used in this study were freeze dried in ampoules when received. A new ampoule was opened for each experiment.

METHODS

Determination of clump-size

The expression 'clump-size' is used to indicate the number of viable bacteria in a clump or chain. The broth cultures used in the experiments were diluted 10-fold, thoroughly mixed using a pipette and o.1 ml of this dilution allowed to dry on a microscope slide. The cocci were fixed and stained and about 200 aggregates counted, noting the number of cocci in each aggregate. From this date the clump-size may be found thus:-

Staphylococcus PS 80

Number of cocci in clump	1	2	3	4	5	6
Number of such clumps	108	101	11	9	0	2

total cells = 391 total clumps = 231

mean = 1.69

Clump-size = 1.69 cocci per clump

It is recognised that this method ignores the fact that some of the cocci may be dead but it seems reasonable to suppose that this will be a constant fraction for cultures of staphylococci grown in the same batch of broth at the same temperature for the same period.

MERCURY RESISTANCE

The method of Green (1962) was followed. Phenyl-mercuric nitrate was dissolved in hot ethyl alcohol to give a concentration of 1 part in 150,000. 0.02 ml of the solution was dropped on to blotting paper discs 0.6 cm in diameter and allowed to dry, this gave about 1.3×10^{-7} gm of the salt on each disc. These discs were used in the same manner as antibiotic sensitivity discs.

Antibiotic resistance

Antibiotic resistance was determined using paper discs impregnated with standard concentrations of antibiotics on seeded agar plates. The concentrations of antibiotics was as follows:-

penicillin, 1 unit per disc; tetracycline, 25 mcgm per disc; streptomycin, 10 mcgm per disc; chloramphenicol, 25 mcgm per disc; erythromycin, 5 mcgm per disc.

Sensitivity to Bacteriophage

Phage typing was carried out at the Staphylococcus Reference Laboratory, Colindale, and at the Wright-Fleming Institute, St. Mary's Hospital Medical School. A phrase such as 'phage type 52A/79' is used to indicate that the strain was sensitive to those two phages only at the routine test dilution. Lysis by phages at 1000 times RTD is indicated by underlining the phage numbers.

Cotton dust

C4 white cotton flock which is pure cellulose and corresponds to a bleached cotton fabric. I am grateful to the Shirley Institute, Didsbury, Manchester and to Messrs. Hutchinson (Ramsey) Ltd. for this material.

Mice

The Wright-Fleming Institute strain of mice is derived from a cross of 'P strain' and 'Strong A'. The mice used in the experiments are the F_2 generation of the cross.

Depilator

The formula for the depilating powder is as follows:-

Barium chloride	7 parts
Flour	7 parts
Talcum powder	7 parts
Soap powder	1 part

The animals' hair was cut as short as possible with clippers. The powder made into a smooth paste with water and applied to the shorn hair. After 2 - 3 minutes the paste, and hair, was removed by washing the animal. The animals were dried and kept in a warm box until the fur had dried completely.

Preparation of vaccine and vaccoid from PS 80

The medium used was a semi synthetic one containing amino acids, sodium lactate, sodium chloride, casamino acids, Difco yeast extract and glucose (Hoit, L.B., unpublished).

Staphylococci were grown in this medium in Thompson bottles, laid flat, 400 ml per bottle, at 34°C for 3 days. The cells were killed by heating to 80°C for 30 minutes, centrifuged and washed twice with saline, re-suspended at a concentration of about 1.5×10^{11} and counted.

Vaccine: The cells suspension was diluted in phosphate buffered saline to 10^9 organisms per ml, and 0.5 ml injected intraperitoneally into each mouse.

Vaccoid: The cell suspension was diluted to 10^{10} cells per ml, 0.3 ml of this added to 10 ml of alpha toxin containing 5Lf per ml. One ml of this mixture was injected intraperitoneally into each mouse.

Modification of the Reticulo-endothelial system

Glycerol trioleate was made up as a 20% v/v suspension in dextrose water containing 1% Tween 80. The mixture was homogenised in a M.S.E. blendor and 0.05 ml volumes (equivalent to 11.5 mgm of glycerol trioleate) injected into the tail vein of each mouse.

Ethyl stearate was made up as a 20% suspension in dextrose water containing 1% Tween 80. All manipulations had to be carried out at 40°C to prevent the stearate from solidifying. The suspension was homogenised and 0.1 ml (equivalent to 20 mgm of ethyl stearate) injected into the tail vein of each mouse.

The methods and dosages followed were those of Cooper and Stuart (1962).

Measurement of sensitivity to antibiotics produced by viridans streptococci

The streptococci were grown in Todd-Hewitt broth for 24 hours and then incorporated into double strength nutrient agar at a temperature of about 45°C. The agar was then poured to form either a complete agar plate or to form a 'ditch' cut in a serum agar plate. In the former type, the staphylococci were inoculated as spots on the streptococcus agar and onto serum agar as a control. In the latter type the staphylococci were streaked across the ditch. Results were read at 24 hours as "complete", "partial" or "no" inhibition.

APPENDIX 3

PUBLICATIONS SUBMITTED IN SUPPORT OF CANDIDATURE

PUBLICATIONS SUBMITTED IN SUPPORT OF CANDIDATURE

The following publications illustrate an epidemiological approach to investigation of the spread of staphylococci and staphylococcal disease in hospital wards.

1) Williams, R. E. O., Noble, W. C., Jevons, M. P., Lidwell, O. M., Shooter, R. A., White, R. G., Thom, B. T. & Taylor, C. W. (1962) Isolation for the control of staphylococcal infection in surgical wards. Brit. Med. J. ii 275.
2) Noble, W. C. (1962) The dispersal of staphylococci in hospital wards. J. clin. Path. 15 552.
3) Davies, R. R. & Noble, W. C. (1962) Dispersal of bacteria on desquamated skin. Lancet ii 1295.
4) Davies, R. R. & Noble, W. C. (1963) Dispersal of staphylococci on desquamated skin. Lancet i 1111.
5) Noble, W. C., Lidwell, O. M. & Kingston, D. (1963) The size distribution of airborne particles carrying micro-organisms. J. Hyg. (Camb.), 61 385.
6) Noble, W. C. & Clayton, Yvonne M. (1963) Fungi in the air of hospital wards. J. gen. Microbiol. 32 397.

The first paper submitted here (Williams, et al., 1963) contains analyses of the rate of spread of staphylococcal disease in hospital wards over a period of about 5 years. These

investigations, of necessity, involved a large number of people in carrying out the various sections of the work. During much of this period (1957-1961) I was mainly responsible for the collection and assessment of air-samples taken in the wards. The 2nd, 5th and 6th papers (Noble, 1962; Noble, Lidwell & Kingston, 1963; Noble & Clayton, 1963) were the result of observations and specific investigations made in the wards during this period. The actual mode of spread of staphylococci was not known although it was felt that 'dust' must play some part, since the observations recorded in Noble, Lidwell & Kingston (1963) showed that the staphylococci were present in the air as particles of about 13 microns equivalent diameter.

Dr. R. R. Davies, Lecturer in Mycology at St. Mary's Hospital Medical School, suggested that since all persons disseminate small particles of desquamated skin which forms a considerable proportion of household dust, these skin fragments might be the particles on which staphylococci were dispersed. In investigations on these lines (Davies & Noble, 1962, 1963) I carried out the bacteriology on which this work was based. Further work on these lines (Noble & Davies, in preparation) has shown that the dispersal of staphylococci is

dependent on the degree of skin colonisation with staphylococci and that patients with skin diseases disseminate more particles of skin and have a greater susceptibility to skin colonisation with staphylococci than do normal subjects.

REFERENCES

AKINLADE, N. D. 1962 A trial of mercuric chloride for rapid identification of epidemic strains of Staphylococcus aureus. West African Med. J. 11 35.

ALAMI, S. Y. & KELLY, F. C. 1960 Influence of coagulases and route of injection on staphylococcal virulence in mice. Proc. Soc. Exp. Biol. Med. Sci. 105 589.

ALDER, V. G., GILLESPIE, W. A. & HERDAN, G. 1953 Production of opacity in egg-yolk broth by staphylococci from various sources. J. Path. Bact. 66 205.

ALDER, V. G., GILLESPIE, W. A. & THOMPSON, M. E. M. 1955 Virulence and phage-patterns of antibiotic resistant staphylococci in a hospital. J. Path. Bact. 70 503.

ALPER, T. & GILLIES, N. E. 1960 The relationship between growth and survival after irradiation of Escherichia coli strain B and two resistant mutants. J. gen. Microbiol. 22 113.

ANDERSON, K. 1956 A survey of toxicity in staphylococci. J. clin. Path. 9 257.

BANFFER, J. R. J. 1962 Antileucocidin and mastitis puerperalis. Brit. med. J. 2 1224.

BARBER, M. 1955 Pigment production by staphylococci. J. gen. Microbiol. 13 338.

BARBER, M., DUTTON, A. A. C., BEARD, M. A., ELMES, P. C. & WILLIAMS, R. 1960 Reversal of antibiotic resistance in hospital staphylococcal infections. Brit. med. J. 1 11.

BARBER, M. & KUPER, S. W. A. 1951 Identification of Staphylococcus pyogenes by the phosphatase reaction. J. Path. Bact. 63 65.

BARBER, M. & WHITEHEAD, J. E. M. 1949 Bacteriophage types in penicillin resistant staphylococcal infections. Brit. med. J. 2 565.

BARBER, M. & WILDY, P. 1958 A study of the antigenic specificity of staphylococcal coagulase in relation to bacteriophage group. J. gen. Microbiol. 18 92.

BARBOUR, R. G. H. & EDWARDS, A. 1953 Mutation of different bacteriophage types of staphylococci to streptomycin resistance. Austral. J. exper. Biol. med. Sci. 31 561.

BASS, J. A. & HIGGINBOTHAM, R. D. 1960 Virulence of staphylococci in mice: A comparison of three methods of challenge. Tex. Rep. Biol. Med. 18 364.

BEINING, P. R. & KENNEDY, E. R. 1963 Characteristics of a strain of Staphylococcus aureus grown in vivo and in vitro. J. Bact. 85 732.

BLAIR, J. E. 1948 (in) Bacterial and mycotic infections of man (Ed. R. J. Dubos) p.331. Lippincott.

BLAIR, J. E. 1958 Factors determining the pathogenicity of staphylococci. Ann. Rev. Microbiol. 12 491.

BLOBEL, H. &. BERMAN, D. T. 1961 Further studies on the in vivo activity of staphylocoagulase. J. infect. Dis. 108 63.

BOAKE, W. C. 1956 Antistaphylocoagulase in experimental staphylococcal infections. J. immunol. 76 89.

BOYER, F. & LAMENSANS, A. 1961 Action du jeûne et de la cortisone sur la sensibilité de la souris à l'infection staphylococcique. Ann. Inst. Pasteur. 100 814.

BRODIE, J., GUTHRIE, W. & SOMMERVILLE, T. 1958 An attenuated variant of Staphylococcus aureus with specific immunizing properties. Brit. J. exp. Path. 39 199.

BROWN, A. E. & FARUQUE, A. A. 1963 The clumping of Staphylococcus pyogenes by serum and fibrinogen free plasmas. J. clin. Path. 16 457.

BROWN, A. E. & HARRIS, H. R. 1963 The effect of carbohydrates on the production of staphylococcal pigment. J. clin. Path. 16 261.

BROWN, J. 1960 Some properties of staphylococci related to pathogenicity. J. Path. Bact. 79 257.

BROWN, R. W. & SCHERER, R. K. 1958 A study of the necrotising action of staphylococcal alpha toxin. Am. J. Vet. Res. 19 354.

BRYCE, L. M. & ROUNTREE, P. M. 1936 The production of beta toxin by staphylococci. J. Path. Bact. 43 173.

BURKE, J. F. & MILES, A. A. 1958 The sequence of vascular events in early infective inflammation. J. Path. Bact. 76 1.

BURNET, F. M. 1929 The exotoxins of Staphylococcus pyogenes aureus. J. Path. Bact. 32 717.

BURNS, J. & HOLTMAN, D. F. 1959 Correlation between desoxyribonuclease activity and other properties of staphylococci. Bact. Proc. p.61.

CANNON, F. D. & Hawn, V. Z. 1963 Phosphatase activity of Staphylococcus aureus. Correlation of enzyme production with resistance to penicillin and phage pattern. J. Bact. 86 1052

CHAPMAN, G. H. 1943 Determination of the chromogenic property of staphylococci. J. Bact. 45 405.

CHAPMAN, G. H. & STILES, M. H. 1940 Carbohydrate fermentation reactions of staphylococci. Amer. J. clin. Path. 10 380.

CHLOUVERAKIS, K., GRIFFITHS, L. L. & SWIFT, P. N. 1963 The relation of the staphylococcal alpha antihaemolysin titre to infection in the newborn. Arch. Dis. Childh. 38 500.

CHRISTIE, R. & NORTH, E. A. 1941 Observations on staphylococcal beta toxin and its production by strains isolated from staphylococcal lesions in man. Austral. J. exp. Biol. med. Sci. 19 323.

CHRISTIE, R., NORTH, E. A. & PARKIN, B. J. 1946 Criteria of pathogenicity in staphylococci. Austral. J. exp. Biol. med. Sci. 24 73.

CLARKE, S. K. R. 1957 Sepsis in surgical wounds with particular reference to Staphylococcus aureus. Brit. J. Surg. 44 592.

CLAUSEN, J. & ROSENKAST, P. 1962 Acid mucopolysaccharides in Micrococcus (Staphylococcus aureus). Acta. Path. Microbiol. Scand. 56 188.

COHN, Z. A. 1962 Determinants of infection in the peritoneal cavity. (1) Response to and fate of Staphylococcus aureus and albus. Yale J. Biol. Med. 35 12.

COHN, Z. A. & MORSE, S. I. 1959 Interaction between rabbit polymorphonuclear leucocytes and staphylococci. J. exp. Med. 110 419.

COLBECK, J. C. 1960 The hospital environment - its place in the hospital staphylococcus infections problem. Amer. J. publ. Hlth. 50 468.

COMMITTEE ON FORMALDEHYDE DISINFECTION 1958 Disinfection of fabrics with gaseous formaldehyde. J. Hyg. Camb. 56 488.

CONTI, C. R., CLUFF, L. E. & SCHEDER, E. P. 1961 Studies on the pathogenisis of staphylococcal infection. (4) The effect of bacterial endotoxin. J. exp. Med. 113 845.

COOPER, G. N. & STUART, A. E. 1962 Susceptibility of mice to pneumococcal infection after modification of the reticulo-endothelial-system with simple lipids. J. Path. Bact. 83 227.

COUTINHO, C. B. & NUTINI, L. G. 1963 Correlation between the essential amino-acid requirements of Staphylococcus aureus, their phage types and antibiotic patterns. Nature 198 812.

COWAN, S. T. 1962 An introduction to chaos, or the classification of micrococci and staphylococci. J. appl. Bact. 25 324.

DAVIES, R. R. & NOBLE, W. C. 1962 Dispersal of bacteria on desquamated skin. Lancet 2 1295.

DAVIES, R. R. & NOBLE, W. C. 1963 Dispersal of staphylococci on desquamated skin. Lancet 1 1111.

DERBYSHIRE, J. B. 1963 Immunization against staphylococcal infections. Recent Progress in Microbiology 8 583. University of Toronto Press.

DINEEN, P. 1961 A period of unusual microbial susceptibility in an experimental staphylococcal infection. J. inf. Dis. 108 174.

DINEEN, P. 1962 Further studies on the period of microbial susceptibility. J. inf. Dis. 111 169.

DISALVO, J. W. 1958 Desoxyribonuclease and coagulase activity of micrococci. Med. Techn. Bull. Suppl. U.S. Armed Forces Med. J. 9 191.

DUBOS, R. J. & SCHAEDLER, R. W. 1958 Effect of dietary proteins and amino-acids on the susceptibility of mice to bacterial infections. J. exp. Med. 108 69.

DUBOS, R. J. & SCHAEDLER, R. W. 1959 Effect of nutrition on the resistance of mice to endotoxin and on the bactericidal power of their tissues. J. exp. Med. 110 935.

DUBOS, R. J. & SCHAEDLER, R. W. 1960 The effect of the intestinal flora on the growth rate of mice and on their susceptibility to experimental infections. J. exp. Med. 111 407.

DUTHIE, E. S. 1954 Evidence for two forms of staphylococcal coagulase. J. gen. Microbiol. 10 427.

DUTTON, A. A. C. 1955 The influence of the route of injection in lethal infections in mice. Brit. J. exp. Path. 36 128.

EKSTEDT, R. D. 1956a Further studies on the antibacterial activity of human serum on Micrococcus pyogenes and its inhibition by coagulase. J. Bact. 72 157.

EKSTEDT, R. D. 1956b The effect of coagulase on the antibacterial activity of normal human serum against selected strains of Micrococcus pyogenes. Ann. N. Y. Acad. Sci. 65 119.

EKSTEDT, R. D. 1963 Studies on immunity to staphylococcal infection in mice. (1) The effect of dosage, viability, and the interval between immunization and challenge on resistance to infection following injection of whole cell vaccines. J. inf. Dis. 112 143.

EKSTEDT, R. D. & NUNGESTER, W. J. 1955 Coagulase in reversing antibacterial activity of normal human serum on Micrococcus pyogenes. Prod. Soc. Exper. Biol. Med. 89 90.

EKSTEDT, R. D. & YOTIS, W. W. 1960 Studies on Staphylococci. (2) Effect of coagulase on the virulence of coagulase negative strains. J. Bact. 80 496.

EKBERG, S. S. 1956 Factors affecting resistance to infection. Ann. Rev. Microbiol. 10 1.

ELEK, S. D. 1959 Staphylococcus pyogenes and its relation to disease. Livingstone, Edinburgh & London.

ELEK, S. D. & CONEN, P. E. 1957 The virulence of Staphylococcus pyogenes for man. A study of the problem of wound infection. Brit. J. exp. Path. 38 573.

ELEK, S. D. & LEVY, E. 1950 Diffusible antigens in Staphylococcal cultures. Brit. J. exp. Path. 31 358.

EVANS, D. G., MILES, A. A. & NIVEN, J. S. F. 1948 The enhancement of bacterial infections by adrenaline. Brit. J. exp. Path. 29 20.

FABER, V. & ROSENDAL, K. 1960 Correlation between phage-type and hyaluronidase production of Staphylococcus aureus. Acta. Path. Microbiol. Scand. 48 367.

FISHER, A. M. & GOSHI, K. 1963 Further studies on the pathogenicity of staphylococci. Bull. Johns. Hopkins. Hosp. 112 143.

FISHER, A. M. & THOMPSON, B. W. 1956 Some problems relating to staphylococcal infections. A comparison of mouse virulence with other properties. Bull. Johns. Hopkins. Hosp. 99 341.

FISHER, M. W. 1959 Measurement of a staphylococcal antibody in human serum by a mouse protection test. Nature 183 1692.

FISHER, M. W., DEVLIN, H. B. & ERLANDSON, A. L. 1963 A new staphylococcal antigen. Nature 199 1074.

FISHER, R. A. 1948 Statistical methods for research workers. Oliver and Boyd Edinburgh.

FISHER, S. 1957 Loss of immunizing power of staphylococcal toxin during routine toxoiding with formalin. Nature 180 1479.

FISHER, S. & ROBSON, J. E. 1963 Experimental staphylococcal infection of the subcutaneous tissue of the mouse. (1) Bacterial population changes in relation to tissue response. J. inf. Dis. 113 204.

FISK, A. 1940 The technique of the coagulase test for staphylococci. Brit. J. exp. Path. 21 311.

FLETCHER, S. 1962 The growth of staphylococci in human serum. J. Path. Bact. 84 327.

FORSSMAN, J. 1938. Studies in staphylococci. (2) What role do staphylococcus lysin against rabbit's blood corpuscles and its antilysin play in staphylococcus infections in human beings. Acta. Path. Microbiol. Scand. 15 184.

FOSTER, W. D. 1962a The role of coagulase in the pathogenicity of Staphylococcus aureus. J. Path. Bact. 83 287.

FOSTER, W. D. 1962b The comparative pathogenicity of strains of Staphylococcus aureus with particular reference to coagulase production. J. Path. Bact. 84 253.

FOSTER, W. D. 1963 The role of alphahaemolysin in the pathogenicity of Staphylococcus aureus. J. Path. Bact. 86 535.

FOSTER, W. D. & H UTT, M. S. R. 1960 Experimental staphylococcal infection in man. Lancet 2 1373.

FRAPPIER, A. & SONEA, S. 1953 Staphylococcie expérimentale de l'embryon de poulet, de gravité constante et proportionelle aux doses de bactéries. Rev. cand. Biol. 12 86.

FRAPPIER, A. & SONEA, S. 1954 L'infection staphylococcique de l'embryon de poulet inoculé par voie sous-cutanée. Un. med. Can. 83 382.

FUSILLO, M. H. & WEISS, D. L. 1963 Effect of human serum on fatality of staphylococcal infections in mice. J. Bact. 86 212.

GAUSE. G. F., KOCHETKOVA, G. V. & VLADIMIROVA, G. B. 1961 Biochemical changes accompanying impaired respiration in staphylococci. Nature 190 978.

GELLENBECK, S. M. 1962 Aerobic respiratory metabolism of Staphylococcus aureus from an infected animal. J. Bact. 83 450.

