

112

STUDY OF PATHOGENESIS OF

STAPHYLOCOCCAL INFECTION

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ABSTRACT

The dynamics of staphylococcal infection in mice has been studied by estimating the inflammatory response and the bacterial population changes in the lesion at intervals after the subcutaneous inoculation of cocci mixed with cotton-dust. The infection-enhancing activity of cotton-dust was found to be correlated with its ability to cause a slight delay in the onset of inflammatory reaction which in its turn led to a rapid growth of cocci in the lesion.

There was found to be some variation in the severity of lesion production in mice by strains of staphylococci injected along with cotton-dust. Studies were made of the inflammatory response of the mice, and the bacterial counts in the lesion, resulting from inoculation with different strains of staphylococci. Strains of Staphylococcus aureus producing severe lesions in mice elicited the inflammatory response with an initial delay which in addition to cotton-dust seemed to be due to some material associated with the cocci themselves. Alpha haemolysin was found to be of importance in the later stages of lesion production.

Mice having a past experience of staphylococcal infection showed an altered reactivity to further infection. These mice showed an accelerated inflammatory response, increased bacterial destruction and reduced necrosis after the injection of staphylococci. The accelerated inflammatory response and the resistance to dermonecrosis was found to be mediated by circulating antibodies

as these could be passively transferred by serum from 'previously-infected' to new mice. Both alpha haemolysin and some material associated with staphylococci seemed to be involved in sensitizing the mice so that the inflammatory response after reinfection was accelerated. Transfer of living lymphoid cells from the 'previously-infected' to normal mice was found to confer some increased susceptibility to staphylococcal infection in the treated mice.

The enhancement of staphylococcal infection by anti-inflammatory agents was to some extent due to a reduction in one or both components of inflammation at 4 hours.

4

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C O N T E N T S

ABSTRACT	2
ACKNOWLEDGEMENTS	4
I INTRODUCTION	9
II DEFINITIONS	11
III REVIEW OF PREVIOUS WORK	12
1. Methods of determining pathogenicity and virulence	12
A. In man	12
(1) Epidemiological studies	12
(2) Experimental studies	13
B. In animals	14
C. Correlation of pathogenicity and virulence with in vitro tests	17
2. Role of structural components of <u>Staphylococcus aureus</u> in pathogenesis	26
3. Host factors influencing the staphylococcal lesion	31
A. Non-specific humoral factors.	31
B. Specific humoral immunity	33

C. Phagocytosis	37
D. Hypersensitivity	38
E. Factors that alter host resistance to infection	39
1. Local trauma	39
2. Hormones and host resistance	40
3. Influence of various disease processes	40
4. Nutrition	41
5. Inflammation and its modification	41
Chemotaxis	43
Anti-inflammatory agents	45
IV AIMS OF THE PRESENT INVESTIGATION	51
V MATERIALS AND METHODS	53
Method of producing subcutaneous abscess .	53
Method of scoring lesion severity	55
Study of acute inflammatory response	56
Bacterial counts in the lesion	61
Anti-inflammatory agents	62
Passive transfer of immunity and hypersensitivity	64

VI	EXPERIMENTAL RESULTS	..	..	..	..	..	66
	1. The infection-enhancing effect of cotton..						66
	2. The effect of varying dosage of <u>Staphylococcus aureus</u> PS 80 with cotton-dust						71
	3. Response to different strains of staphylococci and micrococci						76
	4. Staphylococcal factor causing initial delay in inflammation						84
	5. The response of 'previously-infected' mice to staphylococcal infection						86
	6. Passive transfer experiments						93
	7. Further investigations into the response of 'previously-infected' mice						97
	8. The effect of anti-inflammatory drugs on the course of staphylococcal infection in mice.						100
	9. The effect of anti-inflammatory drugs on subsequent reinfection with staphylococci.						106
	10. The effect of anti-inflammatory drugs on 'previously-infected' mice						107
	11. The effect of endotoxin on staphylococcal infection						110
VII	DISCUSSION	..	..	..	..	..	113
	The role of cotton-dust in producing staphylococcal infection						114
	The response to different strains of staphylococci.						118
	Response of 'previously-infected' mice to staphylococci						126