GILBERT, I. 1931 Dissociation in an encapsulated staphylococcus. J. Bact. 21 157.

GILLESPIE, W. A. & ALDER, V. G. 1952 Production of opacity in egg-yolk media by coagulase positive staphylococci. J. Path. Bact. 64 187.

GILLISSEN, G. & RUDA, M. 1958 Die Korrleation zwischen Phosphatasen Aktivität und Penicillin-resistenz bei M. pyogenes var aureus. Zent. f. Bakt. 1 Abt. Orig. 171 281.

GLADSTONE, G. P. & GLENCROSS, E. J. G. 1960 Growth and toxin production of staphylococci in cellophane sacs in vivo. Brit. J. exp. Path. 41 313.

GLADSTONE, G. P., MUDD, S., HOCHSTEIN, H.D. & LENHART, N. A. 1962 The assay of antistaphylococcal leucocidal components (F & S) in human serum. Brit. J. exp. Path. 43 295.

GLADSTONE, G. P. & van HEYNINGEN, W. E. 1957 Staphylococcal leucocidin. Brit. J. exp. Path. 38 123.

GORRILL, R. H. 1951 Experimental staphylococcal infections in mice. Brit. J. exp. Path. 32 151.

GORRILL, R. H. & McNEILL, E. M. 1963 Staphylococcal infection in the mouse. (1) The effect of route of injection. Brit. J. exp. Path. 44 404.

GOSHI, K., CLUFF, L. E. & JOHNSON, J. E. 1961 Studies on the pathogenesis of staphylococcal infection. (3) The effect of tissue necrosis and antitoxic immunity. J. exp. Med. 113 259.

GOSHI, K., CLUFF, L. E., JOHNSON, J. E. & CONTI, R. C. 1961 Studies on the pathogenesis of staphylococcal infection. (2) The effect of non-specific inflammation. J. exp. Med. 113 249.

GRAY, J. E., WILKINS, J. R., PRESTRUD, M. C. & NIKITAS, C. T. 1957 Further characterisation of an experimental staphylococcal infection in mice. J. infect. Dis. 101 137.

GREEN, S. M. 1962 Mercury sensitivity of staphylococci. J. clin. Path. 15 249.

GREENBERG, L. & COOPER, M. Y. 1960 Polyvalent somatic antigen for the prevention of staphylococcal infection. Canad. Med. Ass. J. 83 143.

GREENBERG, L., COOPER, M. Y. & HEALY, G. M. 1961 Staphylococcus polyvalent somatic antigen vaccine. (2) An improved method of preparation. Canad. Med. Ass. J. 84 945.

GROGAN, J. B. & ARTZ, C. P. 1961 The correlation between mouse virulence and the phage-type, antibiotic sensitivity or source of Staphylococcus aureus. J. Lab. Clin. Med. 58 268.

HALBERG, F. 1963 Circadian (about 24 hour) rhythms in experimental medicine. Proc. Roy. Soc. Med. 56 253.

HALE, J. H. & SMITH, W. 1945 The influence of coagulase on the phagocytosis of staphylococci. Brit. J. exp. Path. 26 209.

HARE, R. & RIDLEY, M. 1958 Further studies on the transmission of Staphylococcus aureus. Brit. med. J. 1 69.

HARRISON, K. J. 1964 The protection of rabbits against infection with staphylococci by immunization with staphylocoagulase toxin or toxoid. J. Path. Bact. 87 145.

HENDERSON, A. & BRODIE, J. 1963 Investigations on staphylococcal coagulase. Brit. J. exp. Path. 44 524.

HERTZ, C. S. & WAITE, B. F. 1961 Observations on the ammonium-molybdate test for virulence of staphylococci. Amer. J. clin. Path. 35 472.

HINTON, N. A. & ORR, J. H. 1957 The distribution of toxins in coagulase positive staphylococci isolated from infections and carriers. J. Lab. Clin. Med. 50 901.

HINTON, N. A., MALTMAN, J. R. & ORR, J. H. 1960 The effect of dessication on the ability of Staphylococcus pyogenes to produce disease in mice. Amer. J. Hyg. 72 343.

HOEPRICH, P. D., CROFT, G. F. & WEST, L. M. 1960 Tellurite reduction as an indicator of potentially pathogenic staphylococci. J. Lab. Clin. Med. 55 120.

HOFFMAN, H. 1957 Oral microbiology. Ann. Rev. Microbiol. 11 183.

HOWARD, J. G. 1954 Diffusible antigens in relation to mouse virulence of Staphylococcus aureus. J. Path. Bact. 68 177.

HOYT, A., MOORE, F. J., KNOWLES, R. G., & SMITH, C. R. 1957 Sex differences of normal and immunized mice in resistance to experimental tuberculosis. Amer. Rev. Tubercl. 75 618.

ILLES, J. 1964 Die beste geeigneten Plasmen für die Staphylokoagulasereaktion. Path. Microbiol. 27 117.

JACKSON, J. R., GIBBONS, R. J. & MAGNER, D. 1958 The effect of staphylococcal toxin on the lungs of rabbits. Amer. J. Path. 34 1051.

JACOBS, S. I., WILLIS, A. T. & GOODBURN, G. M. 1963 Significance of desoxyribonuclease production by staphylococci. Nature 200 709.

JACOBS, S. E., WILLIS, A. T., LUDLAM, G. B. & GOODBURN, G. M. 1963 Studies on newly recognised strains of Staphylococcus aureus associated with hospital infection. Lancet 1 972.

JAMES, R. C. & MACLEOD, C. J. 1961 Induction of staphylococcal infections in mice with small inocula introduced on sutures. Brit. J. exp. Path. 42 266.

JESSEN, O., FABER, V., ROSENDAL, K. & ERIKSEN, K. R. 1959a Some properties of Staphylococcus aureus possibly related to pathogenicity. I. Acta. Path. Microbiol. Scand. 47 316.

JESSEN, O., FABER, V., ROSENDAL, K. & ERIKSEN, K. R. 1959 b Some properties of Staphylococcus aureus possibly related to pathogenicity. II. Acta. Path. Microbiol. Scand. 47 327.

JESSEN, O., ROSENDAL, K., FABER, V., HOVE, K & ERIKSEN, K. R. 1963 Some properties of Staphylococcus aureus possibly related to pathogenicity. III. Acta. Path. Microbiol. Scand. 58 85.

JOHANOVSKY, J. 1958 The significance of delta lysin for pathogenicity of staphylococci. Folia Biologica 4 21.

JOHNSON, J. E., CLUFF, L. E. & GOSHI, K. 1961 Studies on the pathogenesis of staphylococcal infection. (1) The effect of repeated skin infections. J. exp. Med. 113 235.

JONES, J. H. & CAMPBELL, P. J. 1962a The effect of artificial reduction of deep body temperature (hypothermia) on the course of an acute infection in the rat. J. Path. Bact. 84 428.

JONES, J. H. & CAMPBELL, P. J. 1962b Penicillin therapy of experimental staphylococcal septicaemia in mice exposed to cold. J. Path. Bact. 84 433.

JONES, M. 1946 The production of staphylococcus toxin in the chorio-allantoic fluid of the embryonated egg. J. Bact. 51 789.

KAPRAL, F. A. & LI, I. W. 1960 Virulence and coagulases of Staphylococcus aureus. I. Proc. Soc. Exp. Biol. Med. 104 151.

KAPRAL, F. A. & SHAYEGANI, M. G. 1959 Intracellular survival of staphylococci. J. exp. Med. 110 123.

KARAS, E. M. & KAPRAL, F. A. 1962 Virulence and coagulases of Staphylococcus aureus. III. J. Inf. Dis. 111 209.

KASS, E. H. & FINLAND, M. 1953 Adrenocortical hormones in infection and immunity. Ann. Rev. Microbiol. 7 361.

KEDZIA, W. 1962a Respiration endogene de Staphylococcus pyogenes et son rapport avec l'origine des sourches. Ann. Inst. Pasteur 103 939.

KEDZIA, W. 1962b Acetoin production by coagulase positive Staphylococcus aureus isolated from patients and healthy carriers. J. Path. Bact. 84 243.

KIENITZ, M. & PREUNER, R. 1958 Über den Nachweiss des Staphylokokken Enterotoxins (2) Untersuchungen zur Wirkung enterotoxinbildener Staphylokokken auf Hühnerembryonen. Zent. f. Bakt. 1 Abt. Orig. 173 203.

KIMLER, A. 1961 Fluorescein amine media for rapid differentiation of staphylococci. J. Bact. 82 106.

KNIZHNIKOV, V. A. & KASATKINA, I. L. 1957 Criteria of the pathogenicity and enterotoxicity of staphylococci. J. Microbiol. Epidemiol. Immunobiol. 28 79.

KNOTHE, H. 1952 Das verhalten von Staphylokokken, Meningokokken, Streptokokken, Pneumokokken, Bakterium tularense, Pasteurella pestis

und Brucella abortus Bang in bebrüteten Hühnerei. Zent. f. Bakt. 1 Abt. Orig. 158 453.

KOENIG, M. G. 1962 Factors relating to the virulence of staphylococci .I. Comparative studies on two colonial variants. Yale J. Biol. Med. 34 537.

KOENIG, M. G., MELLY, M. A. & ROGERS, D. E. 1962 Factors relating to the virulence of staphylococci. III. Antibacterial versus antitoxic immunity. J. exp. Med. 116 601.

KRYNSKI, S., KEDZIA, W., BECLA, E. & KRASOWSKI, A. 1962 Differences d'activite biochimique chez Staphylococcus pyogenes prelevé dans les produits pathologiques et chez les sujets sains. Ann. Inst. Pasteur 102 231.

LACK, C. H. 1956 Biological characteristics of staphylococci recovered from pathogenic material. Ann. N. Y. Acad. Sci. 65 103

LACK, C. H. & TOWERS, A. G. 1962 Serological tests for staphylococcal infections. Brit. med. J. 2 1227.

LACK, C. H. & WAILLING, D. G. 1954 A study of 435 strains of Staphylococcus pyogenes with reference to factors which may contribute to virulence. J. Path. Bact. 68 431.

LAM, G. T., SWEENEY, F. J., WITMER, C. M. & WISE, R. I. 1963a Abscess forming factor(s) produced by Staphylococcus aureus. (2) Abscess formation and immunity by a staphylococcus and its mutants. J. Bact. 86 87.

LAM, G. T., SWEENEY, F. J., WITMER, C. M. & WISE, R. I. 1963b Abscess forming factor(s) produced by Staphylococcus aureus (1) Collodian bag implantation technique. J. Bact. 86 611.

LAMBERT, H. P. 1960 Gamma globulin in experimental staphylococcal infections. J. Lab. Clin. Med. 56 701.

LAURELL, G. & TOTH-GYULAI, H. 1961 A study of the pathogenicity of Staphylococcus aureus isolated from dust and hospital environment. Acta. Path. Microbiol. Scand. 53 393.

LEVY, E. 1952 Diffusible products of staphylococci. Ph.D. Thesis, University of London.

LI, I. W. & KAPRAL, F. A. 1962 Virulence and coagulases of Staphylococcus aureus (2) Survival of certain coagulase negative mutants in the organs of intravenously infected rabbits. J. inf. Dis. 111 204.

LIDWELL, O. M., NOBLE, W. C. & DOLPHIN, G. W. 1959 The use of radiation to estimate the numbers of micro-organisms in air-borne particles. J. Hyg. Camb. 57 299.

LITHANDER, A. 1961 Antitoxic immunity as protection against staphylococcic infection. Acta. Path. Microbiol. Scand. 53 356.

LOMINSKI, I., SMITH, D. D., SCOTT, A. C., ARBUTHNOT, J. P., GRAY, S., MUIR, D., TURNER, G. H. & HEDGES, C. K. 1962 Immunization against experimental staphylococcal infections with coagulase rich preparations. Lancet 1 1315.

LYONS, C. 1937 Antibacterial immunity to Staphylococcus pyogenes. Brit. J. exp. Path. 18 411.

MACKANESS, G. B. 1960 The phagocytosis and inactivation of staphylococci by macrophages of normal rabbits. J. exp. Med. 112 35.

MACLEOD, C. M., HALL, C. A. & FROHMAN, L. A. 1963 Relationship of abscess formation in mice, guineapigs and rabbits to antistaphylococcal activity of their tissues and blood serum. Brit. J. exp. Path. 44 612.

MADOFF, M. A. & WEINSTEIN, L. 1962 Studies of staphylococcal coagulase reacting factor and anticoagulase in man. Amer. J. Hyg. 75 212.

MALTMAN, J. R., ORR, J. H. & HINTON, N. A. 1960 The effect of dessication on Staphylococcus pyogenes with special reference to implications concerning virulence. Amer. J. Hyg. 72 335.

MARKS, J. & VAUGHAN, A. C. T. 1950 Staphylococcal delta-haemolysin. J. Path. Bact. 62 597.

MAY, J. R. 1961 Experimental staphylococcal infections in man. Lancet 1 340.

McCABE, W. R. 1962 Lethality of pathogenic and non-pathogenic staphylococci in embryonated eggs. Proc. Soc. exp. Biol. Med. 110 452.

McDADE, J. J. & HALL, L. B. 1963 An experimental method to measure the influence of environmental factors on the viability and pathogenicity of Staphylococcus aureus. Amer. J. Hyg. 77 98.

McKENNA, G. F. & TAYLOR, A. 1953 Use of yolk-sac cultivated bacteria in bacterial studies. Texas Rep. biol. Med. 11 283.

MELLY, M. A., THOMISON, J. B. & ROGERS, D. E. 1960 Fate of staphylococci within human leucocytes. J. exp. Med. 112 1121.

MENKIN, V. 1929 Studies on inflammation (1) Fixation of vital dyes in inflamed tissue. J. exp. med. 50 171.

MENKIN, V. 1930a Studies on inflammation (3) Fixation of a metal in inflamed areas. J. exp. Med. 51 879.

MENKIN, V. 1930b Studies on inflammation (4) Fixation of foreign protein at the site of inflammation. J. exp. Med. 52 201.

MENKIN, V. 1931a Studies on inflammation (5) The mechanism of fixation by the inflammatory reaction. J. exp. Med. 53 171.

MENKIN, V. 1931b Studies on inflammation (6) Fixation of trypan blue in inflamed areas of frogs. J. exp. Med. 53 179.

MENKIN, V. 1931c Studies on inflammation (7) Fixation of bacteria and of particulate matter at the site of inflammation. J. exp. Med. 53 647.

MENKIN, V. 1932 Studies on inflammation (8) Inhibition of fixation by urea. A further study on the mechanism of fixation by the inflammatory reactions. J. exp. Med. 56 157.

MENKIN, V. 1933 Studies on inflammation (9) A factor in the mechanism of invasiveness by pyogenic bacteria. J. exp. Med. 57 977.

MENKIN, V. & MENKIN, M. F. 1930 Studies on inflammation (2) A measure of the permeability of capillaries in an inflamed area. J. exp. Med. 51 285.

MILES, A. A. 1955 The meaning of Pathogenicity. (in) Mechanisms of Microbial Pathogenicity. 5th Symposium of the Society for General Microbiology. pp.3, 4.

MILES, A. A. & MILES, E. M. 1943 The fixation of foreign material in inflamed tissue with especial reference to the action of Cl. welchii toxin and antitoxin. Brit. J. exp. Path. 24 95.

MILES, A. A. & MILES, E. M. 1958 The state of lymphatic capillaries in acute inflammatory lesions. J. Path. Bact. 76 21.

MILES, A. A., MILES, E. M. & BURKE, J. 1957 The value and duration of defence reactions of the skin to the primary lodgement of bacteria. Brit. J. exp. Path. 38 79.

MILES, J. A. R. 1951 Observations on the course of infection with certain natural bacterial pathogens of the rat in rats on protein deficient diets. Brit. J. exp. Path. 32 285.

MIRAGLIA, G. J. & BERRY, L. J. 1962 Enhancement of salmonellosis and emergence of secondary infection in mice exposed to cold. J. Bact. 84 1173.

MIRAGLIA, G. J. & BERRY, L. J. 1963 Possible source of the secondary invading staphylococci in mice exposed to acute cold. J. Bact. 85 345.

MOORE, B. 1960 A new screen test and selective medium for the rapid detection of epidemic strains of Staph. aureus. Lancet 2 453.

MORSE, S. I. 1960 Isolation of a phagocytosis inhibiting substance from culture filtrates of an encapsulated Staphylococcus aureus. Nature 186 102.

MORSE, S. I. 1962 Isolation and properties of a surface antigen of Staphylococcus aureus. J. exp. Med. 115 295.

MORSE, S. I. 1963 Distribution of a surface antigen of Staphylococcus aureus. Nature 200 1126.

MYERS, D. M. 1959a Observations on a chemical test related to the virulence of Staphylococcus aureus. Amer. J. clin. Path. 31 128.

MYERS, D. M. 1959b An antibiotic effect of viridans streptococci from the nose, throat and sputum, and its inhibitory effect on Staphylococcus aureus. Amer. J. clin. Path. 31 332.

NOBLE, W. C. 1961 The survival of bacteria under hospital and domestic conditions. M.Sc. Thesis. University of London.

NOBLE, W. C. 1962 The dispersal of staphylococci in hospital wards. J. clin. Path. 15 552.

NOBLE, W. C., WILLIAMS, R. E. O., JEVONS, M. P. & SHOOTER, R. A. 1964 Some aspects of nasal carriage of staphylococci. J. clin. Path. 17 79.

NORDBRINGE, F. 1963 Strains of Staphylococcus aureus with increased tolerance to gentian violet. Acta. Path. Microbiol. Scand. 58 114.

PANTON, P. N. & VALENTINE, F. C. O. 1929 Staphylococcal infection and re-infection. Brit. J. exp. Path. 10 257.

PARKER, M. T. 1958 Some cultural characteristics of Staphylococcus aureus strains from superficial skin lesions. J. Hyg. Camb. 56 238

PARKER, M. T. & JEVONS, M. P. 1963 Hospital strains of staphylococci. C.I.O.M.S. Symposium. Infection in Hospitals. p.55 Blackwell.

PENN, G. B. & ASHFORD, A. 1963 The inflammatory response to implantation of cotton pellets in the rat. J. Pharmacy. Pharmacol. 15 798.

PREVITE, J. J. & BERRY, L. J. 1962 The effect of environmental temperature on the host-parasite relationship in mice. J. infect. Dis. 110 201.

PRICE, K. M. & KNEELAND, Y. 1954 A mucoid form of Micrococcus pyogenes var aureus which shows capsular swelling with specific immune serum. J. Bact. 67 472.

PRICE, K. M. & KNEELAND, Y. 1956 Further studies on the phenomenon of capsular swelling of Micrococcus pyogenes var aureus in the presence of immune serum. J. Bact. 71 229.

PUBLIC HEALTH LABORATORY SERVICE 1960 Incidence of surgical wound infection in England and Wales. Lancet 2 659.

RAMSEY, H. H. 1962 Endogenous respiration of Staphylococcus aureus. J. Bact. 83 507.

REBUCK, J. W. & YATES, J. L. 1954 The cytology of the tuberculin reaction in skin windows in man. Amer. Rev. Tubercle. 69 216.

RIGDON, R. H., JOYNER, A. L. & RICKETTS, E. T. 1934 A study of the action of filterable staphylococcal toxin on the kidneys of normal rabbits. Amer. J. Path. 10 425.

ROBERTSON, H. R. 1958 Wound infection. Ann. Roy. Coll. Surg. Engl. 23 141.

ROGERS, D. E. 1963 The phagocytosis of staphylococci. (in) Recent Progress in Microbiology. 8. p.575 University of Toronto Press.

ROGERS, D. E. & MELLY, M. A. 1960 Further observations on the behaviour of staphylococci within human leucocytes. J. exp. Med. 111 533.

ROGERS, D. E. & THOMPSETT, R. 1952 The survival of staphylococci within human leucocytes. J. exp. Med. 95 209.

ROSENDAL, K. 1963 Correlation of virulence of staphylococci from patients with bacteraemia with various laboratory indices. (in) Recent Progress in Microbiology. 8 570. University of Toronto Press.

ROUNTREE, P. M. 1947 Some observations on fibrinolysin and beta toxin of staphylococci. Austral. J. exp. Biol. med. Sci. 25 359.

ROUNTREE, P. M. 1963a Hospital strains of Staphylococcus aureus. C.I.O.M.S. Symposium. Infection in Hospitals. p.67 Blackwell.

ROUNTREE, P. M. 1963b The effect of desiccation on the viability of Staphylococcus aureus. J. Hyg. Camb. 61 265.

ROUNTREE, P. M. 1963c The origin and spread of virulent staphylococci. (in) Recent Progress in Microbiology. 8 561. University of Toronto Press.

ROUNTREE, P. M. & ASHESHOV, E. H. 1961 Further observations on changes in the phage-typing pattern of phage-type 80/81 staphylococci. J. gen. Microbiol. 26. 111.

ROUNTREE, P. M. & FREEMAN, B. M. 1955 Infections caused by a particular phage-type of Staphylococcus aureus. Med. J. Austral. 2 157.

SALL, T., MUDD, S. & TAUBLER, J. 1961 Concerning the surface of cells of Staphylococcus pyogenes. (1) A pseudocapsulation phenomenon under certain experimental conditions. J. exp. Med. 113 693.

SANDERS, E. 1963 Inhibition of coagulase reaction of pathogenic staphylococci by heparin in vitro. J. Bact. 86 1350.

SCHAEDLER, R. W. & DUBOS, R. J. 1959 Effect of dietary proteins and amino-acids on the susceptibility of mice to bacterial infections. J. exp. Med. 110 921.

SCHUBERT, J. & JOHANOVSKY, J. 1957 The possibility of immunological prevention of staphylococcal infection in mothers and their infants. Casopsis. Lekaru. Ceskych. 96 4 (Reviewed in Bull. Hyg. 32 403 1957.)

SCHWABACHER, H., CUNLIFFE, A. C., WILLIAMS, R. E. O. & HARPER, G. J. 1945 Hyaluronidase production by staphylococci. Brit. J. exp. Path. 26 124.

SEBEK, V., SCHUBERT, J. & JOHANOVSKY, J. 1958 Possibilities of immunological prevention of Staphylococcus infection of mothers and newborns. Casopsis. Lekaru. Ceskych. 97 583. (Reviewed in Bull. Hyg. 34 13 1959.)

SELBIE, F. R. & SIMON, R. D. 1952 Virulence to mice of Staphylococcus pyogenes: its measurement and its relation to certain in vitro properties. Brit. J. exp. Path. 33 315.

SELLERS, T. F. & LeMAISTRE, C. A. 1960 Experimental staphylococcal infection in mice: Observations on the effects of inoculum size, strain variation, penicillin and cortizone. J. Lab. Clin. Med. 55 199.

SERAG, M. G. & GREEN, M. N. 1944 The role of carbohydrates in the development of pigment by Staphylococcus aureus. J. Bact. 48 496.

SHAH, D. B., RUSSEL, K. E. & WILSON, J. B. 1963 Comparison of two media for the detection of the egg-yolk factor of Staphylococcus aureus. J. Bact. 85 1181.

SHAW, C., STITT, J. M. & COWAN, S. T. 1951 Staphylococci and their classification. J. gen. Microbiol. 5 1010.

SHILO, M. 1959 Non-specific resistance to infection. Ann. Rev. Microbiol. 13 255.

SHILO, M. 1962 Resistance increasing activity of bacterial lipopolysaccharides and their subfractions towards levan-enhanced infections. Brit. J. exp. Path. 43 153.

SHOOTER, R. A., SMITH, M. A., GRIFFITHS, J. D., BROWN, M. E. A., WILLIAMS, R. E. O., RIPPON, J. E. & JEVONS, M. P. 1958 Spread of staphylococci in a surgical ward. Brit. med. J. 1 607

SIEGEL, S. 1956 Non-parametric statistics. McGraw-Hill.

SIMON, H. J. 1963 Effect of tetracycline on a standardised intracutaneous staphylococcal infection in guinea-pigs. Proc. Soc. exp. Biol. Med. 113 518.

SMITH, D. D. 1962 Experimental staphylococcal infection in mice. J. Path. Bact. 84 359.

SMITH, D. D. 1963 Mouse virulence and coagulase production in Staphylococcus aureus. J. Path. Bact. 86 231.

SMITH, D. D. & JOHNSTONE, J. M. 1958 Staphylocoagulase activity in vivo. Brit. J. exp. Path. 39 165.

SMITH, E. W., GOSHI, K., NORMAN, P. S. & CLUFF, L. E. 1963 Studies on the pathogenesis of staphylococcal infections. (8) The human cutaneous reaction to injection of alphahaemolysin. Bull. Johns. Hopkins. Hosp. 113 247.

SMITH, I. M., WILSON, A. P., HAZARD, E. C., HUMMER, W. K. & DEWEY, M. F. 1960 Death from staphylococci in mice. J. infect. Dis. 107 369.

SMITH, J. M. & DUBOS, R. J. 1956a The behaviour of virulent and avirulent staphylococci in the tissues of normal mice. J. exp. Med. 103 87.

SMITH, J. M. & DUBOS, R. J. 1956b The effect of nutritional disturbances on the susceptibility of mice to staphylococcal infection. J. exp. Med. 103 109.

SMITH, M. L. 1937 Circulating antitoxin and resistance to experimental infection with staphylococci. J. Path. Bact. 45 305.

SMITH, P. B. 1962 Growth of staphylococci on mercuric chloride agar. J. Bact. 84 1016.

SMITH, W. & HALE, J. H. 1944 The nature and mode of action of staphylococcus coagulase. Brit. J. exp. Path. 25 101.

SMITH, W., HALE, J. H. & SMITH, M. M. 1947 The role of coagulase in staphylococcal infections. Brit. J. exp. Path. 28 57.

SOMPOLINSKY, D. 1962 Chromogenesis by Staphylococcus aureus and typing according to pigmentation. J. Lab. Clin. Med. 60 439.

SORU, E., NESTORESCU, N., POPOVICI, M. & IONESCO, M. 1957 The arginase test, a new method for differentiating pathogenic staphylococci. Studii si Cercetari. Inframicrobiol. Microbiol. si Parazitol. 8 395. (Reviewed in Bull. Hyg. 33 987 1958.)

LORD STAMP 1961 Antibacterial immunity to Staphylococcus pyogenes in rabbits. Brit. J. exp. Path. 42 30.

STUART, A. E., BIOZZI, G., STIFFEL, C., HALPERN, B. N. & MOUTON, D. 1960 The stimulation and depression of reticulo-endothelial phagocytic function by simple lipids. Brit. J. exp. Path. 41 599

STUART, A. E. & COOPER, G. N. 1962 Susceptibility of mice to bacterial endotoxin after modifications of reticulo-endothelial function by simple lipids. J. Path. Bact. 83 245.

THAL, A. P. 1955 Selective renal vasospasm and ischemic renal necrosis produced experimentally with staphylococcal toxin. Observations on the pathogenesis of bilateral cortical necrosis. Amer. J. Path. 31 233.

THAL, A. P. & EGNER, W. 1956 The mechanism of shock produced by means of staphylococcal toxins. Amer. med. Ass. Arch. Path. 61 488.

THAL, A. P. & EGNER, W. 1961 The site of action of the staphylococcus alpha toxin. J. exp. Med. 113 67.

TAUBLER, J. H., KAPRAL, F. A. & MUDD, S. 1963 Role of alpha-toxin in lesion formation by Staphylococcus aureus on sutures subcutaneously implanted in mice. J. Bact. 86 51.

TAYLOR, G. W., SHOOTER, R. A., FRANDSEN, P. H., FIELDER, W. R., & KERTH, W. J. 1962 Staphylococcal wound infection. An experimental study in guinea-pigs. Brit. J. Surg. 49 569.

TOMLINSON, A. J. H. & PARKER, M. T. 1956 Production of opacity on serum agar plates by some strains of Staphylococcus aureus. Nature 177 895.

TURNER, G. C. & WILLIS, A. T. 1962 Staphylococcal invasion of a new surgical ward. J. Path. Bact. 84 349.

WARREN, G. H. & GRAY, J. V. 1963 Production of a polysaccharide by Staphylococcus aureus. (2) Effect of temperature and antibiotics. Proc. Soc. exp. Biol. Med. 114 439.

WASZ-HÖCKERT, O., KOSUNEN, T. & KOHONEN, J. 1958 Effect of ethyl alcohol on the susceptibility of mice to staphylococcal infection. I. Ann. Med. exp. Biol. Fenn. 36 360.

WASZ-HÖCKERT, O., KOSUNEN, T. & KOHONEN, J. 1959 Effect of ethyl alcohol on the susceptibility of mice to staphylococcal infection. II. Ann. Med. exp. Biol. Fenn. 37 121.

WHEATER, D. W. F. & HURST, E. W. 1961 The effect of sex on bacterial infections in mice and on the chemotherapy of one of them. J. Path. Bact. 82 117.

WILEY, B. B. 1961 A new virulence test for Staphylococcus aureus and its application to encapsulated strains. Canad. J. Microbiol. 7 933.

WILEY, B. B. & WONNACOTT, J. C. 1962 Isolation and partial characterisation of a capsular material from Staphylococcus aureus. J. Bact. 83 1169.

WILLIS, A. T. & TURNER, G. C. 1962 Staphylococcal lipolysis and pigmentation. J. Path. Bact. 84 337.

WILLIS, A. T., JACOBS, S. I. & GOODBURN, G. M. 1964 Pigment productio enzymatic activity and antibiotic sensitivity of staphylococci: subdividion of the pathogenic group. J. Path. Bact. 87 157.

WILLIAMS, R. E. O. 1959 Epidemic staphylococci. Lancet 1 190.

WILLIAMS, R. E. O., BLOWERS, R., GARROD, L. P. & SHOOTER, R. A. 1960 Hospital infection; causes and prevention, London, Lloyd-Luke.

WILLIAMS, R. E. O. & HARPER, G. J. 1946 Determination of coagulase and alphahaemolysin production by staphylococci. Brit. J. exp. Path. 27 72.

WILLIAMS, R. E. O. & HARPER, G. J. 1947 Staphylococcal haemolysins on sheep-blood agar with evidence for a fourth haemolysin. J. Path. Bact. 59 69.

WILLIAMS, R. E. O. & JEVONS, M. P. 1961 Lysotypen von Staphylococcus aureus verschiedener Herkunft. Zent. f. Bakt. 181 349.

WILLIAMS, R. E. O., NOBLE, W. C., JEVONS, M. P., LIDWELL, O. M., SHOOTER, R. A., WHITE, R. G., THOM, B. T. & TAYLOR, G. W. 1962 Isolation for the control of staphylococcal infection in surgical wards. Brit. med. J. 2 275.

WOODIN, A. M. 1961 Assay of the two components of staphylococcal leucocidin and their antibodies. J. Path. Bact. 81 63.

YOTIS, W. W. & EKSTEDT, R. D. 1959 Studies on staphylococci. (1) The effect of serum and coagulase on the metabolism of coagulase positive and coagulase negative strains. J. Bact. 78 5(

YOTIS, W. W. & EKSTEDT, R. D. 1960 Studies on staphylococci (3) Further studies on purification and mechanism of action of an antibacterial human serum factor. J. Bact. 80 719.

YOUNG, G. 1954 Experimental staphylococcal infections in the hamster cheek pouch. The process of localisation. J. exp. Med. 99 299.

No. 1.

Reprinted from the BRITISH MEDICAL JOURNAL
August 4, 1962, *vol. ii, pp.* 275–282

ISOLATION FOR THE CONTROL OF STAPHYLOCOCCAL INFECTION IN SURGICAL WARDS

BY

R. E. O. WILLIAMS,* M.D., M.R.C.P.

W. C. NOBLE,* M.Sc.

M. PATRICIA JEVONS, M.D.

O. M. LIDWELL, D.Phil.

Public Health Laboratory Service, Colindale, London

R. A. SHOOTER, M.D., M.R.C.P.

R. G. WHITE, M.B., B.S.

B. T. THOM, M.B., B.S.

G. W. TAYLOR, M.S., F.R.C.S.

St. Bartholomew's Hospital, London

In a study of the natural history of staphylococcal infection we have observed a total of 3,986 patients treated in one or other of three surgical wards at St. Bartholomew's Hospital during the past five years. It was soon apparent that one factor of great importance in the causation of sepsis was the difference that exists between the pathogenic activities of different strains of *Staphylococcus aureus* (Shooter *et al.*, 1958). We also found that those of our patients whose noses were colonized with staphylococci during their stay in the ward suffered a much higher sepsis rate than those who remained non-carriers (Williams *et al.*, 1959).

From the start of our investigations in two of the wards, A and B, we had, on empirical grounds, isolated patients infected with tetracycline-resistant staphylococci in side-wards whenever possible. The observation that relatively few strains of staphylococci had epidemic propensities supported the idea that isolation of the carriers of dangerous strains might reduce the incidence of sepsis, and in 1959 four isolation cubicles were constructed in one of the wards (B). This

*Now at the Wright-Fleming Institute of Microbiology, St. Mary's Hospital Medical School, London.

enabled the isolation policy to be pursued with greater ease and completeness.

We have now tried to assess the value of our isolation policy in preventing post-operative wound sepsis and in controlling the spread of staphylococci generally. Most of the analyses are based on the records from wards B and C, but results for ward A, which was studied only in the earlier part of our investigation, are included when possible. We also report here the results of a comparison of the use of cotton blankets in place of wool in one ward (B).

Materials and Methods

The Wards.—The general arrangement of the wards (A, B, and C) was described in our previous papers. All three were general male surgical wards, but there were especially large numbers of patients undergoing rectal surgery in ward A, vascular surgery in ward B, and urinary tract surgery in ward C. All three wards had a total of three beds in two side-wards, and in addition ward B had, from July, 1959, four beds in four single "cubicles" built into the ward. These cubicles were constructed of wood and glass and the walls reached from floor to ceiling. Each had a wash-basin, and an extract-fan was fitted in the window so that air from the cubicle did not return to the ward; the exhaust fans were run continuously, and the cubicle door was kept closed whenever the patient in the cubicle needed isolation. The side-wards did not have controlled ventilation, but were 25 feet (7.5 metres) from the main ward along a corridor.

Nursing Routine.—In wards A and B all patients who were infected with or were carriers of tetracycline-resistant staphylococci were nursed in isolation whenever there was a bed available in a side-ward or a cubicle. Since admission to isolation depended on the results of bacteriological examination, there was inevitably a delay between the time when a patient became infected and the time he was transferred to isolation. In ward C infected patients were not isolated. Strict non-touch dressing techniques were used in all the wards; gowns were worn when nursing patients in isolation in ward A but not in ward B. Cleaning equipment used in the isolation rooms was not used in the main ward. Woollen blankets, when soiled, were laundered after the discharge of a patient who was known to have an

infected lesion and, otherwise, at irregular intervals. The cotton blankets introduced into ward B were laundered after the discharge of each patient and, in addition, at monthly intervals for long-stay patients.

Bacteriological Routine.—Nasal swabs were taken from patients on the day of admission or as soon after as possible ; thereafter all the patients in the ward had their noses swabbed on one day each week. Bacteriological examination was made of material from all septic lesions, suspected chest infections, and so forth. During the latter four years swabs were also taken from healthy wounds at the time of the first post-operative dressing. All cultures were examined for staphylococci, and coagulase-positive strains were tested for antibiotic sensitivity and phage type. The air of the wards was examined for staphylococci with slit-samplers, the usual volume examined being 216 cubic feet (5.8 cubic metres) collected over a two-hour period on each of three days in the week. Bedding and curtains were sampled by the sweep-plate method as necessary. Nasal swabs from the ward staff were examined as a routine only during the first eight months of the study.

Definition of Sepsis.—Wounds were regarded as " septic " only if there was visible pus ; the sepsis was attributed to staphylococci if these bacteria were isolated from a septic wound. The term " other sepsis " is used to cover all other clinically recognizable infective complications ; most of the infections referred to were in the chest. Infections of the urinary tract have not been considered, because urine samples were not received from all patients.

Chemoprophylaxis.—During one six-month period neomycin nasal prophylaxis was used in ward B, a small amount of 1% neomycin or later " naseptin " cream being administered to all patients twice daily, and soap containing 2% hexachlorophene was used for washing. This period has been excluded from all analyses, except when mentioned specifically.

Calendar of Experiments.—Fig. 1 shows the periods during which the various investigations were carried out in the wards. For brevity we refer to A and B as the " isolation " wards and C as the " non-isolation " ward.

Effect of Introducing Cotton Blankets.—As will be seen from Fig. 1, ward B was supplied with cotton blankets in October, 1958. There was surprisingly little difference between the wool- and cotton-blanket periods

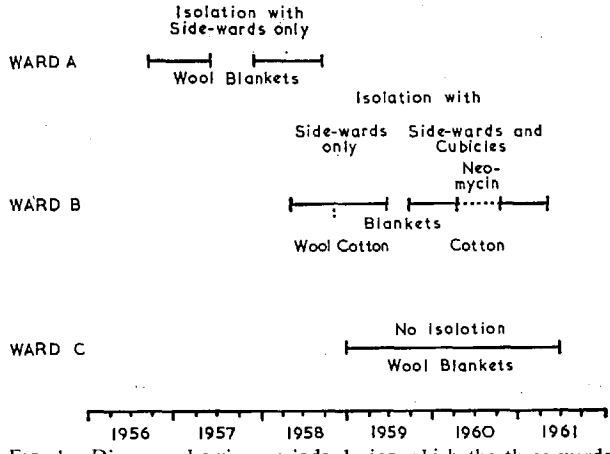


FIG. 1.—Diagram showing periods during which the three wards were under the various investigations.

in the frequency with which the blankets in use on the beds yielded staphylococci, in the numbers of staphylococci found in air samples, in the frequency with which patients acquired staphylococci in the nose, or in the incidence of staphylococcal sepsis (Table I). Nor do the figures for the cotton-blanket period in B show any notable difference from those derived from ward C, which used wool blankets throughout. In all the

TABLE I.—*Effect of Cotton Blankets*

	Ward B Cotton Blankets	Ward B Wool Blankets	Ward C Wool Blankets
No. weeks of survey	41	20	119
,, patients	386	181	1,604
No. of blankets	230	52	127
Percentage of blankets sampled at random yielding staphylococci	49	58	46
Airborne staphylococci (cols. per c.ft.) ..	0·15	0·12	0·18
Patients acquiring staphylococci in nose, percentage of all admissions	20·5	18·8	16·7
Staphylococcal post-operative sepsis, as percentage of all operations	3·5	3·9	4·5

subsequent analyses the results from the wool and cotton periods have been taken together.

Methods of Analysis

We have had to use a number of different methods to measure the effectiveness of our isolation policy in preventing the spread of infection.

(1) *Direct Comparison of Total Sepsis Rates in the Isolation and Non-isolation Wards.*—This is the simplest method, but it has the disadvantage that there were substantial differences in the operations commonly performed on the patients in the different wards, and the different operations had very different sepsis rates. This difficulty can be partially overcome either by confining the analysis to the group of operations performed in reasonable numbers in both units or by some method of statistical "standardization" to allow for the differing distribution of operations in the two wards.

(2) *Carrier Rates.*—The acquisition of hospital staphylococci in the nose is another measure of the extent to which cross-infection occurs with a ward.

(3) *Source of Staphylococci Causing Sepsis.*—We have tried to separate those cases of sepsis that appeared to result from cross-infection from those apparently due to self-infection and to compare the experience of the wards in this respect. This separation could be done simply by counting the number of cases due to "epidemic types" of staphylococci, or by trying to determine the most probable source of infection for each individual case of sepsis.

(4) *Spread from Recognized Carriers.*—A completely different approach was to measure the extent to which carriers of recognized types of staphylococci spread their strains to other patients in the ward and to compare spread from patients nursed in the open ward with that from patients nursed in isolation.

Method 4

The analysis was confined to types of staphylococci that seemed able to spread in the ward, shown by their isolation from two or more patients within a period of 14 days. Each staphylococcal type was then considered separately, and for each day of the period during which it was present in the ward we noted whether (*a*) all carriers and infected patients were being nursed in isolation, or (*b*) some were present in the open ward. We also recorded the number of patients who were found to have acquired the specific staphylococcus on each day of observation. The figures from all the different staphylococcus types and for the three wards were summated. We could thus express the number of acquisitions of staphylococci on days when all the known sources of the specific types were in isolation as a ratio to the total number of days on which there were such carriers in the isolation rooms and not in the open ward, and so compare the frequency of spread in the various circumstances.

A number of assumptions and conventions had to be adopted ; most are concerned with the interpolation needed because after admission the patients had nasal swabs taken at weekly intervals. The conventions are illustrated in Fig. 2.

Patients were assumed to be infected from the day midway between a negative and a positive culture provided the interval between the cultures was not more than seven days ; and from a positive culture to the day of discharge from the ward provided this was not more than seven days after the day of the culture (patients A and B in Fig. 2). Patients not swabbed on admission were also regarded as infected from the day

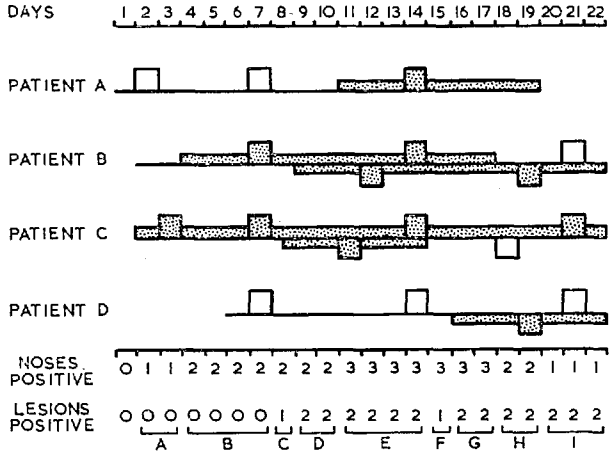


FIG. 2.—Diagram showing the conventions adopted for estimating cross-infection rates. Horizontal lines show the period for which the patient was in hospital. Square blocks represent cultural results: above line from the nose, below line from lesion. Stippled blocks represent cultures yielding *Staph. aureus*. Horizontal stippled bands show period during which patient was assumed to be infected.

midway between admission and the day of the first culture (B, nasal cultures). A few patients whose nasal swabs taken on admission yielded a staphylococcus currently epidemic in the ward were regarded as the victims of cross-infection unless the strain belonged to a type known to be common in the normal population. Half-days of carriage were counted as whole days.