	Type of blood vessels affected during staphylococcal inflammation	131
	The effect of anti-inflammatory drugs and endotoxin on staphylococcal infection..	132
VIII	CONCLUSIONS	136
IX	APPENDIX	138
	Media	138
	Depilating powder	138
	Glycerine jelly	139
	Ink-gelatine mixture	139
	Staining procedures.. .. .	139
	REFERENCES	141

I INTRODUCTION

The characteristic staphylococcal lesion in man is the subcutaneous or soft tissue abscess. The mechanism by which Staphylococcus aureus establishes itself and produces a lesion is not properly understood and although this organism produces a large number of biologically active extracellular toxins and enzymes, the part they play in the evolution of a staphylococcal abscess remains unknown. One important factor that influences the outcome of the staphylococcal lesion is the reaction of the host. If we are to understand the factors in the host and in the staphylococci that determine the pathogenesis of infection, a suitable experimental model has to be used for the study. The ideal method for determining the mechanism of staphylococcal infection in man would be to infect man experimentally. But there are severe limitations in doing such experiments and usually animals have been used. However, great caution is needed in interpreting the results obtained from animal experiments as relevant for human infections.

For a study of the pathogenesis of the commonest type of staphylococcal lesion, the cutaneous abscess, techniques that involve the injection of very large numbers of cocci by intravenous or intraperitoneal route are not suitable. But in order to produce a cutaneous abscess in experimental animals it is necessary to inject more than a million cocci. Quite clearly, it is doubtful if such a high dose of Staphylococcus aureus can be initially introduced

in most natural infections. However in the presence of cotton dust, subcutaneous abscesses can be produced in mice with as few as 100 staphylococci (Noble 1965). Noble's experimental model is particularly suitable because some correlation has been shown to exist between the "virulence" of the staphylococci for mice as tested by this method, and their "epidemicity" for man.

This thesis describes a study of the inflammatory response of the mouse to subcutaneous staphylococcal infection. Particular attention has been given to the early stages of the inflammatory response, because of the considerable body of evidence indicating that the period immediately following infection is the most critical in deciding the final outcome of the lesion.

In order to analyse the progress of the lesion, various components of the acute inflammatory response have been studied in relation to: differences between strains of staphylococci, the animal's past experience of staphylococcal infection and the effect of anti-inflammatory drugs. The components of acute inflammation selected for analysis were fluid exudation, leucocyte emigration, damage to blood vessels and the bacterial population changes in the local lesion at various intervals after infection.

II DEFINITIONS

Before discussing the published work on the pathogenesis of staphylococcal infection, it is desirable to clarify the meaning of some of the terms used in the text.

The term pathogenesis refers to the mechanism by which a microorganism causes disease in a particular host. The terms pathogenicity and virulence have commonly been used as synonyms, but, as Miles (1955) has pointed out, a useful distinction can be made between the terms. Pathogenicity may be regarded as a general attribute of a species or genus or some other grouping of microorganisms and refers to the capacity of microorganisms to cause disease in a given host species. Virulence indicates the degree of pathogenicity of a given stable homogenous strain of a microbe in a particular host species under defined conditions.

The term Staphylococcus aureus has been used for those Gram-positive and catalase-positive cocci that are able to produce coagulase, phosphatase, acid from glucose when incubated anaerobically and from mannitol both aerobically and anaerobically. When the term "staphylococcus" is used in the text without qualification, it is taken to mean Staphylococcus aureus. The term "micrococcus" is used without qualification for the Gram-positive, catalase-positive cocci that are unable to form acid from glucose anaerobically (Baird-Parker 1963, 1965). The term "Coagulase-negative staphylococcus" is used for those Gram-positive and catalase-positive cocci that form acid from glucose anaerobically but are unable to produce coagulase.

III REVIEW OF PREVIOUS WORK

1. Methods of determining pathogenicity and virulence

(A) In man

(1) Epidemiological Studies

There is epidemiological evidence that strains of Staphylococcus aureus differ in their ability to cause disease in man. Some phage types of staphylococci are more frequently responsible for epidemics in hospitals than others (Williams 1959, Williams and Jevons 1961, Parker and Jevons 1963); these strains are designated as "hospital" or "epidemic" strains. Phage typing can thus be of value in recognizing "epidemic" staphylococci. However, virulence judged from epidemiological studies may not be necessarily due to differences in the ability to produce disease. There is a possibility that a strain may be fully virulent but may lack some factors leading to "communicability" (Williams 1963a).

In order to discover the "virulence" factor, the properties of staphylococcal strains from carrier and lesion sites have often been compared (Williams 1963a,b). Statistically there seems to be some difference in the distribution of phage types amongst staphylococcal strains from nose and lesion sites. But epidemic types occur frequently amongst patients' noses without the production of any septic complication so that the straightforward comparison of strains from lesions with strains from carrier sites can not be easily interpreted.

(2) Experimental Studies

Experimental staphylococcal infection in man requires either an injection of large numbers of staphylococci or tissue damage at the site of inoculation. Garre (1885) seems to have been the first to test the virulence of staphylococci for man. The procedure was, however, dangerous before antibiotics were available.

With the advent of antibiotic therapy the question of pathogenicity of staphylococcus for man was restudied (Elek 1956, Elek and Conen 1957). It was shown that the skin of normal humans is highly resistant to staphylococcal infection. The average pus forming dose by intradermal injections was 2 to 8 x 10⁶ staphylococci. Strains from lesions in man or from healthy carriers did not clearly differ in virulence. On the other hand, the minimum pus-forming dose in normal skin could be reduced to 10² cocci if organisms were impregnated on a silk suture that was tied through the skin and left in place.

That local tissue damage reduces the infecting dose of staphylococci for man is also shown by another experiment (Foster and Hutt 1960). An overnight broth culture of pathogenic staphylococcus was inoculated into a scarified area of skin by means of a platinum loop and the inoculum was covered under a sealed cover slip. With this technique as few as 15 organisms produced a lesion. Foster and Hutt could not detect any difference in the

virulence of 5 strains of coagulase positive staphylococci by this test; coagulase negative staphylococci produced seropurulent lesions without erythema.

Virulence tests in man are, however, fraught with dangers. Even with the use of antibiotics, severe complications may arise. Therefore for detailed studies animals have been used.

(B) In animals

Most laboratory animals are relatively resistant to infection with Staphylococcus aureus. The animals commonly employed for the experimental staphylococcal infection include guinea pigs, rabbits and mice. The nature of the effect chosen to indicate virulence varies in different experimental models but has very commonly been death of the animal. Although death usually provides the most secure end point, death from staphylococcal infection produced by injecting cocci intraperitoneally, intravenously or intracerebrally (Cohn 1962a, Fisher and Goshi 1963, Smith 1962 and Corrill and McNeil 1963) is due to the lethal toxin and these tests may well throw little light on the mechanism of abscess formation.

Similarly experimental procedures, such as the intraperitoneal implantation of sacs (Gladstone and Glencross 1960, Lam et al. 1963) or diffusion chambers (Houser and Berry 1961), injection of the chick embryo (Sonea and Frappier 1954, McCabe 1964, Lyles et al. 1965) or injection of cocci in mouse testis (Blumenthal 1964) give very

little information relevant to the production of cutaneous abscesses.

More realistic experimental procedures include the injection of cocci by intramuscular, intradermal and subcutaneous routes.

(1) Intramuscular inoculation

Intramuscular injection of more than 10^6 staphylococci in mice induces local edema and sometimes ulceration through the skin. The intensity of the reaction, as determined by the degree of swelling has been used as a criterion of the virulence (Selbie and Simon 1952).