A few patients had single cultures taken from their wounds ; if these yielded staphylococci the wound was regarded as infected for the seven-day period centred

on the day of the culture (patients C and D). No patient was regarded as suffering more than one " acquisition " with a particular type—that is, for this purpose the lesions in patients B and C of Fig. 2 were assumed to be infected from the patients' own noses.

The date taken for the acquisition of a staphylococcus, for correlation with the state of the ward at the time, is the day on which the strain is presumed to have been acquired—that is, day four in patient B of Fig. 2. There were sometimes a few days during the period of prevalence of a particular staphylococcus type when no carriers were known to be present in the ward. Patients becoming infected during such periods are entered separately in Tables VII and VIII. An epidemic was regarded as continuing over these periods when the staphylococcus was not shown to be present in the ward only if one or more of the patients known to have been infected during the earlier part of the epidemic was still present.

Incidence of Sepsis

Of the 2,555 patients included in the analysis, 1,677 had surgical operations ; 100 (6%) of the operation wounds showed post-operative sepsis, in 72 cases (4.3% of all wounds) due to *Staph. aureus*. An additional 20 wounds (1.2%) were found to be colonized with staphylococci but were not diseased.

Of 72 wounds with post-operative staphylococcal sepsis, only five showed signs of the sepsis within five days of the operation ; in 27 sepsis was first recognized between 6 and 10 days after operation, and in the remaining 40 it was not seen until 11 days after operation. Of the 72 wounds, 62 yielded staphylococci from their first post-operative swab and 10 are known to have been free of staphylococci at the first swabbing.

Sepsis in Relation to Nasal Carriage

Table II shows the relation of staphylococcal sepsis—wound infection and other septic lesions—to the patients' carrier state. It is based on patients in wards B and C, and includes the records of some of the patients analysed in our previous report (Williams *et al.*, 1959). Analysis of the available records from ward A shows close similarity. There was a striking difference between the frequency of staphylococcal wound sepsis in patients who were never nasal carriers (1.3%) and those who entered the ward as non-carriers and became carriers

while in the ward (11.9%) or who changed their type of staphylococcus while in the ward (12.4%). The rate for the permanent carriers was intermediate (3.4%). "Other" staphylococcal sepsis was also more frequent in the patients who acquired staphylococci in the ward.

There were 38 cases of staphylococcal wound sepsis in nasal carriers with staphylococci of the same type isolated from nose and lesion. Twenty of these patients were nasal carriers of the lesion type six or more days before the sepsis developed, and a further eight were found to be nasal carriers between one and five days before the appearance of sepsis. In only 4 of the 38 was the staphylococcus not isolated from the nose until more than five days after the appearance of sepsis.

Table II also shows, however, that the patients who acquired staphylococci in their noses in the ward had more non-staphylococcal sepsis than the non-carriers. We therefore sought other differences between those who acquired staphylococci in the ward and those who did not. The most striking difference was in length of stay in the ward (Table III): the mean stay for the patients who acquired nasal staphylococci while in the ward was 22.0 days compared with 11.6 days for those who remained non-carriers. The mean stay in hospital for patients with non-staphylococcal sepsis was also

TABLE II.—*Relation of Wound and Other Sepsis to Nasal Staphylococcal Carrier State (Wards B and C)*

Carrier State	No. of Patients	Percentage Developing			
		Staphylococcal Sepsis		Non-staphylococcal Sepsis	
		Wound	Other	Wound	Other
Patients having operations:					
Never carrier	758	1·3	0·3	0·9	1·3
Non-carrier on admission, acquired staphylococcus in ward	235	11·9	6·0	4·3	4·3
Carrier on admission, changed staphylococcus in ward	121	12·4	7·4	3·3	0·8
Carrier on admission, no change	563	3·4	2·7	1·2	1·8
Subtotal	1,677	4·3	2·4	1·7	1·8
Patients not having operations*	878	0·1†	0·8	0	0·2
Total	2,555	2·8	1·8	0·95	1·3

* 57% of these patients had only one nasal swab examined.
† Patient admitted with traumatic lesion.
Excluded from this table are 137 patients from whom no nasal swab was obtained and 484 patients who were in ward B during the period of the type 53/77 epidemic and subsequent neomycin prophylaxis.

TABLE III.—*Length of Stay in Hospital in Relation to Carrier State (Wards B and C)*

Carrier State	Percentage of Patients in Various Carrier Categories among those whose Total Stay in Hospital was:				Mean Stay (Days)
	1–10 Days	11–20 Days	21–30 Days	31 + Days	
Never carriers	56·0	46·0	36·5	22·5	11·6
Carrier on admission, no change of strain ..	38·5	33·0	20·5	18·5	11·6
Changed strain or acquired staphylococcus in hospital ..	5·5	21·0	43·0	59·0	22·0
Total patients	897	747	223	157	

This table is based on those patients who had no sepsis.

longer than for those who had no sepsis, and it seems likely that the prolonged stay in the ward of the patients with non-staphylococcal sepsis resulted in some of them becoming nasal carriers of ward staphylococci. It is interesting to note that only 32% of the 44 patients with non-staphylococcal sepsis acquired their ward staphylococcus before the onset of sepsis, compared with 57% of 84 of the patients with staphylococcal sepsis (d = 25, SEd = 9.3).

Sepsis in Relation to Age and Type of Operation

Sepsis was more frequent in the older patients (Table IV) and in those having certain operations, especially genito-urinary surgery and operations on ischaemic limbs (see Table V). The effect of age may, in part at least, reflect the slower rate of healing in old age, with consequent increase in exposure to infection.

TABLE IV.—*Sepsis in Relation to Age (Wards B and C)*

Age (Years)	No. of Patients having Operations	Percentage Developing Wound Sepsis		Percentage Developing Other Sepsis		Percentage Acquiring Nasal Staphylococci while in Ward
		Staphylococcal	Non-staphylococcal	Staphylococcal	Non-staphylococcal	
1–30	286	1·1	1·4	1·1	1·1	14·5
31–50	482	2·7	1·5	2·1	1·9	16·7
51–70	748	6·1	1·9	3·3	2·3	25·7
71 +	161	6·3	1·9	1·2	1·2	26·6

Assessment of Value of Isolation

Functioning of the Isolation Rooms

The isolation cubicles in ward B were planned so that air from the cubicle should never return into the open

ward. The success achieved in this was assessed in three ways. Smoke tests carried out on several occasions showed that when the fans were working and the doors only slightly open there was no flow out of the cubicles into the ward. This was confirmed by liberation of nitrous oxide into a cubicle with subsequent sampling outside, using the equipment described by Lidwell (1960). When the door was widely opened there was some reflux from the cubicle into the ward.

Bacteriological testing by means of simultaneous air-sampling in main ward and isolation cubicle was not entirely satisfactory. Thus on 3 out of 10 occasions staphylococci of the same type as in the cubicle were found in the ward air, but it was not possible to exclude the possibility of undetected carriers in the main ward. On a further four occasions high air counts were obtained in the cubicles by shaking the patient's blankets; despite concentrations up to 53 staphylococci per cubic foot (1,870 per cubic metre) in the cubicle, no staphylococci of the particular type were found in the main ward.

In wards A and B the side-wards along the corridor were also used for isolation. These had no mechanical ventilation, but the structure of the building had the effect, in ward A, of providing a steady outflow of air from the main ward. The ventilation pattern in the side-rooms of ward B was variable and there must sometimes have been reflux of air from these rooms into the main ward; the side-rooms were not, however, often used for the isolation of infected patients after the construction of the cubicles in the ward.

Some difficulty was experienced at first in persuading patients to remain in the isolation cubicles in ward B with the doors closed, for they could see the other patients in the ward around them but could not converse with them. On one occasion at least we believe that cross-infection from an isolated patient was attributable to the patient having switched the fan off. This was at a time when we had a rapidly spreading epidemic of staphylococci of type 53/77 which led us to attempt control by neomycin prophylaxis. The period of this epidemic and of the neomycin prophylaxis is omitted from the subsequent analyses because early in the epidemic there were too many infected patients for isolation to be practicable.

Sepsis Rates

The incidence of post-operative wound infection in ward B (excluding the 53/77 epidemic just mentioned and the period during which neomycin prophylaxis was in use) was 3.8% ; that in the non-isolation ward (C) was 4.5%. As we have already indicated, a simple direct comparison is not satisfactory because of the differing nature of the operations. Table V presents the results in more detail. The operations entered in the top half

TABLE V.—*Incidence of Staphyloccocal Sepsis in Isolation Ward (B) and Non-isolation Ward (C)*

	No. of Operations Performed		Percentage Wound Sepsis		Percentage Other Sepsis*	
	B	C	B	C	B	C
Gastrectomy ..	57	39	1·8	5·1	7·0	2·6
Appendicectomy	39	58	0	0	0	1·7
Repair of hernia	110	151	0	6·0	0	0·7
Neck and head ..	33	39	3·0	0	3·0	2·6
Recto-anal ..	35	55	2·9	3·7	0	1·8
Nephrectomy ..	10	35	40·0	26·5	0	0
Miscellaneous abdominal ..	47	83	4·3	7·2	6·4	4·8
Total ..	331	460	2·7	6·1	2·7	1·95
Varicose vein† ..	52	58	1·9	0	1·9	0
Minor limb ..	84	55	3·6	0	1·2	1·8
Major limb and arterial graft ..	72	2	12·5	50	8·3	0
Genito-urinary operations ..	55	320	9·0	4·7	1·8	2·8
Other operations‡	114	112	0	0·9	2·6	0·9
Grand total	708	1,007	3·8	4·5	2·8	1·1

* " Other " sepsis includes chest sepsis, boils, etc.; infections of urinary tract are excluded altogether.

† The mean stay in hospital for varicose-vein operation is 9·7 days in the isolation ward, but only 4·8 days in the non-isolation ward.

‡ Other operations were as follows: lumbar sympathectomy (B, 58; C, 3), cervical sympathectomy (B, 9; C, 0), eye operations (B, 0; C, 62), mastectomy (B, 5; C, 4), cholecystectomy (B, 15; C, 17), thyroidectomy (B, 9; C, 5), thoracic operation (B, 6; C, 0), mixed operations (B, 12; C, 21).

of the table were performed in reasonable numbers in both wards ; those below were done in much larger numbers in one of the wards than in the other, or were, like minor limb operations, different in nature; or, as in the case of operations on varicose veins, there was a major difference in the routine of post-operative management. For the common list of operations the sepsis rate was 2.7% in the isolation ward and 6.1% in the non-isolation ward ; this difference is not statistically significant (SEd = 3.6).

There was no striking difference between the two wards in the incidence of other staphylococcal sepsis.

Carrier Rates

Of the patients swabbed on admission to ward B (isolation) 1.9% were carrying tetracycline-resistant staphylococci in the nose compared with 3.2% of the patients admitted to the non-isolation ward C. In both wards the patients acquired staphylococci in their noses, but the rate of acquisition was much slower in ward B than in ward C (Fig. 3): among patients who had been in the ward six weeks the proportion carrying tetra-

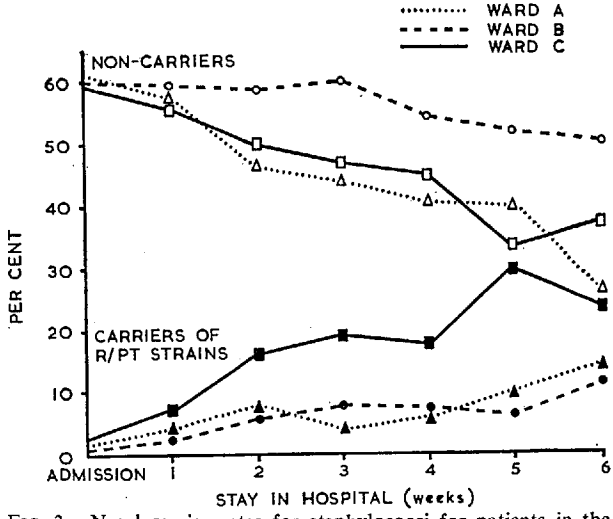


FIG. 3.—Nasal carrier rates for staphylococci for patients in the wards. Non-carriers=Patients with no *Staph. aureus* in nose; R/PT strains=Staphylococci resistant to penicillin and tetracycline.

cycline-resistant strains was 11% in ward B and 23% in ward C. A similar analysis for the other ward (A), which had been studied earlier and which had only sideward isolation rooms, showed a rate of acquisition of resistant staphylococci very similar to that of ward B.

Presumed Source of Infection

There was an indication that epidemic spread of staphylococci was less in ward B than in ward C (Table VI). Thus there were fewer epidemics due to tetracycline-resistant staphylococci, a lower proportion of all the septic lesions were due to "epidemic" types of staphylococci—that is, strains causing more than two

cases of sepsis in one episode—and a rather higher proportion of the sepsis was attributable to staphylococci carried by the patient on admission. The numbers of cases or incidents on which these proportions are based are, however, rather small, so that by themselves they would have little significance.

The second isolation ward (A) was in several respects intermediate between wards B and C (Table VI).

TABLE VI.—*Sources of Staphylococcal Infection*

	Isolation				Non-isolation	
	B		A		C	
Frequency of epidemic incidents per month of observation	0·3		0·4		0·4	
Frequency of incidents due to strains resistant to penicillin and tetracycline	0·1		0·3		0·3	
Percentage of all septic lesions due to:						
Tetracycline-resistant staphylococci	52		30		89	
"Epidemic" types of staphylococci	38		43		66	
	No.	%	No.	%	No.	%
Wound sepsis due to staphylococci:						
Carried on admission	4	*15*	9	*26*	3	*7*
Acquired in ward:						
Probably first in nose	6	*37*	8	*65*	15	*42*
First in lesion	4		14		4	
Wound sepsis in patients not carriers of wound strain:						
Due to "epidemic" staphylococci	4	*15*	1	*3*	11	*24*
Due to "non-epidemic" staphylococci	8	*30*	2	*6*	11	*24*
Source not known	1		—		1	
Total patients with wound sepsis	27		34		45	

Cross-infection from Particular Patients

For this analysis we have used the records of all three wards and have not distinguished side-ward from cubicle isolation. Since even in the isolation wards there were often days when patients harbouring tetracycline-resistant strains were actually present in the open ward, many of the "non-isolation" days in fact refer to the isolation wards. Furthermore, since the analysis was based on all episodes in which a particular type of staphylococcus infected two or more patients, regardless of its tetracycline-resistance, some of the incidents concern staphylococci for which isolation was not considered.

Comparison of Wounds and Nasal Carriers as Source of Infection

It was first necessary to discover whether nasal carriers were more or less liable to give rise to cross-infection than patients with septic lesions. The results of an analysis based simply on cross-infection from patients in the open ward are presented in Table VII. There was a steep increase in the frequency with which patients acquired the staphylococci in the ward with the number of carriers present to serve as sources. Patients with septic lesions gave rise to cross-infections only slightly more often than nasal carriers.

The material was re-analysed to see whether antibiotic-resistant staphylococci behaved differently, and to see whether patients with severe septic lesions were any more active in generating cross-infection than all those with lesions. In neither case was any noteworthy difference from the figures of Table VII detected.

From these results it seemed justifiable to treat nasal carriers and septic lesions as equivalent for the measurement of cross-infection from patients in isolation.

It will be noted that the cross-infection rate on days when no sources were known to be present in the ward was practically the same as that on days when there was one known source (Table VII). These days with no known source were all from the middle of recognized incidents, and we presume that there must in fact have been some unrecognized source of infection present. Investigations of perineal carriage made during one epidemic revealed the existence of a number of patients with staphylococci on the skin but not in the nose. These would have been missed by our routine examinations, but might well have been important as a source of cross-infection.

Cross-infection from Patients in Isolation

With sources of the specific staphylococci present in the open ward the acquisition rate for other patients in the ward was 10.5 per 100 days ; when all the recognized sources of infection were in isolation it was 7.3 (Table VIII). When there were no known sources of infection in the ward (as in the previous analysis on occasional days during the run of a particular type episode) the rate was 7.3. This again suggests that there may often have been an unrecognized source of infection in the ward on these days.

TABLE VII.—*Nasal Carriers and Septic Lesions as Sources of Cross-infection (Wards A, B, and C)*

	Sources of Infection in Ward								
	No Known* Source	Carriers Only			1 Lesion Only	1 Lesion + Carriers			2 Lesions + Carriers
		1	2	3+		1	2	3+	
Total days' experience ..	793	1,242	487	375	168	267	181	149	130
No. patients acquiring infection in ward:									
Nasal carriage only ..	41	70	38	45	10	19	26	21	21
Septic lesion, not carrier ..	8	10	6	3	2	1	1	1	0
Lesion and carriage ..	9	9	7	8	2	4	3	8	5
Total acquisitions	58	89	51	56	14	24	30	30	26
Acquisitions per 100 days ..	7·3	7·2	10·5	15·0	8·3	9·0	16·6	20·0	20·0

* In this table "no known source" includes days when patients in isolation carried the strain, but there was no known source in the main ward.

TABLE VIII.—*Cross-infection from Patients in Isolation (Wards A, B, and C)*

	Source of Infection			
	None Known	In Main Ward	In Isolation	In Main Ward and Isolation
Total days' experience ..	478	2,730	315	269
No. of patients in open ward acquiring infection:				
Nasal carriage only ..	23	225	18	25
Septic lesion, no carriage	5	22	3	2
Lesion and carriage ..	7	45	2	1
Total acquisitions	35	292	23	28
Acquisitions per 100 days ..	7·3	10·5	7·3	9·8

Analyses from the three wards taken separately gave very similar figures.

Table VIII omits the period of one staphylococcal epidemic due to phage type 53/77. This strain was introduced by an unrecognized carrier transferred from another ward and had a very great tendency to spread and colonize the nose. It is certain that the isolation rules were not properly followed at the time when the first recognized patient was segregated, and several secondary cases arose. It subsequently became impossible to isolate all the known carriers, and neomycin nasal cream and hexachlorophene soap were therefore given to all patients in the ward ; this regime continued for various reasons for a total of six months (Fig. 1). If the period of the type 53/77 outbreak were included in Table VIII the figure for acquisition from isolated patients would be increased from 7.3 to 9.5 per 100 days ; those for sources in main ward, or main ward and isolation, would become 10.5 and 11.5 respectively.

In Table VIII there are recorded 23 infections acquired at a time when the only known sources were in isolation. For nine of these cases a carrier had been present in the main ward one to five days before the presumed date of acquisition, so the infection may have been acquired in the ward but remained unrecognized for a longer period than allowed for by our conventions. There was no known carrier within the previous seven days for the remaining 14, but six of them were part of an outbreak due to type 52/55 and occurred within a few days of each other ; the staphylococcus was found in the air of the main ward even when all the carriers were in isolation, so that it is possible there was an undetected carrier among the patients or staff.

Cross-infection in Relation to Propinquity to a Carrier

Using methods of analysis similar to those employed for testing the effect of isolation, we tried to find out whether cross-infection was commoner in patients nursed in beds close to a carrier. No clear relation could be demonstrated (Table IX), but the regression coefficients of acquisition rate on bed-spacing are all negative, as would be expected if the closer patients were at greater risk, though none of them is statistically

TABLE IX.—*Effect of Distance from Carrier on Cross-infection*

No. of Bed Spaces between Patient Acquiring Infection and Nearest Carrier	Acquisition Rate per 1,000 Patient Days with		
	1 Carrier in Ward	2 or More Carriers or Patients with Lesions	Any Type of Source
1	6·3 (8)	9·3 (38)	8·6 (46)
2	4·1 (7)	8·6 (32)	7·2 (39)
3	8·2 (15)	3·4 (9)	5·3 (24)
4	6·7 (11)	7·8 (11)	7·2 (22)
5 and 6	4·1 (7)	8·5 (6)	5·3 (13)

Numbers in parentheses = No. of acquisitions.
N.B.—Owing to the layout of the ward, no patient could be more than six bed spaces away from any other patient.

significant. The results are consistent with the notion that the most remote patients experience as much as half the acquisition rate of the patients nursed adjacent to a carrier. It is interesting to note that, in a few experiments in which a tracer gas was liberated at one point in the ward, samplers at the far end of the ward (which because of the ward layout is equivalent to six bed spaces distant) collected about half the quantity of tracer collected 6–8 ft. (1.8–2.4 m.) from the point of liberation. Such a reduction in transfer has, however, a limited effect on cross-infection because so few patients are as much as five or six beds away from one another.

Neomycin and Hexachlorophene Prophylaxis

Neomycin and hexachlorophene prophylaxis for all patients admitted to ward B was started in April, 1960, because of the widely spreading epidemic due to a staphylococcus of phage type 53/77 and the lack of sufficient isolation accommodation. The prophylaxis was continued until October, 1960. The wound sepsis rate during the whole period April to October was 5.6% (on 356 operations). Eight of the 20 cases of sepsis were due to type 53/77, so that the sepsis rate for other types was about 3.5%—that is, the same as the overall rate for the non-neomycin period. Moreover, the sepsis

rate on 96 operations performed after the end of the 53/77 epidemic in mid-May was as high as 7.3%. The introduction of the neomycin and hexachlorophene prophylaxis appeared to eliminate the type 53/77 staphylococcus and terminate that epidemic. However, during the subsequent period it had no detectable effect in reducing the total post-operative sepsis rate; it is, however, curious that of the seven septic wounds occurring in patients on prophylaxis, four were infected with staphylococci sensitive to penicillin and tetracycline.