(2) Intradermal inoculation

By the intradermal route, not less than 10^7 Staphylococcus aureus are needed to produce small necropurulent lesions in the mouse or rabbit (Johnson et al. 1961, Fisher 1962).

(3) Subcutaneous inoculation

Like the skin and muscle the subcutaneous tissue of mice exhibits a high degree of resistance to infection with Staphylococcus aureus (James and MacLeod 1961, Fisher and Robson 1963). More than 10^6 are needed to produce a lesion. Similar doses are required for guinea pigs (MacLeod, Hall and Freeman 1963).

The above experimental procedures are open to the criticism that large numbers of cocci are required to cause infection. It

seems unlikely that, in the production of natural staphylococcal disease, such a high dose of cocci can be introduced initially to the site of lesion. Hence, many artifices have been designed to enhance the pathogenicity of Staphylococcus aureus and thereby reduce the infecting dose of cocci.

(4) Potentiating action of a foreign body

The suture technique used for enhancing Staphylococcus aureus infection in man is also applicable to experimental animals. James and MacLeod (1961) were able to produce cutaneous abscesses consistently in mice with as few as 10 - 100 coagulase positive staphylococci by introducing them on sutures. Of the suture materials used silk, silicone-treated silk, cotton and dacron were most effective in potentiating the infection. No difference in virulence could be detected amongst 22 coagulase-positive staphylococci, but the number of mice used to test individual strains was too small to yield firm conclusions. There were also some drawbacks in this experimental procedure; the sutures were sometimes lost during the experiment thereby affecting the severity of lesions, and there was difficulty in assessing the dose of bacteria inserted on the suture especially when a small inoculum was used. Noble (1965) overcame these difficulties with a technique whereby subcutaneous abscesses could be produced in mice using as little as 10^2 staphylococci inserted on plugs of cotton dust. This

is the method used in the present study.

(5) Other potentiating procedures

Necrosis of the rabbit skin produced by a thermal burn was found to result in a striking increase in local infectivity of Staphylococcus aureus when the infecting dose was given within 2 or 3 days after burning (Goshi, Cluff and Johnson 1961, Goshi, Cluff, Johnson and Conti 1961).

Administration of adrenaline (Evans, Miles and Niven 1948, Miles and Niven 1950), non-lethal doses of endotoxin (Conti, Cluff and Scheder 1961) and levan (Shilo 1962, Shilo 1964) have also been used to increase the infectivity of staphylococci.

(C) Correlation of pathogenicity and virulence with in vitro tests.

There are certain characters which are regularly present in all strains of Staphylococcus aureus and absent from coagulase negative staphylococci and micrococci. These characters have been thought to be associated with pathogenicity. Let us examine these characters one by one.

(i) Pathogenicity and in vitro tests

(1) Coagulase

That coagulase plays a role in the pathogenesis of infection was suggested by the observation that the correlation between coagulase production and pathogenicity holds only when the

plasma of the animal concerned is coagulable (Smith and Hale 1944, Smith 1963). However, it has subsequently been shown that strains of Staphylococcus aureus varying in their ability to clot guinea pig plasma do not vary in their capacity to produce disease in this animal (Foster 1962).

The role of coagulase in temporarily interfering with phagocytosis by contributing to the fibrin barrier has been both affirmed (Hale and Smith 1945) and denied (Rogers and Tempestt 1952). Injection of large amounts of coagulase intravenously in rabbits has been shown to produce fibrinogen depletion and intravascular clotting (Smith and Johnstone 1958, Blobel and Berman 1961). However, extensive intravascular clotting does not accompany severe or fatal staphylococcal disease (Fisher 1956).

Coagulase has also been shown to counteract the action of an antistaphylococcal factor present in the normal human serum, thereby permitting the growth of pathogenic strains (Ekstedt 1956, Yotis 1962, 1963). Although there is a possibility that more coagulase is produced in vivo than in vitro, this was not supported by the work of Gladstone and Glencross (1960). Moreover, no close relation has been established between amount of coagulase produced and the relative virulence of different strains of Staphylococcus aureus (Elek 1959).