Of 92 patients under neomycin prophylaxis after the end of the 53/77 epidemic, 37 (40%) were at some time nasal carriers of staphylococci. This may be compared with 55% for similar patients not given chemoprophylaxis.

Discussion

On the face of it the results of our attempt to control staphylococcal cross-infection by isolation are disappointing: a post-operative wound-infection rate of 3.8% in a ward in which all patients known to be harbouring tetracycline-resistant strains were isolated, compared with a rate of 4.5% in a ward where no isolation was practised. But we need to consider not only whether the isolation was actually effective in restraining the spread of staphylococci but also how much we could have hoped to achieve by our isolation methods at their best, and the extent to which a similar system could ever be applied in a general hospital routine.

The effect of an alteration in technique is necessarily difficult to establish by comparing the experience of patients in only two wards, and none of our conclusions can be more than tentative. Apart from the difference in isolation policy between the wards, there may have been other unrecognized differences in management which affected our results. This proviso has to be borne in mind in all our comparisons between the wards when we attribute the observed differences to the isolation policy.

There was one important difference, which we can allow for—namely, the different operations performed on the patients in the two wards. There were 331 operations on patients in the isolation ward and 460 on patients in the non-isolation ward having one or other of seven common operations. The sepsis rate on these

operations was 6.1% in the non-isolation ward and 2.7% in the isolation ward. The sepsis rate to be "expected" in the isolation ward, calculated by applying to each group of operations the rate observed in the non-isolation ward, was 5.1%. Though not statistically significant, the figures suggest a halving of the sepsis rate.

In the isolation ward the operations excluded from the common list had a higher-than-average sepsis rate (4.7%). If we assume that the sepsis had been reduced among these operations by the same proportion as among the common operations, the sepsis rate in a control ward with a similar distribution of operations and with the conditions of our non-isolation ward might have been about 8.2% (58/708). If these rather tenuous assumptions are accepted we can estimate that the isolation policy may have prevented 31 cases of sepsis.

One also needs to ask how much an isolation policy could have achieved. Four of 26 cases of sepsis in the isolation ward were due to staphylococci carried by the patient on admission (information on one further patient is lacking); this is equivalent to a sepsis rate of 0.6%, which could certainly not be prevented by isolation. A further eight cases were due to staphylococci not isolated from any other septic lesion; in so far as these were derived from known carriers, they were mostly tetracycline-sensitive, so that the sources of these cross-infections would not have qualified for isolation under our rules. On these grounds the incidence of "unavoidable" sepsis might have reached 1.8% of the 708 patients; this is about half the sepsis actually observed.

An alternative approach is to consider what might have happened if we had been able to isolate effectively all patients harbouring "epidemic strains" of staphylococci—that is, those giving two or more cases of sepsis—from the time of admission to the ward. Elimination of all cross-infection with epidemic strains would have prevented 11 of the 27 cases of sepsis in ward B, again reducing the sepsis rate from 3.8% to 2.3%.

In sum, then, the records suggest that if the ward had not followed an isolation policy at all there might have been 58 cases of wound sepsis, and if it had been completely successful in preventing cross-infection from patient to patient there might have been only 16. The observed number was 27.

A part of the sepsis regarded as unavoidable in the previous analysis is due to the patients' own staphylococci. Part is probably also due to cross-infection in the operating-theatre, though very few of our patients developed their sepsis with the signs of fever soon after operation and deep pus that are characteristic of theatre infections ; in over half the cases the appearance of sepsis was delayed for more than 10 days. Nevertheless we cannot exclude infection in the operating-theatre or soon afterwards as quite unimportant. Some cases of infection may also be due to infection from the medical and nursing staff in the wards. These were outside our investigation, but the small proportion of acquisitions by patients at times in our epidemics when we knew of no carrier or lesion among patients (58/378, Table VII) does not point to a large reservoir in the staff.

That the isolation rooms were probably reasonably effective in preventing the escape of staphylococci from patients nursed in them is shown not only by the tracer experiments but also by the fact that when all known infected patients were in isolation the cross-infection rate was reduced to the same level as that found during periods in the middle of epidemics when there were no known carriers present in the ward. One must presume that the residual cross-infection is attributable to undetected carriers present in the ward.

Although our observations were not continued long enough to provide unequivocal results, it seems that the isolation rooms with ventilation, when properly used, were probably effective in limiting the spread of staphylococci from patients nursed within them, and this was as true of the isolation rooms within the ward as of the side-wards. If confirmed this is important, since isolation rooms within an open ward are sometimes the only facilities available. We have the strong impression that some of our "escapes" from isolation resulted from the switching off of the exhaust fan ; this certainly allowed the contaminated air from within the room to reach the main ward.

The main difficulty of an isolation policy became clear during our study ; it is the difficulty, in many epidemics of ward infection, of detecting carriers who serve as reservoirs from which the spread is maintained. We could detect hardly any difference between carriers and patients with septic lesions as sources of cross-infection,

and, though there might be greater differences in other circumstances, it seems to us inescapable that a control policy based simply on the isolation of patients with septic lesions must often fail. To detect all the dangerous carriers by swabbing is an expensive procedure and will frequently give the information too late—as it often did in our own wards. The implication seems to be that, unless we perfer to protect patients against the acquisition of staphylococci in their nose by chemoprophylaxis, hospitals ought to be constructed so as to isolate as many as possible of the patients from one another, and in particular to protect newly admitted patients from exposure to those who have been in hospital long enough to acquire hospital staphylococci.

Summary

The natural history of staphylococcal infection was studied in three male surgical wards, in two of which an attempt was made to isolate all patients known to carry or be infected with tetracycline-resistant staphylococci. One of the wards was provided with four exhaust-ventilated cubicles for isolation purposes.

The introduction of cotton blankets, washed at frequent intervals, into one of the wards had no detectable effect.

Patients who acquired staphylococci in their nose during their stay in hospital developed staphylococcal sepsis more than five times as often as those who did not. In most cases the nasal colonization preceded the sepsis. The patients who acquired staphylococci also had a higher incidence of non-staphylococcal sepsis, but this appeared to be associated with prolonged stay in hospital.

The post-operative sepsis rates were compared in patients having operations performed with comparable frequency in one of the isolation wards, and in the non-isolation ward. The rates were 2.7 and 6.1% respectively ; the difference of 3.4% does not reach the level of statistical significance.

Patients in the isolation wards became nasal carriers of tetracycline-resistant staphylococci more slowly than those in the non-isolation ward.

There was some evidence that epidemic spread of staphylococci was less in the isolation wards than in the non-isolation ward.

When individual sources of infection were considered, it was found that nasal carriers were almost as important in generating cross-infection as patients with septic lesions. When all patients known to be sources of infection in the ward were isolated the incidence of cross-infection was apparently reduced. The exact magnitude of the reduction is difficult to determine because of the almost certain presence of undetected sources of infection in the ward.

The limitations of any study of this sort based on three wards is stressed, but, taken at their face value, the results suggest that the isolation policy might have reduced the sepsis rate by about one-half. The demonstrable importance of nasal carriers as sources of cross-infection implies that such an isolation policy can be applied only if continuous bacteriological surveillance is available. Since this is seldom practicable, the conclusion is reached that, to prevent the transfer of staphylococcal infection, hospitals need to be constructed so as to isolate as many patients as possible from one another.

For permission to study their patients, we are grateful to our surgical colleagues, Mr. C. Naunton Morgan, Mr. D. F. Ellison Nash, Mr. A. W. Badenoch, Mr. I. P. Todd, and Sir James Paterson Ross, Bt. Our thanks are also due to the sisters and nursing staff of the wards; to the Treasurer and Governors of St. Bartholomew's Hospital for the construction of the cubicles, and to Mr. John Weeks for help in their design; to the Medical Research Council for a grant for scientific assistance; and to Miss Olive Duke and Miss Susan Green, B.Sc., for assistance in the laboratory.

REFERENCES

Lidwell, O. M. (1960). *J. Hyg. (Lond.)*, **58**, 297.
Shooter, R. A., Smith, M. A., Griffiths, J. D., Brown, Mary E. A., Williams, R. E. O., Rippon, Joan E., and Jevons, M. Patricia (1958). *Brit. med. J.*, **1**, 607.
Williams, R. E. O., Jevons, M. Patricia, Shooter, R. A., Hunter, C. J. W., Girling, J. A., Griffiths, J. D., and Taylor, G. W. (1959). Ibid., **2**, 658.

Printed in Great Britain by Fisher, Knight and Co., Ltd., Gainsborough Press, St. Albans.

No. 2

THE DISPERSAL OF STAPHYLOCOCCI IN HOSPITAL WARDS

BY

W. C. NOBLE

Reprinted from Journal of Clinical Pathology
Volume 15, page 552, 1962

LONDON
BRITISH MEDICAL ASSOCIATION
TAVISTOCK SQUARE, W.C.1

J. clin. Path. (1962), **15**, 552

The dispersal of staphylococci in hospital wards

W. C. NOBLE[1]

From the Air Hygiene Laboratory, Central Public Health Laboratory, Colindale, London

SYNOPSIS The air of three hospital wards was examined frequently for staphylococci for a period of nearly four years using slit samplers. 'Broadcasts' of staphylococci into the air were observed with the air count rising well above the mean of 0·2 staphylococcal particle per cubic foot and these broadcasts often appeared to be due to single patients.

Eight of the 3,675 patients admitted to the wards possessed an ability to disperse staphylococci into the air of the wards which was markedly greater than normal.

The actual dispersal about the ward appeared to be mediated by the bedclothes, for, when these were disturbed, large numbers of staphylococci were disseminated into the air.

During investigations of post-operative staphylococcal sepsis in surgical wards at St. Bartholomew's Hospital, London, the air of the wards was frequently examined for staphylococci. Shooter, Smith, Griffiths, Brown, Williams, Rippon, and Jevons (1958) observed 'broadcasts' of staphylococci into the air from time to time and some of these broadcasts appeared to be associated with particular patients. The study was continued and the more extensive records now available have been analysed in an attempt to discover more about the spread of staphylococci by such patients.

METHODS

The study was carried out in three male surgical wards (A, B, C). The work of the units was general surgery, although a proportion of specialized work was carried out in each ward. Wards A and C used wool blankets throughout but ward B used cellular cotton blankets for much of the investigation. Patients harbouring staphylococci resistant to penicillin and tetracycline were nursed in isolation in wards A and B but not in ward C (Williams, Noble, Jevons, Lidwell, Shooter, White, Thom, and Taylor, 1962).

The wards, which measure 56 ft. by 43 ft., have a volume of about 30,000 cu. ft. Each has 22 beds, spaced about 6 ft. (1·8 m.) apart, with three more beds in two side wards. There are cotton curtains, suspended from rails 7 ft. (2·1 m.) from the floor, which can be pulled out to surround each bed. During the course of the study one of the wards, B, had four single-bed cubicles built in the body of the ward.

[1]Present address: The Wright-Fleming Institute of Microbiology, St. Mary's Hospital Medical School, Paddington, London, W.2.

Received for publication 16 May 1962.

A total of 3,675 patients was seen during this study. They had nasal swabs taken on admission to the wards and thereafter at weekly intervals. No systematic attempt was made to examine other possible carrier sites for staphylococci.

During the four years of the survey, the air of the wards was examined on three days each week, usually Monday, Wednesday, and Friday, using a slit sampler running for two consecutive hours. During the first year the total volume sampled daily was 48 cu. ft.; later it was 216 cu. ft. For observing the effect of such procedures as bed-making and wound dressing, a slit sampler drawing 6·3 or 12·6 cu. ft. per minute was used so that adequate volumes could be sampled in short periods.

The colony count obtained using the slit sampler gives a measure of the number of airborne particles carrying staphylococci and in accordance with general usage the counts are given here as colonies per cubic foot of air sampled. For the routine ward samples the counts are given as the average for the two-hour sampling period.

The bedding was examined qualitatively for staphylococci using the sweep plate technique (Williams, Blowers, Garrod, and Shooter, 1960). For quantitative estimation of staphylococcal contamination a cellular cotton blanket was held, 7 sq. in. at a time, clamped over the mouth of a tube through which air was drawn directly into a slit sampler. The blanket was covered with a filter paper and gently percussed, the organisms thus released being collected on agar plates exposed for successive one-minute periods in the slit sampler. Experience showed that about half the recoverable staphylococci appeared on the first six plates exposed, and so the total count of recoverable staphylococcal particles has been estimated by doubling the count on six successive plates.

One colony from each positive nasal culture and two colonies from each bed sweep were phage-typed. As about 75% of the air samples yielded fewer than 40 colonies of staphylococci from the 216 cu. ft. sample (less than 0·2 colony per cu. ft.), staphylococci from the air

were typed as a routine only when the count rose above this level. However, up to 12 colonies from each air sample were preserved whether or not typing was indicated on the basis of the count observed. When more than two-thirds of the staphylococci recovered from the air were of the same phage type, that type was referred to as being predominant in the air.

The medium used throughout this study was Oxoid blood agar base containing 5% horse serum and 0·01% phenolphthalein-phosphate (Barber and Kuper, 1951). Phosphatase-positive colonies were tested for coagulase and were phage-typed at the Staphylococcus Reference Laboratory, Colindale. Throughout this paper the word staphylococcus is used to mean the coagulase-positive *Staphylococcus aureus*.

RESULTS

ROUTINE COUNTS OF AIRBORNE BACTERIA Although the total airborne flora and the number of staphylococci recovered varied from day to day, the average was remarkably similar for the three wards (Table I). During the 732 ward days of observation there were 177 days when the count of staphylococcal particles in the air was greater than 0·2 per cu. ft. (Fig. 1). On 21 days the count was greater than 1·0 per cu. ft. and on four days greater than 2·0. The highest count recorded for a two-hour period was 3·4 staphylococcal particles per cubic foot.

An analysis of variance was carried out on the air counts of ward C over a period of 83 weeks with two samples on each of the three days each week. The variance between days and that between weeks was significant at the 0·001 level. (Variance ratio, between days $F = 8{\cdot}7$, $n_1 = 2$, $n_2 = 413$; variance ratio, between weeks $F = 4{\cdot}1$, $n_1 = 82$, $n_2 = 413$.) Variance between the two counts on one day was not significant.

TABLE I

MEAN NUMBER OF BACTERIA IN AIR SAMPLES FROM THE THREE WARDS

Ward	*Colonies per Cubic Foot*		*Number of Days of Sampling*
	Total Bacterial Flora	*Staphylococci*	
A	27·0	0·22	114
B	26·4	0·18	317
C	32·7	0·18	321

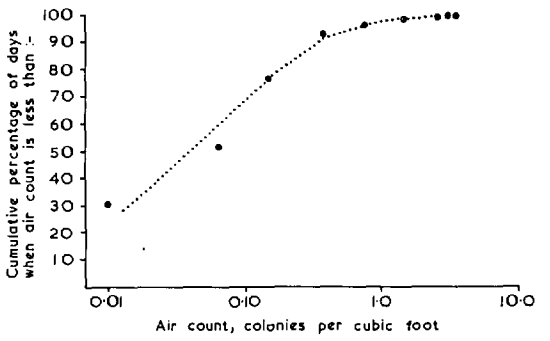


FIG. 1. *Counts of airborne staphylococci. On 3% of the days no staphylococci were recovered from the air (count less than 0·01 particle per cubic foot).*

RELATION OF AIRBORNE BACTERIAL COUNTS TO CARRIERS On 162 of the 177 days when the count of airborne staphylococci was greater than 0·2 per cu. ft., there was one type of staphylococcus predominant in the air. The phage types of the staphylococci recovered from the air were compared with those isolated from the noses or wounds of the patients at the previous weekly swabbing. For 64 of the 162 days, only one patient was found to carry the strain predominant in the air. Thirty-eight patients contributed to these 64 days; 16 patients were associated with high air counts on two or more days and eight patients were associated with high air counts throughout much of their hospital stay. These eight patients are referred to for convenience as 'dispersers'.

On a further 45 of the 162 days, one of the 38 patients referred to above and one or more other carriers were present and on 47 days several carriers were present, but it was not possible to attribute the spread of the staphylococci to any one of them. On only six of the days when the count in the air was greater than 0·2 particle per cu. ft. was no carrier known to be present.

CHARACTERISTICS OF DISPERSERS Some of the characteristics of the eight dispersers observed are given in Figure 2. Six of these patients were active dispersers on admission to the wards and two became dispersers of the staphylococci that they acquired in hospital. Dispersal of the staphylococci acquired by patient H apparently resulted in an outbreak of staphylococcal sepsis. Dispersal from patient G led only to nasal colonization of other patients.

Patient A carried and disseminated the same strain on three successive admissions whereas patient C was not a carrier on his first admission but on readmission two years later carried and dispersed the type 6/53/75 referred to below. Patient D was a disperser on his first admission but not on his second four months later, although carrying the same types of staphylococci. The remaining dispersers were observed only once.

Although none of these patients had an infected lesion on admission to the wards six of them subsequently developed some infection with the same staphylococcus as was carried nasally. Patient A

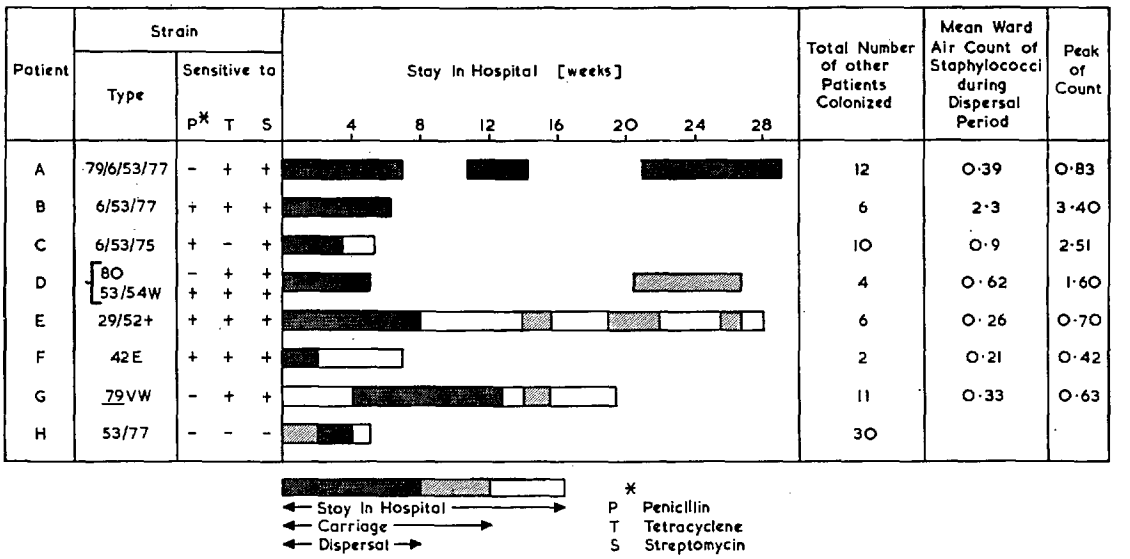


FIG. 2. *Characteristics of dispersers in relation to their stay in hospital. Patient H acquired his strain in another ward. As he was isolated in a cubicle the total number of patients infected with his strain and the air counts in the main ward are not directly related to his ability to disperse organisms.*

had an infected colostomy in the sixth week of his first admission and a boil in the third week of his second admission. Patient B developed infection of an abdominal laparotomy wound during the fourth week of his stay in hospital and patient C had an infected ulcer in the third week of his stay. Patient E had an infected sputum in the second week and patient G a boil in the eighth week of his stay. Patient H had an infected sputum a few days after his admission to the ward. Only one of these infections, that of patient G, could be classed as severe. Patients D and F had no infections. Most of the infections developed some time after admission and it seems probable that they were the result of the large numbers of staphylococci found in the patients' environment rather than the cause of the dispersal.

ENVIRONMENTAL STAPHYLOCOCCI

RECOVERY OF STAPHYLOCOCCI FROM BLANKETS When the number of airborne staphylococci in the ward rose markedly above 0·2 per cu. ft. an attempt was made to locate the source of the organisms by taking bedsweeps from the top blanket near the head of each bed. There was normally a delay of two to three days between the day on which the high air count occurred and the day on which the bedsweeps were taken.

Although the strain in the air was often recovered in small numbers from some or many of the beds in the ward, it was common to find one bed that was heavily contaminated and it is presumed that this bed was the source of the airborne organisms. From Table II it can be seen that over the 28 occasions on which bedsweeps were taken when a disperser was present, 76 (12·4%) of the 615 beds examined yielded more than 20 colonies of staphylococci. These 76 beds were distributed amongst the 28 occasions, but there was a tendency to find more beds heavily contaminated when the disperser had been in the ward some time than soon after his arrival.

In the absence of a high air count more than 95% of the blankets yielded six or fewer colonies of staphylococci by the sweep plate method.