In order to define the role of coagulase, some workers have used laboratory-derived mutants of Staphylococcus aureus lacking

coagulase (Li and Kapral 1962, Karas and Kapral 1962).

Coagulase negative mutants of staphylococci were shown to be as pathogenic for rabbits and mice as the parent strains. Great caution is needed in such experiments and the coagulase negative "mutants" described by Korman (1963) which differed from parent strains in coagulase production, fermentation of mannitol and galactose and the structure of cell wall cannot readily be accepted as true "mutants" without much more evidence.

Finally coagulase negative strains of staphylococci may be responsible for disease processes (Jones, Deibel and Niven 1963 and Torres Pereira 1962). These observations do not exclude coagulase as a pathogenic factor, but probably emphasize the fact that virulence is due to several factors whose relative importance is governed by the circumstances of the infection.

(2) Alpha haemolysin

The classical biological effects of alpha haemolysin are lethality, dermonecrosis and in vitro haemolysis of some red blood cells. It has now been demonstrated that these activities are due to one substance (Kumar et al. 1962, Bernheimer and Schwartz 1963).

There has been a discussion as to whether alpha haemolysin produces necrosis of cutaneous tissue directly (Elek 1959) or

through production of prolonged spasm of blood vessels (Thal and Egner 1954). But the fact that necrotic changes have been observed in explants of skin (Lawrence 1959) indicates that necrosis is probably not produced by circulatory occlusion. This is in agreement with the work of Goshi, Smith, Cluff and Norman (1963).

Alpha haemolysin produces spasm and paralysis of both smooth and skeletal muscles (Thal and Egner 1961, Lominski et al. 1962); it can damage a variety of tissue culture cells (Korbecki and Jeljaszewicz 1965) as well as platelets (Siegel and Cohen 1964; Bernheimer and Schwartz 1965) and lysosomes (Bernheimer and Schwartz 1964). It kills rabbit leucocytes, but human leucocytes in contrast are resistant (Gladstone et al. 1957). There is evidence to suggest that the primary action of alpha haemolysin is on cell membranes (Bernheimer 1965) and the damage to the cell results in a decrease in glucose utilization and lactic acid production (Jeljaszewicz et al. 1965). Korbecki and Jeljaszewicz (1965) noted that alpha haemolysin has a synergic effect in producing cytotoxic effect on tissue culture cells by staphylococcal deoxyribonuclease. This observation suggests that simultaneous action of several staphylococcal toxins may play an important role in the pathogenicity of Staphylococcus aureus.

The effect of alpha toxin on various tissue cells would seem

to indicate that it should be involved in abscess formation. But there is no direct experimental evidence to suggest that it plays any important role in the pathogenesis of lesions characterized only by abscess formation (Goshi, Cluff and Johnson 1961 and Foster 1963). It does not increase the ability of a given inoculum to produce a pyogenic lesion (Elek and Conen 1957), and organisms suspended in saline have been found to be no less virulent than those suspended in culture medium containing alpha toxin (Noble 1965). However it does seem to be the cause of skin necrosis in staphylococcal lesions (Goshi, Smith, Cluff and Norman 1963, Noble 1964). Noble (1964) found that the severity of the staphylococcal lesions in mice was not correlated solely with the production of alpha haemolysin in vitro and other enzymes particularly beta lysin and fibrinolysin seemed to play a supporting role. However, it has been shown that staphylococci cultivated in vivo produce much higher quantities of alpha toxin than in vitro (Gladstone and Glencross 1960, Houser and Berry 1961).

(3) Beta haemolysin

Beta haemolysin production is typical of staphylococci isolated from animals (Burns and Holtman 1960). The fact that beta haemolysin production is uncommon in human strains (Williams and Harper 1947, Elek and Levy 1950) means it can not be an essential factor in pathogenicity.