TABLE II

PERCENTAGE OF PATIENTS' BLANKETS YIELDING SPECIFIED NUMBER OF STAPHYLOCOCCI

	% Blankets Yielding Specified No. of Staphylococci						*No. of Days on which Blankets Were Sampled*	*No. of Blankets Sampled*
	0	*1-3*	*4-6*	*7-9*	*10-20*	*21-*		
Disperser present	32·8	32·0	7·5	6·5	7·8	12·4	28	615
No disperser present	58·0	34·9	2·7	0·7	1·8	2·0	23	450

TABLE III

RECOVERY OF STAPHYLOCOCCI FROM BLANKETS

Patient	*No. of Staphylococcal Particles Recovered per Square Inch*	*Calculated No. on Entire Blanket*
E, disperser	56, 76, 77, 78, 83, 100, 127, 146, 160, 175	640,322
G, disperser	3, 32, 40	148,500
1, non-disperser	6, 7	38,016
2, non-disperser	4, 5	27,240
3, non-disperser	4, 4	24,354
4, non-disperser	1, 2	8,190

The figures given above are the values of separate determinations carried out on different areas of blanket.

Quantitative examination for staphylococci of blankets taken from the beds of two dispersers showed very large numbers of organisms, but examination of blankets from the beds of four non-dispersers yielded only small numbers of staphylococci (Table III).

RATE OF CONTAMINATION OF BEDDING Once bedclothes were put on the beds in the wards they became contaminated with staphylococci very quickly. Table IV shows the number and percentage of blankets from which staphylococci were recovered in relation to the length of time that the bed had been occupied by the patient.

The rapidity with which an active disperser can contaminate his bedding is shown by investigations on patient C. This patient was given clean pyjamas, bedding, and curtains. After two hours, a sweep plate of the sheet on which he lay yielded more than 700 colonies of his staphylococcus and after five hours these organisms were recoverable from the curtains around his bed. The sheet became contaminated more rapidly than the blankets, suggesting that the organisms were derived from his body rather than from his nose.

Patient C was, by chance, observed from the moment of his arrival in the ward and he carried a rather distinctive type of staphylococcus (phage type 6/53/75, resistant to tetracyclines but sensitive to penicillin). On the day of his admission, none of the beds in the ward carried type 6/53/75. During the act of undressing he disseminated large numbers of organisms into the air and two days after his admission his type was recovered from 14 of the 22 beds. After one week, 20 of the 22 beds, 28 of the 30 curtains, 12 out of 12 window ledges, and three of the three sinks sampled were contaminated with this type of staphylococcus. The number of patients and beds from which this strain was subsequently isolated is shown in Figure 3.

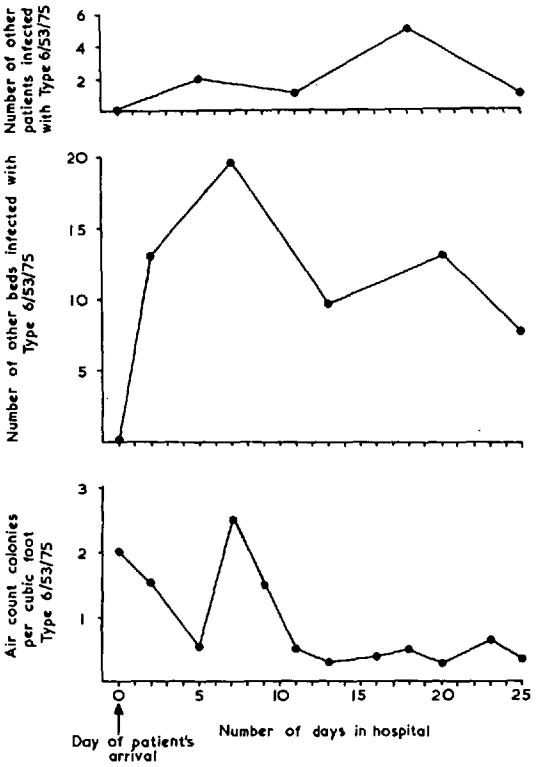


FIG. 3. *Dispersal of staphylococci by patient C. On the day of this patient's arrival in the ward the first air sample yielded no colonies of his staphylococcus but the second sample yielded four colonies per cubic foot.*

TABLE IV

RECOVERY OF STAPHYLOCOCCI FROM BLANKETS ON BEDS IN WARD B

	No. of Days Bed Occupied							*Blankets from Linen Cupboard*
	0	*1-5*	*6-10*	*11-15*	*16-20*	*21-25*	*>26*	
No. of beds sampled	23	66	40	29	22	15	58	182
% Beds yielding staphylococci	35	42	38	59	41	53	62	14
% Beds yielding >4 colonies of staphylococci	8·5	12	17·5	28	23	20	41	0
% Beds yielding >4 colonies of staphylococci carried by occupant of bed	—	4·5	12·5	21	18	13	17	—

Clean blankets were put on the bed before the admission of all patients, after any patient had been in his bed for four weeks, and when indicated by soiling, etc.

RELATION OF NUMBER OF AIRBORNE BACTERIA TO NUMBER OF CARRIERS When a disperser was present in the ward the number of staphylococci in the air bore little or no relation to the numbers of carriers of the strain, although the presence of large numbers of staphylococci in the air usually resulted in some other patients becoming carriers, as with the case illustrated in Figure 3.

For 11 episodes lasting more than six weeks when a strain was spreading amongst the patients but no disperser could be found, the number of carriers of the strain was plotted against the air count on the day of swabbing as a scatter diagram. Altogether there was little correlation between the number of carriers and the air count.

DISPERSAL FROM FOMITES

In an attempt to define the mechanism of dispersal rather more precisely, air sampling was carried out close to the beds of patients during bedmaking and wound dressing. The two groups of patients selected for these investigations were (a) six of the eight dispersers and 10 non-dispersers and (b) 23 patients suspected of having staphylococcal wound infection, staphylococci being isolated from the wounds of 11 of these patients.

Investigation of a further 10 patients with staphylococcal pneumonia showed that only two of them were disseminating organisms and then in very small numbers.

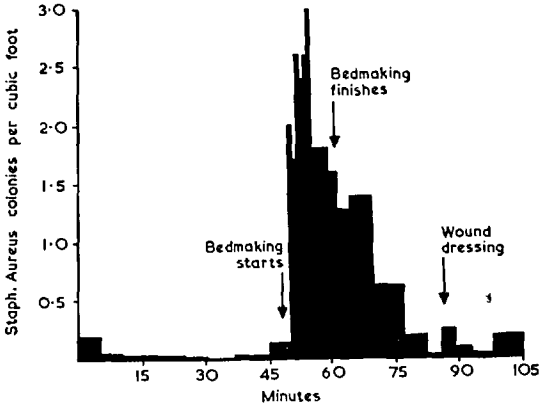


FIG. 4. *Dispersal of staphylococci during bedmaking.*

DISPERSAL DURING BEDMAKING Bedmaking was studied on 24 occasions. On 19 of these occasions there was an increase in the number of airborne staphylococci and on the remaining five occasions an increase in the total bacterial flora only. The recognized dispersers liberated more staphylococci than the non-dispersers (Table V). An example of the timing of the dispersal in relation to bedmaking is given in Figure 4. It can be seen that few staphylococci were liberated until the bedclothes were disturbed even though a blood sample was taken from the patient in the period before bedmaking.

TABLE V

INCREASE IN NUMBERS OF AIRBORNE STAPHYLOCOCCI DURING BEDMAKING

Patient	*Staphylococci in Nose*	*Staphylococci in Wound*	*No. of Colonies of Staphylococci in Air*	
			Before Bedmaking	*Peak of Count*
Dispersers				
A	+	−	0·08	2·20
A	+	+	0·64	2·10
A	+	+	0·10	1·60
A	+	−	1·00	1·30
B	+	−	0·40	9·50
D	+	−	9·00	36·00
D	+	+	0·10	5·40
D	+	−	0·50	4·50
D	+	+	0·20	3·10
D	+	−	0·06	3·00
E	+	+	12·00	53·00
E	+	+	0·10	20·60
F	+	+	0·09	2·40
G	+	+	0·50	7·90
Non-dispersers				
a	−	+	1·00	2·40
b	+	+	0·10	1·70
c	−	−	0·10	0·90
d	+	−	0·09	0·80
e	+	+	0·10	0·40
f	−	−	0·19	0·38
g	−	−	0·20	0·13
h	−	+	0·40	0·09
i	+	+	0·11	0·06
j	+	−	0·05	0·00

One of the investigations was carried out in an exhaust-ventilated cubicle of about 1,000 cu. ft. capacity. A peak count of 53 staphylococcal particles per cu. ft. was obtained, suggesting that rather more than 50,000 staphylococcal particles must have been liberated during the process of bedmaking.

DISPERSAL FROM CURTAINS Disturbance of bedside curtains was observed on 41 occasions. On 32 of these occasions there was an increase in the total airborne flora and on 26 occasions there was an increase in the staphylococcal flora. On 17 of these 26 occasions some or all of the staphylococci recovered were of the same phage type as carried by a patient in an adjoining bed.

DISPERSAL DURING WOUND DRESSING PROCEDURES When investigating the spread of staphylococci from the wound itself, it is necessary to eliminate as far as possible all other sources of organisms for, as shown above, disturbance of the bedding can result in dispersal. In our experiments the bedding was therefore changed and the patient put in clean pyjamas before the dressing was done. Where these experiments were carried out in the main ward, care was taken not to disturb the curtains after the start of the sampling. Many of the experiments were, however, performed in the isolation rooms. There was, of course, still the possibility that the wound dressing was contaminated on the outside with organisms from the nose or other carrier site, but this could hardly be avoided.

Wound dressing procedures were observed on 25 occasions involving 23 patients. On 12 of these occasions the wound was found to be infected with staphylococci (Table VI). An increase in the number of staphylococci in the air was seen during the dressing of six of the 12 infected wounds and two of the 13 uninfected wounds.

PERSISTENCE OF ORGANISMS IN THE WARD In experiments with five blankets removed from the beds of patients and kept in the laboratory, staphylococci were recovered for periods up to 203 days. However, staphylococci seem to disappear from the air of the ward very rapidly after the last-known carrier is discharged. Thus for 20 episodes when a strain was spreading amongst the patients, a search was made amongst the strains isolated from the air of the ward in the week after the discharge of the last-known

TABLE VI

DISPERSAL OF STAPHYLOCOCCI DURING DRESSING OF INFECTED WOUNDS

Patient	*State of Wound*		*Whether Patient is Nasal Carrier of Wound Staphylococcus*	*No. of Colonies of Staphylococci in Air*	
	Bacteriological	*Clinical*		*Before Dressing*	*Peak of Dressing*
F	Staphylococci	Healthy	+	0·10	2·40
2	Staphylococci	Healthy	−	0·02	0·40
F	Staphylococci	Healthy	+	0·13	0·80
4	Staphylococci	Slight infection	+	0·03	0·16
5	Staphylococci	Healthy	−	1·00	2·40
6	Staphylococci	Healthy	+	0·30	0·80
7	Staphylococci	Healthy	−	0·08	0·13
A	Staphylococci	Slight infection	+	0·10	0·12
E	Staphylococci	Healthy	+	1·00	1·10
D	Staphylococci	Slight infection	+	0·20	0·20
11	Staphylococci	Slight infection	+	0·60	0·60
A	Staphylococci	Slight infection	+	0·15	0·15
13	No staphylococci	Healthy	Nasal carrier	0·10	0·24
14	No staphylococci	Healthy	Nasal carrier	0·03	0·22
15-25	No staphylococci	Healthy	Some nasal carriers		No rise in count

Slight wound infection is used to indicate the appearance of some pus and inflammation at the wound edges.

TABLE VII

PERSISTENCE OF STAPHYLOCOCCI IN AIR OF WARDS AFTER DISCHARGE OF LAST-KNOWN CARRIERS OF STRAINS SPREAD AMONGST PATIENTS

Phage Type	*No. Strains Typed*	*No. Strains of Index Type Found*	*Count per cu. ft of Index Type*	*Count per cu. ft. on Last Day of Carrier's Admission*	*No. Days Since Last Carrier Present*
52A/79	16	1	0·01	0·05	1
52/52A/80	17	1	0·01	0·03	2
52A/79	29	1	0·01	0·02	3
52/52A/79/80/7/42E	11	1	0·01	0·10	4
79	13	1	0·01	0·05	5
Various strains taken from 15 other episodes	259	0	<0·01	0·02-0·17	1-7

carrier. In only five of these cases were such staphylococci recovered (Table VII), four from ward B and one from ward C. A total of 259 staphylococci was examined from the air samples taken after the end of the remaining 15 episodes without any strains of the appropriate type being recovered.

DISCUSSION

During this study, which lasted nearly four years, 3,675 patients were admitted to the wards and 1,488 of these patients carried staphylococci in the nose on admission but we detected only six patients who had the ability to contaminate their environment heavily with staphylococci. About 18% (662) of the patients acquired a staphylococcus in the nose during their stay in hospital but only two of these had the ability to disperse this staphylococcus to any marked extent.

Although 81 other patients became colonized with the same types of staphylococci as carried by the eight dispersers, none of them were observed to become dispersers themselves. Hence it seems likely that the ability to disperse is a property of the patient rather than of the staphylococcus. There seems to have been no common factor such as age, disease, or type of staphylococcus carried in this series of patients.

Four factors have been recognized in other work on the dispersal of organisms. Eichenwald, Kotsevalov, and Fasso (1960) found that their 'cloud babies' apparently disseminated organisms from the respiratory tract and that the ability to disseminate staphylococci was sometimes associated with adenovirus infection, but this factor was not investigated in this study. White (1961) showed that patients from whom large numbers of staphylococci could be recovered by nasal swabbing carried more staphylococci on the skin and disseminated more staphylococci from their bedding than non-carriers or carriers with only slight nasal growth. We have no information as to the heaviness of nasal carriage of staphylococci amongst the patients in this survey but when there was no disperser present in the ward staphylococci were recovered from the beds of 59% of the nasal carriers and from 36% of the non-carriers. The same phage type as was carried in the nose of the patient was isolated from 39% of the beds. Hare and Cooke (1961) found that patients with eczema or mycosis fungoids carried many staphylococci on the skin and contaminated their bedding with staphylococci. Patients with lesions which could be covered, such as wound infection, boils, etc., liberated few staphylococci unless there was a profuse discharge from the lesion. In the investigations reported above, only one of the patients had a severely infected lesion and this developed four weeks after he had begun dispersing the organism. Hare and his colleagues have emphasized the importance of perineal carriage of staphylococci in relation to dispersal. It was not possible, however, in our study to carry out routine skin swabbing but perineal swabs taken from three of the dispersers (A, B, and D) all yielded staphylococci of the same type as carried in the nose. Skin swabbing was not carried out on the other five dispersers.

Although the strains disseminated by the dispersers were recovered from the noses of the other patients and the lesions on the dispersers themselves, only one strain caused any sepsis amongst other patients in the ward. This was the strain with which patient H was admitted to the ward under investigation from another ward. Thus it appears that the dispersal of staphylococci, even in enormous numbers, does not lead to cross-infection unless the strain itself has epidemic propensities; this same strain was able to spread through another ward without any dispersers being apparent.

The studies reported in this paper constituted part of the larger investigation referred to above (Williams *et al.*, 1962) and arose as a result of the discussions held by the workers involved in that investigation, to whom I am extremely grateful. I should like to record my indebtedness to the Treasurer and Governors of St. Bartholomew's Hospital for permission to work in the wards.

REFERENCES

Barber, Mary, and Kuper, S. W. A. (1951). *J. Path. Bact.*, **63**, 65.
Eichenwald, H. F., Kotsevalov, O., and Fasso, L. A. (1960). *Amer. J. Dis. Child.*, **100**, 161.
Hare, R., and Cooke, E. M. (1961). *Brit. med. J.*, **2**, 333.
Shooter, R. A., Smith, M. A., Griffiths, J. D., Brown, Mary E. A., Williams, R. E. O., Rippon, Joan E., and Jevons, M. Patricia (1958). *Ibid.* **1**, 607.
White, A. (1961). *J. Lab. clin. Med.*, **58**, 273.
Williams, R. E. O., Blowers, R., Garrod, L. P., and Shooter, R. A. (1960). *Hospital Infection*: Lloyd-Luke, London.
——, Noble, W. C., Jevons, M. Patricia, Lidwell, O. M., Shooter, R. A., White, R. G., Thom, B. T., and Taylor, G. W. (1962). *Brit. med. J.*, **2**, 275.

No. 3

DISPERSAL OF BACTERIA ON DESQUAMATED SKIN

R. R. DAVIES
M.Sc. Wales, Ph.D. Lond.

W. C. NOBLE
M.Sc. Lond.

Reprinted from THE LANCET, *December* 22, 1962, *pp.* 1295-1297

DISPERSAL OF BACTERIA ON DESQUAMATED SKIN

R. R. DAVIES
M.Sc. Wales, Ph.D. Lond.

W. C. NOBLE
M.Sc. Lond.

From the Wright-Fleming Institute of Microbiology, St. Mary's Hospital Medical School, London, W.2

MUCH attention has been paid to the way in which microorganisms, especially staphylococci, are spread in hospital wards. Noble (1961) reported that the mean equivalent diameter of air-borne particles carrying staphylococci was 13·5 μ*; and Lidwell et al. (1959) showed that, on average, these particles each carried about 4 viable units. These results suggest that the bacteria are not suspended freely in the air but are carried on other, larger particles. It has been widely assumed that textile fibres act as the carriers; although Rubbo et al. (1960) showed that the presence of bacteria in the air was not necessarily associated with complete textile fibres.

That bacteria can be recovered from the skin has been reported by many workers; moreover, in man, a complete layer of skin is desquamated every one or two days (Rothman 1954). Skin scales were found in the air of hospital wards and barracks as early as 1855 (Thomson 1855, Rainey 1855, Hewlett et al. 1861, Temple-Wright 1869, de Chaumont 1867). More recently Davies (1958) observed that the number of skin scales in the air was increased during bedmaking; and the possibility that bacteria are carried on scurf and skin scales was suggested by Gordon (1905) and Allison and Gunn (1932).

It seemed reasonable, therefore, to examine the air and dust of hospital wards to determine whether skin scales were present and whether viable bacteria could be detected on them.

* The equivalent diameter of a particle is the diameter of a sphere of unit density which has the same settling-rate in still air (calculated on the basis of Stoke's Law) as the particle in question (Bourdillon et al. 1948).

Fig. 1—Photomicrograph showing skin scales and textile fibres impacted from the air of a hospital ward. (×250.)

Methods

Air samples were taken by means of a slit-sampler (Bourdillon et al. 1941) and a cascade impactor (May 1945). Slides for the impactor were coated with petroleum jelly or silicone grease as an adhesive. Dusty surfaces were sampled by pressing similar slides upon them, and dust was also collected by picking it up on ' Sellotape ', which was then mounted on a microscope slide. Since there is no specific stain for keratin, the deposits were stained by the following methods: Gram's stain, Shorr's stain, iodine, Pressley's stain (Pressley 1958), and Millon's reagent.

Results

More than 50 samples of dust from undisturbed surfaces in obstetric, medical, and surgical wards (where both cotton and woollen blankets were in use) were examined under the microscope, and all showed the same picture: the deposit consisted largely of flake-like particles which morphologically resembled skin scales. A much smaller number of fibres was also seen.

Air samples were taken in these wards by means of a slit-sampler and a cascade impactor, and the deposits obtained showed a very similar picture; again the bulk of the deposit resembled skin scales in appearance (fig. 1).

Deposits sampled from the air and dust, and also skin scales from our arms, were impacted on to sticky slides and stained as reported above. The flake-like particles

Fig. 2—Photomicrograph of a skin scale in a slit-sampler deposit after 3 hours' incubation on nutrient agar showing a microcolony. (×1000.)

from both air and dust gave the same staining reactions as the skin from our arms. It seemed reasonable therefore to conclude that these flakelike particles were desquamated-skin scales.

Owing to the uneven and sometimes granular surface of the squames, it is difficult to identify individual micro-organisms on them with certainty. Skin scales from the air of hospital wards were therefore impacted on to the surface of nutrient agar, using a slit-sampler with the plate stationary, and the culture plates were incubated at 37°C for 3 to 5 hours. Coverslips on which a drop of methylene-blue in alcohol had been placed were laid on the traces and these were examined under the microscope. The micro-colonies seen were normally associated with the skin scales (fig. 2); more than 80 deposits on serum agar were examined and, of 45 micro-colonies which were sufficiently clear to photograph, only 1 was not associated with skin.

In contrast to the samples obtained in the hospital wards, samples taken from the air of a room which had been contaminated by a " simulated sneeze " (Bourdillon et al. 1948) showed a different picture. Many micro-colonies of streptococci and micrococci were observed that were not associated with particulate matter, although some were observed which were clearly associated with squamous cells. In a simulated sneeze, 25% of the particles which bear salivary streptococci have an equivalent diameter of less than 4 μ and only about 12% are greater than 18 μ (Lidwell et al. 1959). One might therefore

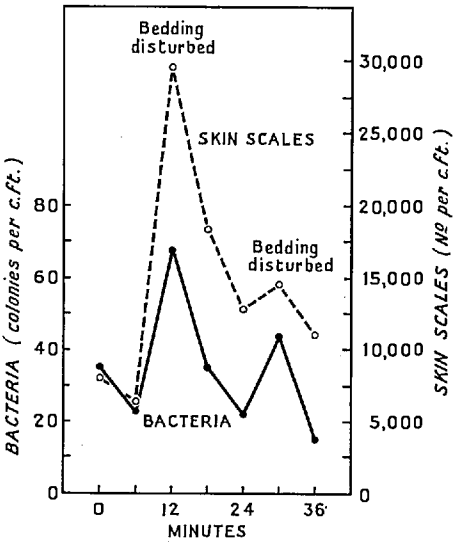


Fig. 3—The number of airborne skin scales and bacteria during disturbance of patients' bedding.

expect to find considerable numbers of streptococci unassociated with any particulate matter.