It has now been purified (Chesbro et al 1965, Wiseman 1965) and the purified toxin is not lethal for rabbits and mice. It is strongly cytotoxic for tissue culture cells (Korbecki and Jeljaszewicz 1965) and damages rabbit platelets (Bernheimer and Schwartz 1965). Beta Haemolysin seems to affect the lipid metabolism of susceptible cells (Magnuson et al. 1962, Gulasekharan et al. 1963). It has also been suggested that it damages the cellular membrane (Korbecki and Jeljaszewicz 1965). The exact role of beta haemolysin in the development of staphylococcal lesion is not known. However beta haemolysin has some potentiating effect on other staphylococcal toxins, e.g., it renders red cells more susceptible to staphylococcal delta lysin (Williams and Harper 1947); enhances the leucolytic action of delta haemolysin (Gladstone and van Heyningen 1957) and accelerates skin necrosis in rabbits when injected along with alpha haemolysin (Chesbro et al. 1965). Although beta haemolysin production is a characteristic of animal strains, recently strains of a new "epidemic" type of Staphylococcus aureus from human lesions have been shown to produce beta haemolysin (Willis et al. 1963).

(4) Delta haemolysin

Almost all human strains of Staphylococcus aureus produce delta haemolysin (Williams and Harper 1947, Elek and

Levy 1950). Besides its action on red blood cells, it produces dermonecrosis (Marks and Vaughan 1950) and lyses human leucocytes (Gladstone and van Heyningen 1957). It is possible that delta haemolysin may be involved in the pathogenicity of Staphylococcus aureus.

(5) Leucocidins

There are at least three distinct staphylococcal leucocidins (Gladstone and van Heyningen 1957). The alpha haemolysin, sometimes known as the Neisser Wechsberg leucocidin, attacks rabbit leucocytes and has no effect on human leucocytes. Delta haemolysin is active against rabbit, guinea-pig, human and mouse leucocytes. It is not only lethal to leucocytes but lytic as well. The third leucocidin, called as Panton-Valentine (P-V) leucocidin is cytotoxic for human and rabbit leucocytes. Woodin (1959) found that P-V leucocidin consists of F (fast) and S (slow) components and that both fractions are required for leucotoxicity. It is a distinct possibility that the cytotoxic effects of various leucocidins in vivo aids in the progression of staphylococcal disease.

(6) Other extracellular products

The role of various other enzymes of Staphylococcus aureus, e.g. hyaluronidase, deoxyribonuclease,

lipase and fibrinolysin, in pathogenicity is not yet recognized (Elek 1959). Enterotoxin which is responsible for staphylococcal food poisoning is not discussed in this review.

(ii) Virulence and in vitro tests

So far the characters of staphylococci that may be related to pathogenicity have been discussed. Some properties are limited to certain strains of Staphylococcus aureus and these have been tested for correlation with virulence.

Egg-yolk reaction has been claimed to distinguish virulent from non virulent strains. Virulence has been correlated with a positive (Alder, Gillespie and Thompson 1955) as well as a negative egg yolk reaction (Rosendal 1963, Noble 1964). However others regard this test to be of no value in distinguishing virulent strains (Willis and Turner 1962).

Resistance to mercury salts has also been correlated with the "epidemicity" of Staphylococcus aureus (Moore 1960). But it has been claimed that this test merely serves to identify staphylococci that have multiple antibiotic resistance rather than the "epidemic" strains (Willis, Jacobs and Goodburn 1963).

Resistance to antibiotics particularly penicillin and

tetracycline is a characteristic of most of the "epidemic" strains of staphylococci (Williams et al. 1962). It is likely that antibiotic resistance, instead of directly affecting the virulence of a strain, enables it to survive in the hospital environment. Moreover, there are instances of widespread nasal colonization of patients with antibiotic resistant Staphylococcus aureus in the absence of sepsis (Barber et al. 1953, Shooter et al. 1958).

Recently, it has been claimed that the metabolism of "virulent" staphylococci is different from avirulent cocci and the former exhibit a greater biochemical activity than the latter (Krynski, Kedzia and Kaminska 1964, Ivler 1965). Virulence of staphylococci has also been correlated with the nucleic acid content of the bacterial cell and the number of lines of precipitation obtained between antitoxin and staphylococcal strains (Howard 1954, De Repentigny, Sonea and Frappier 1962).

Correlation of in vitro tests with virulence has the essential drawback that bacteria may not produce in laboratory media the same antigens and enzymes as they produce in vivo. It is also very difficult to make an accurate determination of the virulence of a particular strain of Staphylococcus aureus.

2. The role of structural components of *Staphylococcus aureus* in pathogenesis.

The pathogenicity of *Staphylococcus aureus* seems to be due to the combined action of several bacterial factors whose effect is influenced by the reaction of the host and the circumstances of infection. Besides the extracellular factors, structural components of staphylococci also seem to play an important role.

(A) Capsular antigens

The Smith strain of *Staphylococcus aureus* has been extensively used for studying the virulence of staphylococci. This strain was isolated by Dubos (Smith and Dubos 1956a) and it produces two colonial variants in soft agar containing serum or plasma (Hunt and Moses 1958). The "diffuse" variant was found to be highly virulent for mice. These variants possess a definite capsule which can be demonstrated in electron micrographs (Koenig and Nelly 1965) and by negative staining (Mudd 1965). The capsular antigen is a polysaccharide whose major component is 2 - aminoglucuronic acid (Morse 1962a, Fisher, Devlin and Erlandson 1963, Perkins 1963 and Haskell and Hanessian 1963). The increased virulence of encapsulated staphylococci has been attributed to antiphagocytic properties of this surface antigen (Morse 1962a, Koenig 1962, Rogers and Nelly 1962). It also elicits a hypersensitivity reaction when

injected intracutaneously in rabbits immunized with a homologous strain of staphylococcus (Morse 1962a). The antigen has been recognized in only a few strains of Staphylococcus aureus which have, in common with the Smith strain, a mucoid colonial appearance, no recognizable bacteriophage type and absence of bound coagulase (Morse 1963).

Koenig and Melly (1965) screened 1,833 clinical isolates of Staphylococcus aureus and found only one strain of "diffuse variant". Therefore such rare strains may throw little light on the pathogenesis of staphylococcal infection. However, an attractive hypothesis has been put forward that other staphylococci may produce similar antigens during in vivo multiplication (Koenig, Melly and Rogers 1962b). Further support for this is the presence of opsonizing antibodies against the Smith diffuse strain in normal human serum (Rogers and Melly 1962), but the sera of rabbits immunized with strains other than Smith also react with the encapsulated Smith strain (Mudd and DeCourcy 1965) so the serological evidence is equivocal.

(B) Cell wall teichoic acid

The existence of a specific carbohydrate antigen of Staphylococcus aureus was recognized in the mid-1930's (Julianelle and Wiegand 1935a, Wiegand and Julianelle 1935). This

antigen was designated as polysaccharide A. After the elegant studies of Baddiley (1962) had defined the structure of Staphylococcus aureus teichoic acid, it was shown that polysaccharide A and Staphylococcus aureus cell wall teichoic acid are chemically and serologically similar (Haukenes 1962a, b, c). The N-acetylglucosamine of the teichoic acid is the determinant group for serological specificity (Morse 1962b, Nathenson and Strominger 1962) and may occur both in alpha and beta linkages (Baddiley et al. 1962). It is now established that the beta-glucosamine linkage is responsible for the precipitation line of polysaccharide A (Davison et al. 1964). Naturally occurring Staphylococcus aureus strains seem to have both alpha and beta specificities in varying amounts (Torri et al 1964).