Dispersal of Skin Scales during Bedmaking

On 14 occasions samples were taken simultaneously by means of a slit-sampler and a cascade impactor during disturbance of patients' bedding. The rise in the number of airborne bacteria was accompanied by a corresponding rise in the number of skin scales recovered (fig. 3). During these experiments the ratio of skin scales to aerobic bacterial colonies was between 400 and 1700 to 1.

Bedding and clothing was " vacuum-cleaned " using an impactor and the deposits were examined under the microscope; in the deposit, textile fibres were few compared with the large number of skin scales.

Discussion

Our results suggest strongly that desquamation plays a major part in the dispersal of the bacteria which are carried on the surface of the body.

We have not so far identified precisely the bacterial species growing on the skin scales; the vast majority seen were cocci, but occasionally bacilli and chaining cocci were observed. Most of the organisms recovered from the

air of hospital wards by means of a slit-sampler with serum-agar as the culture medium and overnight incubation at 37°C, are cocci; thus of 1066 colonies examined from slit-sampler plates used in the wards, 90% were gram-positive cocci, 2% were gram-positive bacilli, and 7% were gram-negative bacilli.

It seems likely that salivary organisms, such as the majority of the streptococci, may be spread by droplet nuclei rather than on skin scales. The " cloud babies " described by Eichenwald et al (1960) dispersed staphylococcus-carrying particles of a much smaller diameter than is usual for staphylococci even in nurseries for the newborn (Cope et al. 1961), and it seems possible that this spread also was mediated by droplet nuclei. Bang (1961), however, has reported that, during virus infection, increased numbers of cells are desquamated from the upper respiratory tract, which in itself could lead to increased bacterial dispersion; the smaller apparent size of the respiratory-tract particles might be due to shrinkage of the non-cornified cells.

In the spread of staphylococci by dispersers of the type described by Hare and Ridley (1958) and Noble (1962) some factors require further investigation. These patients may have a heavier skin carriage of staphylococci, or they may desquamate more skin than do the non-dispersers.

Samples taken in the wards by means of the cascade impactor showed that the particles of skin had a mean equivalent diameter of approximately 8 μ, which is rather smaller than the diameter found for bacteria-carrying particles, but no estimate has yet been obtained of the mean equivalent diameter of the skin scales that bear viable microorganisms.

Summary

Examination of deposits obtained from the air of hospital wards by means of a slit-sampler and a cascade impactor revealed the presence of large numbers of skin scales. Brief incubation of these deposits showed that bacteria were carried on them.

It is suggested that most of the bacteria dispersed into the air of hospital wards from carriers and bedding are carried on desquamated skin.

REFERENCES

Allison, V. D., Gunn, W. (1932) *Proc. R. Soc. Med.* **25**, 927.
Bang, F. B. (1961) *Bact. Rev.* **25**, 228.
Bourdillon, R. B., Lidwell, O. M., Lovelock, J. E. (1948) *Spec. Rep. med. Res. Coun., Lond.* no. 262; pp. 6, 59.
— — Thomas, J. C. (1941) *J. Hyg., Camb.* **41**, 197.
Cope, A., Shooter, R. A., Green, S. M., Noble, W. C. (1961) *Brit. med. J.* ii, 329.
Davies, R. R. (1958) Moulds in Dust and Air. Ph.D. thesis, University of London.
de Chaumont, F. (1867) Army Medical Department, appendix to report for 1867; p. 265.
Eichenwald, H. F., Kotsevalov, O., Fasso, L. A. (1960) *Amer. J. Dis. Child.* **100**, 161.
Gordon, M. H. (1905) Annual Report of the Medical Officer of the Local Government Board for the Years 1903–4. H.M. Stationery Office.
Hare, R., Ridley, M. (1958) *Brit. med. J.* i, 69.
Hewlett, T. G., Stanley, St. J., Baynes, R. (1861) Army Medical Department. Sanitary Report for 1861; p. 380.
Lidwell, O. M., Noble, W. C., Dolphin, G. W. (1959) *J. Hyg., Camb.* **57**, 299.
May, K. R. (1945) *J. sci. Instrum.* **22**, 187.
Noble, W. C. (1961) *J. Path. Bact.* **81**, 523.
— (1962) *J. clin. Path.* **15**, 552.
Pressley, T. A. (1958) *Lancet*, ii, 712.
Rainey, G. (1855) Board of Health Report: Report on Cholera Epidemic of 1854; p. 134.
Rothman, S. (1954) Physiology and Biochemistry of the Skin; p. 586. Chicago.
Rubbo, S. D., Pressley, T. A., Stratford, B. C., Dixson, S. (1960) *Lancet*, ii, 397.
Temple-Wright, R. (1869) Army Medical Department, appendix to report for 1868; p. 251.
Thomson, R. D. (1855) Board of Health Report: Report on Cholera Epidemic of 1854; p. 119.

The Lancet Office,
7, Adam Street, Adelphi, London, W.C.2

DISPERSAL OF STAPHYLOCOCCI ON DESQUAMATED SKIN

Sir, - In our communication on the dispersal of bacteria upon desquamated skin (Davies, R.R., Noble, W.C. Lancet, 1962, ii, 1295), we stated that we had not determined precisely which bacterial species were carried on the skin-scales, although most of them were cocci.

Since then we have been able to identify the organisms by the micro-pipetting technique of de Fornbrune. Microcolonies seen on the skin-scales were picked off with a micro-pipette and manipulator; the pipette was broken off in broth; and after incubation the bacteria were identified.

We have investigated the aerial dispersal of Staphylococcus aureus by 5 patients. Skin-scales were impacted from the air by the methods previously described, and, in each instance, staphylococci of the same phage type as those recovered from the patients' skin by swabbing were obtained from micro-colonies which developed on skin-scales (see table).

AERIAL DISPERSAL OF Staphylococcus aureus

Case	Source of skin-scales	No. of micro-colonies		
		Subcultured in broth	Yielding growth in broth	Yielding Staph. aureus
1	Disturbance of bedding	22	15	9
2		21	14	9
3		8	8	2
4	Patient undressing	21	20	20
5		20	20	20

The results suggest strongly that the Staph. aureus carried on the surface of the body is dispersed mainly by desquamated skin and that the contaminated skin-scales are spread by bedding and clothing.

R.R. Davies
W.C. Noble

Wright-Fleming Institute of Microbiology,
St. Mary's Hospital Medical School,
London, W.2.

Published in The Lancet 1963 i 1111

J. Hyg., Camb. (1963), **61**, 385
Printed in Great Britain

The size distribution of airborne particles carrying micro-organisms

By W. C. NOBLE,* O. M. LIDWELL and D. KINGSTON
Cross-Infection Reference Laboratory, Central Public Health Laboratory, London, N.W. 9

(*Received* 9 *April* 1963)

The ability of a particle to remain airborne, its ability to pass through filters, the site at which it may be deposited in the respiratory tract and the rate at which it will be removed from the air by sedimentation are all dependent on the size and density of the particle. In the course of a variety of investigations we have determined the size distribution of particles carrying various species of bacteria and fungi, using the size-grading slit-sampler described by Lidwell (1959). A few of the results obtained have already been quoted in part, but the majority have not been published previously.

The air sampler used separates the airborne particles into four size ranges, each of which is deposited on the surface of the agar medium contained in one of four 6 in. Petri dishes. This apparatus is constructed so that the air sample, entering through a slit 7 mm. wide, impinges on to the surface of the first Petri dish at such a velocity that only the larger particles, i.e. those having an equivalent particle diameter† greater than about 18μ, are deposited. The air stream carrying the smaller particles is then caused to impinge in turn on to the surface of the three remaining Petri dishes, each time at an increased velocity and through a narrower slit, so that the minimum particle size for 50 % deposition is about 10μ for the second dish, 4μ for the third and less than 1μ for the fourth and last. When the plates have been incubated the colonies found will be derived from organisms which entered the sampler carried on airborne particles corresponding approximately to the four size ranges, greater than 18μ, between 18 and 10μ, between 10 and 4μ and less than 4μ. These size limits correspond to the value of equivalent particle diameter for 50 % deposition so that there is, in fact, a considerable size overlap between the fractions. In spite of this, however, reasonably good estimations of the particle size distribution within the sample and hence of the median equivalent particle diameter and of the dispersion, expressed either as an interquartile range or, if appropriate, as a standard deviation, can be made by plotting on probability paper the cumulative fraction oversize against the 50 % collection limits, namely 18·2, 9·6 and 4·2μ. As there are internal losses in the instrument it is necessary to correct the numbers of colonies counted in the later stages in order to arrive at a good estimate of the size distribution in the original sample. The numbers found on

* Now at The Wright-Fleming Institute, St Mary's Hospital, London, W. 2.

† The equivalent particle diameter is the diameter of a sphere of unit density which has a settling rate in air equal to that of the particle in question.

plates 2, 3 and 4 should be multiplied by 1·10, 1·20 and 1·25, respectively. These factors are derived from experimental observations.

CULTURAL METHODS

Samples for *Staphylococcus aureus* were collected on nutrient agar containing 5% horse serum and 0·1% phenolphthalein-phosphate (Barber & Kuper, 1951). Phosphatase positive colonies were tested for coagulase. Counts of total flora and of aerobic spore-bearing bacilli were made on the same medium but the phenolphthalein-phosphate was sometimes omitted.

Streptococci were grown on serum-sucrose agar containing tellurite and crystal violet (Williams & Hirch, 1950). The levan-producing colonies of *Str. salivarius* (Williams, 1956) were recognized by their colonial appearance and counted after 40–48 hr. incubation at 37° C. The other streptococci and the enterococci were estimated by picking a random sample of colonies from these plates and examining them by methods previously described (Air Hygiene Committee, 1954, Routine C, pp. 52–4).

Samples for *Clostridium welchii* were collected on a modified Nagler medium containing neomycin. Those colonies showing zones of serum opacity were regarded as *Cl. welchii* (Noble, 1961).

Sabouraud's dextrose agar containing antibiotics to suppress bacterial growth was used for isolating fungi. Cultures were incubated for 3 days at 37° C. for the *Aspergilli* and the *Candida* species and up to 3 weeks at room temperature for the remaining fungi. When sampling for *Candida albicans* or the dermatophyte fungi, Actidione (0·5 g./l.) was added to the medium.

RESULTS

The values deduced for the median equivalent particle diameters and for the inter-quartile ranges are given in Tables 1 and 2. These were obtained in the way described previously (Lidwell, 1959). Estimates of the inter-quartile ranges, the limiting diameters defining the 25% smallest and the 25% largest particles, are given, rather than standard deviations of the diameter, since the forms of the distributions are not known. In most cases an arithmetic-normal distribution appeared to fit the data reasonably well, where the median equivalent particle diameter was greater than 10μ; if the median diameter was smaller than this a log-normal distribution usually appeared the better. We have, however, no information about the tails of the distribution except that these must be truncated at or above about 1μ for the bacteria and at sizes corresponding to that of the single cells for the fungal species. Some environmental factors can be seen to affect the recorded values of median equivalent particle diameter. Ventilation preferentially removes the smaller particles so that the median diameter normally becomes greater when the ventilation is increased. The staphylococcal samples show a small but definite increase in median diameter with increasing activity during the sampling period. A similar, and partly associated, variation with the amount of air contamination is illustrated in Fig. 1.

Table 1. *Airborne bacteria*

Species	Place	Activity, etc.	Colonies counted	Median equivalent diameter of the airborne particles (μ)	Inter-quartile range (μ)
Total aerobic flora (grown at 37° C.)	Offices	Low ventilation	> 15,000	7·7	4–11
	Offices	Good ventilation	> 8,000	10·0	5–15
	Hospital wards	Moderate	> 50,000	12·8	7–18
	Hospital wards	Considerable	> 30,000	13·0	8–18
	Operating rooms	Unoccupied	> 30,000	12·3	7–18
Total mouth streptococci	Offices	Low ventilation	> 800	10·0	4–16
	Offices	Good ventilation	> 300	12·4	6–18
Streptococcus salivarius	Offices	Low ventilation	> 500	11·0	4–18
	Offices	Good ventilation	89	14·4	7–(22)
Beta-haemolytic streptococci	Offices	Low ventilation	29	11·7	8–15
	Offices	Good ventilation	22	12·5	8·5–16·5
Enterococci	Offices	Low ventilation	83	11·0	6–16
	Offices	Good ventilation	50	10·8	4–17
Staphylococcus aureus	Hospital wards	Light–moderate	> 6,000	13·3	8–18
	Hospital wards	Bedding disturbed	> 7,000	14·8	10–(19)
	Hospital wards	Bed-making	> 2,000	15·7	11–(20)
Bacillus sp.	Hospital ward	Moderate	> 300	(3·0)	(?)–8
Clostridium welchii	Outside air	Wet weather	186	11·0	5·5–16·5
	Outside air	Dry weather	> 500	17·2	10–(24)
	Hospital wards	Moderate (wet weather)	299	11·4	4–18

The inter-quartile range is given as the limiting diameters defining the 25% smallest and the 25% largest particles. Where the estimated diameters are below 4μ or above 18μ the values depend on extrapolation and have been given in brackets to indicate the greater possibility of error.

The samples of air from the offices were obtained in several different rooms of a large group of offices over a period of months.

The hospital samples came from a number of different hospitals.

Table 2. *Airborne fungi*

Species	Place and activity, etc.	Colonies counted	Diameter of spore or single cell (μ)	Median equivalent diameter of the airborne particles (μ)	Inter-quartile range (μ)
*Aspergillus fumigatus**	Hospital ward, moderate	> 100,000	2·5–3·5	(3·0)	[3–3]
Penicillium spp.	Hospital ward, moderate	> 750	2·5–4·5	(3·1)	[2–4]
Paecilomyces spp.	Hospital ward, moderate	105	(2·5–3) × 6	(3·4)	(2)–6
Rhodotorula spp.	Hospital ward, moderate	> 4,000	(3–5) × (4–7)	(3·8)	[3·3–4·3]
Aspergillus spp.†	Hospital ward, moderate	159	2·5–4	4·3	(2)–8
Cladosporium spp.	Hospital ward, moderate	> 2,500	(2–6) × (3–20)	4·9	(3)–7
Aspergillus niger	Hospital ward, moderate	125	2·5–10	5·5	(3)–9
Syncephalastrum spp.	Hospital ward, moderate	240	(2·5–5) × (5–20)	6·6	4–10
Rhizopus spp.	Hospital ward, moderate	45	3 × (6–9)	6·8	4–10
Monilia sitophila	Hospital ward, moderate	74	3 × (3–10)	9·5	7–13
Didymocladium spp.	Hospital ward, moderate	> 500	5 × (10–15)	10·7	7–15
Candida albicans	Hospital ward, moderate	67	(3–6) × (3–12)	13	5–(21)
Trichophyton mentagrophytes‡,§	Clinic, scraping and examination of skin	> 300	2 × 4 (*a*) (4–6) × (10–50) (*b*)	16	11–(22)
Rhodotorula spp.‖	Clinic, scraping and examination of skin	118	(3–5) × (4–7)	18	11–(26)
Epidermophyton floccosum§	College dormitories, little	18	(6–10) × (20–30)	19	12–(26)
E. floccosum	Clinic, scraping and examination of skin	172	(6–10) × 20–30)	(21)	15–(26)
Candida albicans	Clinic, scraping and examination of skin	> 1,000	(3–6) × (3–12)	(22)	18–(27)

The inter-quartile range is given as the limiting diameters defining the 25% smallest and the 25% largest particles. Where the estimated diameters are below 4μ or above 18μ the values depend on extrapolation and have been given in brackets to indicate the greater possibility of error involved. Substantial correction has had to be applied to the values of the quartiles for *Asp. fumigatus*, *Penicillium* spp. and *Rhodotorula* spp., found in hospital wards, on account of the diffuse cut-off of the stages in the instrument, and these are enclosed in square brackets. Where the spore or cell concerned is markedly non-spherical the approximate ranges of both the shortest and the largest axes are given in brackets, linked by a multiplication sign. In the case of *Trichophyton mentagrophytes*, (*a*) refers to the microconidia and (*b*) to the macroconidia.

* In seven separate evaluations the estimated median equivalent diameters ranged from 1·8 to 4·1 with an indication that the value tended to be larger in the winter.

† Including *Asp. gracilis*, *Asp. nidulans*, *Asp. terreus* and *Asp. versicolor*.

‡ This was derived from examination of an artificially contaminated arm.

§ Spores of these organisms were never seen in material obtained directly from patients. Only mycelium could be seen in the infected skins.

‖ This organism is not known to have been associated with any patient.

The hospital ward samples were obtained in various places on different occasions.

In comparing the figures for median equivalent particle diameter given here with those deduced from the ratio between volumetric and settling counts (Bourdillon, Lidwell & Lovelock, 1948) it must be remembered that these latter are primarily estimates of the mean settling rate, i.e. of the mean-square diameter. The relation between this quantity and the median diameter as determined here or by data

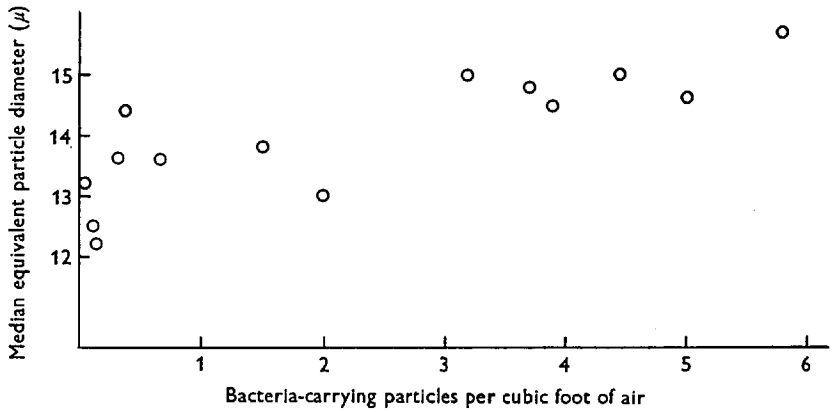


Fig. 1. Median diameters of airborne particles carrying *Staphylococcus aureus*.

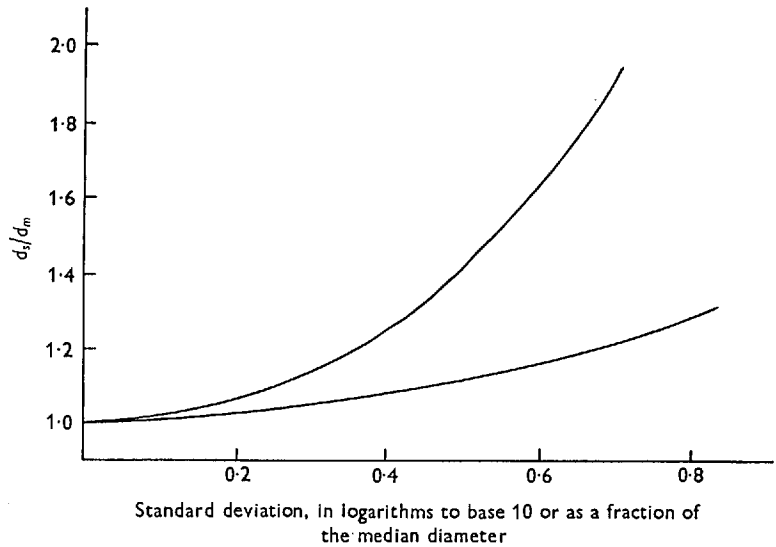


Fig. 2. Relationship between mean settling diameter, d_s, and median diameter, d_m. The upper of the two curves refers to log-normal distributions. The arithmetic-normal distributions, lower curve, are truncated at zero diameter and their standard deviations are expressed as a fraction of the median diameter.

obtained in a similar way, e.g. samples taken with the Andersen or Batelle samplers (Andersen, 1958; Mitchell & Pilcher, 1959), depends on the distribution of particle size in the sample. Fig. 2 gives values for the ratio of the two quantities for both arithmetic-normal and log-normal distributions over a range of values of the standard deviations of these distributions.

The following example illustrates these points.

At the same time as the samples of total flora were collected in the operating theatres with the size-grading sampler (see Table 1), settling plates were also exposed. The mean rate of settling over the whole series of experiments was 5·89 colony-forming particles per minute per square foot of surface exposed. The mean volumetric count over the same period was 4·90 cu.ft. of air sampled. This corresponds to a mean settling rate for the particles concerned of 5·89/4·90 = 1·20 ft./min. Using the figure of $0{\cdot}006d^2$ for the settling rate in feet per minute of a unit density particle d microns in diameter this corresponds to a mean equivalent settling diameter of $(1{\cdot}20/0{\cdot}006)^{\frac{1}{2}} = 14{\cdot}2\mu$.

The median equivalent diameter of the particles as determined by the size-grading sampler was $12{\cdot}3\mu$ and the distribution conformed well to a normal distribution in the arithmetic value of the diameter with a standard deviation of $8{\cdot}0\mu$. The ratio of this standard deviation to the median diameter is 0·65 and from the lower curve in Fig. 2 this would correspond to a median settling diameter 1·18 times the median diameter, i.e. $12{\cdot}3 \times 1{\cdot}18 = 14{\cdot}5\mu$, which agrees closely with the estimate obtained above by direct comparison of the settling and the volumetric counts, namely $14{\cdot}2\mu$.

The estimated standard deviations of the size distributions of the airborne bacteria-carrying particles which we have so far examined have almost always fallen within a fairly narrow range, about 0·3 for a log-normal distribution to the base 10 or about 0·6 times the median diameter for arithmetic-normal distributions. For standard deviations of this magnitude the mean settling diameter is about 15 % greater than the median diameter and this value may be used when comparing particle diameters estimated by the two methods where detailed information on the size distributions are not available.

DISCUSSION

The most striking fact revealed by these data is the size of the median equivalent diameter associated with almost all the bacterial species and with those fungi that are probably derived from a human source, that is, the dermatophyte species, *Trichophyton* spp. and *Epidermophyton* spp. and *Candida albicans*. These diameters, which are much greater than the dimensions of the microbial cell, imply that the organisms are usually disseminated into the air in association with material derived either from the menstruum in which they originally multiplied or from some intermediate resting place. There is some evidence (Williams, Lidwell & Hirch, 1956) that the streptococci are derived directly from the mouth and that the particle therefore largely consists of dried saliva. Davies & Noble (1962) have recently presented evidence that many, if not most, staphylococci are present in the air attached to skin scales which could account for the observed diameters. It also seems likely that infected skin scales are responsible for the dissemination of the dermatophyte fungi. The size distribution of airborne particles carrying micro-organisms is determined by two opposing factors, gravity, which tends to eliminate the large particles, and the chance that a particle will carry a viable organism, which is likely to increase with the size of the particle. These factors combine to

confine the distribution within a relatively narrow range over the main part of which, at any rate, the distribution approximates closely to the arithmetic normal.

In contrast many fungi such as the aspergilli, penicillia, cladosporia, among those included in the observations reported here, possess a mechanism for direct dispersal of their spores into the atmosphere from their natural growth sites. This is reflected in the close correspondence for many of these species between the median equivalent particle diameters found for the air sample and the size of a single spore. Within the observed portion these distributions approximate to a log-normal form.

The most marked exception to the general pattern described above is the particle diameter associated with the aerobic spore-bearing bacilli. This is not much larger than the single cell although there is a very wide spread of particle size. It is possible that this is a result of the capacity of these organisms to multiply in extremely dilute media, in which sporulation commonly occurs, so that when the spores are dispersed by mechanical action they are only very loosely bound together and embedded in only small amounts of dried material.

SUMMARY

Values are given for the median equivalent diameters and for the inter-quartile range, of airborne particles carrying a variety of micro-organisms.

Organisms associated with human disease or carriage were usually found on particles in the range 4–20μ equivalent diameter.

Many fungi appeared to be present in the air as single spores.

We should like to acknowledge the collaboration of Dr Yvonne M. Clayton in the collection and examination of the fungal samples.

REFERENCES

Air Hygiene Committee (1954). Air disinfection with ultra-violet irradiation: its effect on illness among school-children. *Spec. Rep. Ser. med. Res. Coun., Lond.*, no. 283, pp. 52–4.

Andersen, A. A. (1958). New sampler for the collection, sizing, and enumeration of viable airborne particles. *J. Bact.* **76**, 471.

Barber, M. & Kuper, S. W. A. (1951). Identification of *Staphylococcus pyogenes* by the phosphatase reaction. *J. Path. Bact.* **63**, 65.

Bourdillon, R. B., Lidwell, O. M. & Lovelock, J. E. (1948). Studies in air hygiene. *Spec. Rep. Ser. med. Res. Coun., Lond.*, no. 262, Appendices II and IX.

Davies, R. R. & Noble, W. C. (1962). Dispersal of bacteria on desquamated skin. *Lancet*, ii, 1295.

Lidwell, O. M. (1959). Impaction sampler for size grading airborne bacteria-carrying particles. *J. sci. Instrum.* **36**, 3.

Mitchell, R. I. & Pilcher, J. M. (1959). Improved cascade impactor for measuring aerosol particle sizes. *Industr. Engng Chem. (Indust.)*, **51**, 1039.

Noble, W. C. (1961). The size distribution of airborne particles carrying *Clostridium welchii*. *J. Path. Bact.* **81**, 523.

Williams, R. E. O. (1956). *Streptococcus salivarius* (vel *hominis*) and its relation to Lancefield's group K. *J. Path. Bact.* **72**, 15.

Williams, R. E. O. & Hirch, A. (1950). The detection of streptococci in air. *J. Hyg., Camb.*, **48**, 504.

Williams, R. E. O., Lidwell, O. M. & Hirch, A. (1956). The bacterial flora of the air of occupied rooms. *J. Hyg., Camb.*, **54**, 512.

J. gen. Microbiol. (1963), 32, 397–402
Printed in Great Britain

Fungi in the Air of Hospital Wards

By W. C. NOBLE*
Air Hygiene Laboratory,
Central Public Health Laboratory, Colindale, London, N.W. 9
and YVONNE M. CLAYTON
Mycology Research Laboratory, Brompton Hospital, London, S.W. 3

(*Received* 25 *February* 1963)

SUMMARY

The fungal flora of the air of hospital wards was investigated by using slit samplers. Isolations were made on Sabouraud glucose agar and particular attention was paid to the flora which grew at 37°. *Aspergillus fumigatus* was recovered on each of the 78 days of sampling and reached peak of incidence in the autumn and winter months; no other fungal species was recovered at 37° with such regularity. Bed sweeps revealed the presence of these fungi on blankets in the hospital wards. The mean equivalent diameter of the air-borne particles was determined for several fungal species and was found to correspond closely to that of individual spores. No dissemination of fungal particles by four patients with the hypersensitivity type of aspergillosis or by one patient with an aspergillus mycetoma was observed.

INTRODUCTION

Calendars illustrating the incidence of fungal spores in outdoor air at different times of the year have been prepared by several workers (e.g. Hyde, Richards & Williams, 1956). Examination of the fungi that can be isolated from the air of buildings has, however, been largely confined to those situations where specific fungi might be expected. Thus Ibach, Larsh & Furcolow (1954) reported the isolation of *Histoplasma capsulatum* from the air of a chicken house belonging to a farmer who had recently contracted histoplasmosis, and Stallybrass (1961) reported on the incidence of aspergilli in a grain mill. Clayton & Noble (1963) isolated *Candida albicans*, *Epidermophyton floccosum* and *Trichophyton mentagrophytes* from the air in the vicinity of patients being examined for fungal infections of the skin. Howe, Silva, Marston & Woo (1961) found that of 62 hospital blankets examined, 29 (47 %) yielded unspecified fungi. We know of no other studies carried out on the fungi recoverable from the air of hospital wards. The present report deals with such a study and investigates the size distribution of the fungal particles recovered, particular attention being paid to the flora which grew at 37°. The air of wards at St Bartholomew's Hospital was examined on 78 days between March 1960 and April 1961. Air sampling and blanket sweeps were also carried out at the Brompton Hospital around the beds of four patients with the hypersensitivity type of aspergillosis and one patient with an aspergillus mycetoma.

* Present address: Wright-Fleming Institute of Microbiology, St Mary's Hospital Medical School, London, W. 2.

METHODS

Slit-samplers were used which sampled at 5 cu.ft./min. on to 6 in. plates and were run for 1 hr. There was no serious overcrowding of the colonies when the plates were incubated at 37° but very much smaller volumes of air had to be sampled when the plates were to be incubated at 20–25°. The slit sampler gives a measure of the mean number of fungal and bacterial particles in the air and the results are conveniently expressed as the number of colonies obtained/cu. ft. of air sampled. The method estimates only the viable and not the total number of spores in the air.

Special samples were also taken on the size-grading slit-sampler (Lidwell, 1959) which divides the particles sampled into four fractions: particles greater than 18 μ 'equivalent diameter', particles between 10 and 18 μ, particles between 4 and 10 μ and particles less than 4 μ. From these data the 'mean equivalent diameter' can be calculated. The equivalent diameter of a particle is the diameter of a sphere of unit density having the same settling rate in still air (calculated by using Stokes's Law) as the particle in question (Bourdillon, Lidwell & Lovelock, 1948).

Blankets in use in the wards were examined by the sweep-plate technique of Williams, Blowers, Garrod & Shooter (1960).

The medium used was Sabouraud glucose agar containing streptomycin or neomycin to suppress bacterial growth. Preliminary tests with a slit sampler and with 'settle' plates indicated that more colonies were recovered at 37° on the Sabouraud agar than on malt agar or on Czapek-Dox agar. Since the main interest of the study lay in the Aspergillus species, plates were incubated at 37° but sometimes room temperature (20–25°) was also used.

RESULTS

Isolation of Aspergillus fumigatus *from the air*

A total of more than 96,000 cu. ft. of air was sampled over 78 days. *Aspergillus fumigatus* was recovered by culture at 37° on each of these days. During the spring and summer months the numbers of *A. fumigatus* particles isolated ranged from 0·01 to 0·2/cu. ft. In the autumn and winter months counts of up to 35 particles/cu.ft. were encountered for periods of up to 2 hr., whilst peak counts of 70 particles/cu.ft. were encountered during 10 min. sampling periods (Fig. 1).

Isolation of other fungi

In the samples recorded above, the only other fungus isolated at 37° with any regularity was *Aspergillus niger*. It was present on 32 of the 78 days, although never in such large numbers as was *A. fumigatus*, the highest count recorded being 0·2 *A. niger* particles/cu.ft. The other fungi recovered from these samples, apart from *Candida albicans*, are given in Table 1. A report upon the prevalence of *C. albicans* in the air is dealt with in a separate paper.

Some sample plates were also incubated at room temperature (20–25°). The various fungi recovered from these 20–25° plates but not from the 37° plates are given in Table 2.

The fungal colonies growing at 37° on Sabouraud agar generally represented about 1 % of the flora which would grow at 20–25°, which averaged about 10 colonies/cu.ft.

This ratio of 37° to room temperature growth fluctuated greatly, depending on the amount of *Aspergillus fumigatus* currently in the air.

The size-distribution of air-borne fungal particles

By using the size-grading slit-sampler the mean equivalent diameters of various air-borne fungal particles were calculated and are shown in Table 3. The mean equivalent diameter of these particles approximates to the diameter of spores measured microscopically. This suggests that a number of the air-borne particles exist as single spores and that the spores have a density close to unity.

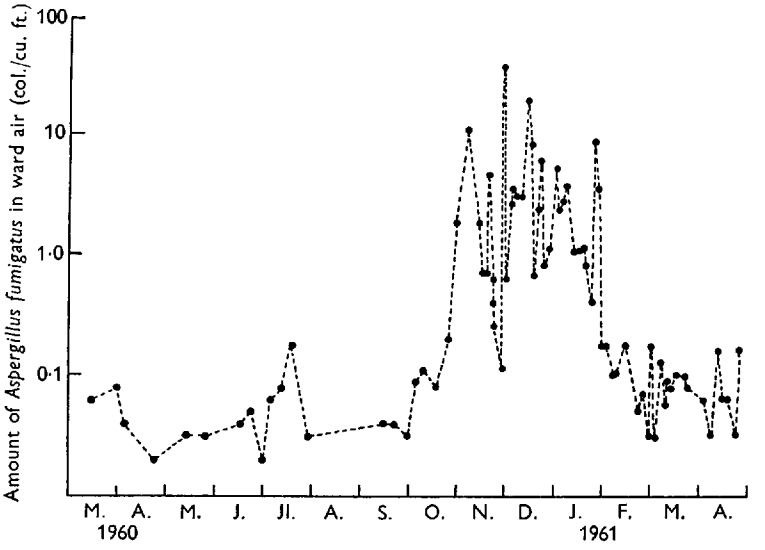


Fig. 1. Number of colonies of *Aspergillus fumigatus* recovered/cu.ft. from ward air over a 2 hr. sampling period

Table 1. *Fungi recovered on a total of* 78 *days' sampling from air samples incubated at* 37°

Fungus	No. of days fungus isolated	Fungus	No. of days fungus isolated
Alternaria	2	*Paecilomyces*	27
Aspergillus candidus	1	*Penicillium*	29
A. flavus	21	*Rhizopus*	13
A. fumigatus	78	*Scopulariopsis*	2
A. gracilis	2	*Sordaria fumicolor*	1
A. nidulans	17	*Syncephalastrum*	20
A. niger	32	*Trichoderma koningii*	5
A. tamarii	3	*Trichothecium*	2
A. terreus	13	*Verticillium*	2
A. ustus	1		
A. versicolor	2	*Chaetomium brasiliensis*	1
Botrytis cinerea	1	*Sporobolomyces*	1
Cunninghamella	1	*Rhodotorula*	1
Epicoccum nigrum	1	*Streptomyces*	3
Fusarium	1	*Mycelia sterilia*	—
Gliocladium	1		
Monilia sitophila	17		
Mucor racemosus	4		

Examination of blankets

Over a period of 6 weeks during the autumn and winter, 200 blankets in use in the wards were examined by the sweep-plate method. *Aspergillus fumigatus* was recovered from 79 (39·5 %) of the blankets examined, although the number of beds which yielded the fungus varied from week to week. Other fungi which grew at 37° were recovered from 83 % of the blankets; the majority of fungi recovered from the air were also obtained in small numbers from blankets.

Table 2. *Fungi recovered from air samples incubated at 20–25° but not recovered at 37°*

Aleurisma	*Phoma*
Aspergillus ochraceus	*Aureobasidium pullulans*
Cephalosporium	*Stysanus*
Cladosporium	*Trichophyton terrestre*
Didymocladium	*Trichosporon cutaneum*
Isaria	
Mucor pusillus	*Candida mycoderma*
M. spinosus	

Table 3. *Mean equivalent diameter of air-borne fungal particles*

Organism	Percentage of particles of given size range (μ)				Mean equivalent diameter of particle (μ)	Size range of single spore (μ)	No. of colonies counted
	> 18	10–18	4–10	< 4			
Aspergillus fumigatus	0·7	5·5	24·9	68·9	2·9*	2·5– 3	113,449
A. niger (group)	4·0	15·6	47·1	33·3	5·5	2·5–10	125
Aspergillus†	6·9	12·1	34·2	46·8	4·3	2·5– 3·5	159
Cladosporium	1·4	15·5	41·1	42·0	5·2	(2–6) × (3–20)	2,651
Didymocladium	14·5	49·2	31·6	4·7	10·2	5 × (10–15)	523
Monilia sitophila	8·3	39·1	49·7	2·9	8·9	3 × (3–10)	74
Paecilomyces	3·9	6·0	35·0	55·1	3·7	6 × (2·5–3)	105
Penicillium	0·7	6·3	26·8	66·2	3·0	2·5–4·5	798
Rhizopus	3·8	31·3	38·7	26·2	6·6	6–9 on long axis	45
Rhodotorula	0·3	5·3	37·9	56·5	3·8	(3–5) × (4–7)	4,039
Syncephalastrum	3·2	26·1	47·2	23·5	6·8	(2·5–5) × (5–20)	240

* This result is based on seven separate evaluations having the following values: 1·8, 2·3, 2·7, 3·2, 3·3, 4·0, 4·1. There is a suggestion that the mean equivalent diameter is greater in winter than in summer.

† These comprise *Aspergillus gracilis, A. nidulans, A. terreus, A. versicolor.*

Investigations in the environment of patients with aspergillosis

Aspergillus fumigatus was isolated from the blankets of four patients with the hypersensitivity type of aspergillosis and one patient with an aspergillus mycetoma. The number of colonies recovered on the sweep plates was no greater than on those from the surgical wards at St Bartholomew's Hospital. No increase in the mean equivalent diameter of the air-borne spores could be demonstrated when the blankets were shaken, as might have been expected if the spores had become

associated with organic debris or blanket fibre. The incidence of *A. fumigatus* in the air in the environment of these five patients with the fungus in their sputa was no greater than in air samples taken elsewhere in the hospital and outside the hospital at the same time. These particular investigations were carried out in the autumn.

DISCUSSION

Many workers have used the spore trap to determine the fungus species present in the air (e.g. Hamilton, 1959). Whilst this method is useful for studying allergens, it has the disadvantage that the Aspergilli and Penicillia cannot be differentiated. Another method which has been used was to expose agar plates and to allow organisms to settle on them ('settle' plates). De Vries (1960) and Stallybrass (1961) isolated Aspergilli in this manner, and Friedman, Derbes, Hodges & Sinski (1960) thus recovered dermatophytes from the air of a clinic. However, the number of spores which settle on the surface is dependent not only on their concentration in the

Table 4. *Terminal settling velocity of spherical particles of unit density in still air*

Mean equivalent particle diamcter (μ)	Settling rate (ft./min.)
30	5·4
20	2·4
15	1·3
10	0·6
5	0·15
2	0·024
1	0·006

The settling rates are calculated from the data given in Bourdillon *et al.* (1948).

air but also on the size of the particles (Table 4). A particle of 20 μ equivalent diameter settles at a rate of 2·4 ft./min. whilst one of 2 μ settles at only 0·024 ft./min. in still air, so that even very small air movements will suffice to keep the smaller particles air-borne. Richards (1954) recorded differences in the recovery of moulds indoors and outdoors which he attributed to differences in settling rates of spores of different sizes.

By using the size-grading slit-sampler we found that the mean equivalent fungus particle diameter was close to the diameter of a single spore. It seems reasonable to conclude that some of the air-borne particles are individual spores, though from their size, they might also be fragments of mycelium. Such fragments have been observed in the Hirst spore trap (Hamilton, 1959) but it is not known whether these were viable. Davies (1957) found that more than 80 % of Cladosporium particles recovered indoors were single spores and a further 13 % existed as groups of two spores. Clayton & Noble (report to be published) found that *Candida albicans* in the air of hospital wards had a mean equivalent diameter greater than that of individual organisms. The same is true for dermatophytes (Clayton & Noble, 1963) and for *Staphylococcus aureus* and *Clostridium welchii* in hospital wards (Noble, 1961). These micro-organisms are derived from patients in the wards and may be associated with organic debris.

The number of spores of any given fungus species recovered from the air varied from day to day and from season to season. The peak prevalence for *Aspergillus fumigatus* recorded in these studies was in the autumn, which corresponds with data collected by Hyde *et al.* (1956) by using settle plates. The failure to demonstrate dissemination of *A. fumigatus* by five patients with aspergillosis may have been due to the fact that any increase in fungal spores in their environment was masked by the large numbers of spores already present in the air in the autumn when examinations were made, but equally, such patients may not disperse the fungus.

We are indebted to the Treasurer and Governors of St Bartholomew's Hospital and to the physicians and surgeons of the Brompton Hospital for permission to work in the wards. One of us (Y.M.C.) was in receipt of a grant from the Asthma Council.

REFERENCES

BOURDILLON, R. B., LIDWELL, O. M. & LOVELOCK, J. E. (1948). Studies in air hygiene. *Spec. Rep. Ser. med. Res. Coun., Lond.* **262**, pp. 6, 249.

CLAYTON, Y. M. & NOBLE, W. C. (1963). Airborne spread of dermatophytes and *Candida albicans*. *Trans. St John's Hospital Derm. Soc.* **48**, 36.

DAVIES, R. R. (1957). A study of air-borne Cladosporium. *Trans. Brit. mycol. Soc.* **40**, 409.

DE VRIES, G. A. (1960). *Aspergillus fumigatus* and actinomycetes in air. *Acta allerg., Kbh.* **15**, 99.

FRIEDMAN, L., DERBES, V. J., HODGES, E. P. & SINSKI, J. T. (1960). The isolation of dermatophytes from the air. *J. invest. Dermat.* **35**, 3.

HAMILTON, ELIZABETH D. (1959). Studies on the air spora. *Acta allerg., Kbh.* **13**, 143.

HOWE, C. H., SILVA, T. F., MARSTON, A. T. & WOO, D. D. B. (1961). Staphylococcal contamination of mattresses and blankets on a surgical ward under non-epidemic conditions. *New Engl. J. Med.* **264**, 625.

HYDE, H. A., RICHARDS, M. & WILLIAMS, D. A. (1956). Allergy to mould spores in Britain. *Brit. med. J.* i, 886.

IBACH, M. J., LARSH, H. W. & FURCOLOW, M. L. (1954). Epidemic Histoplasmosis and air-borne *Histoplasma capsulatum*. *Proc. Soc. exp. Biol., N.Y.* **85**, 72.

LIDWELL, O. M. (1959). Impaction sampler for size-grading air-borne bacteria carrying particles. *J. sci. Instrum.* **36**, 3.

NOBLE, W. C. (1961). The size distribution of air-borne particles carrying *Clostridium welchii*. *J. Path. Bact.* **81**, 523.

RICHARDS, M. (1954). Atmospheric mould spores in and out of doors. *J. Allergy*, **25**, 429.

STALLYBRASS, F. C. (1961). A study of Aspergillus spores in the atmosphere of a modern mill. *Brit. J. industr. Med.* **18**, 41.

WILLIAMS, R. E. O., BLOWERS, R., GARROD, L. P. & SHOOTER, R. A. (1960). *Hospital Infection*. London: Lloyd-Luke.