It has been claimed that the phagocytosis promoting action of human serum against Staphylococcus aureus is removed by absorption with teichoic acid (Mudd et al 1963) and therefore it is suggested that antibodies against Staphylococcus aureus cell wall teichoic acid are required for efficient phagocytosis. Polysaccharide A also elicits an immediate cutaneous hypersensitivity reaction after intradermal injection in most adults (Julianelle and Hartmann 1936).

(c) Cell wall protein antigen

A protein antigen of Staphylococcus aureus was described

by Verway (1940). Later Jensen (1958) observed a group antigen of Staphylococcus aureus which he called antigen A. It has now been shown that Verway and Jensen both were dealing with the same antigen, which is protein in nature and has been purified (Lofkvist and Sjöquist 1962, Yoshida et al. 1963, Grov et al. 1964). Antigen A is present in most strains of Staphylococcus aureus with the notable exception of the Wood 46 strain (Horse 1965).

The part played by this antigen in pathogenicity is not known. Absorption of human serum with antigen A does not in any way reduce the opsonic properties of serum (Leshart et al. 1963). Similarly, high titre rabbit antisera against Smith diffuse strain after absorption with antigen A were still capable of passive protection of mice against infection with Smith strain (Ekstedt 1965). On the other hand, Stamp (1964) reported that antigen A conferred protection against intravenous challenge in rabbits.

(D) Other cellular antigens

Two protective antigens have been demonstrated in the Smith strain of Staphylococcus aureus (Ekstedt 1963 a,b); a polysaccharide antigen which is probably identical to teichoic acid (Ekstedt 1965) and a protein antigen whose identity is not established but is different from antigen A.

A cell wall "aggregasin" of Staphylococcus aureus has

been described by Fisher (1965). The aggressin was found in all of 10 coagulase positive staphylococci, that were examined; it was not present in coagulase negative strains. When 0.9 μ g of the aggressin was injected subcutaneously in mice mixed with coagulase-positive staphylococci, it had definite infection-enhancing activity. The aggressin did not appear to be identical with teichoic acid or with extracellular products present in toxic culture supernatant fluids of strain Wood 46.

There has been a suggestion that an endotoxin like substance is involved in the pathogenicity of Staphylococcus aureus. Staphylococcal extracts obtained by methods similar to those used with Gram-negative bacteria possess biological properties not unlike Gram-negative bacterial endotoxin (Arndt, Young and Ritts 1963). Staphylococcal extracts also produce cutaneous lesions when injected with epinephrine in the manner described for the endotoxin of Gram-negative bacteria (Neter et al. 1960).

Besides the known antigens of Staphylococcus aureus, there is a possibility of the existence of unrecognized antigens that have a role in pathogenicity. Several studies show the presence of uncharacterized antigens in Staphylococcus aureus (Bernheimer and Schwartz 1961, Oeding and Haukenes 1963, Kienitz and Schmelter 1964).

3. Host factors influencing the staphylococcal lesion

There is a considerable body of evidence that humans and animals exhibit a high degree of resistance to staphylococcal infection (Elek and Conan 1957, Johnson, Chaff and Goshi 1961, Fisher 1962, Gorrill and McNeil 1963, MacLeod, Hall and Frohman 1963). The mechanism of this resistance is not properly understood. It seems to depend on a complex system involving non-specific and specific defense factors.

(A) Non-specific humoral factors

It is now well established that human and animal sera possess antistaphylococcal activity (Spink and Vivino 1942, Fisher 1960, MacLeod et al. 1963) though the exact nature of the substance or substances in the serum responsible is unknown. Ekstedt (1956) suggested that it was a nonantibody, heat-stable component probably identical to the "beta-lysin" of Pettersson (1926). He found that, while coagulase-positive strains were serum resistant, coagulase-negative strains were serum susceptible. Later studies revealed that a water soluble globulin was the bactericidal fraction of the serum (Yotia and Ekstedt 1960) and that coagulase-negative staphylococci could be protected from it by treating them with coagulase. However other workers have been unable to show a reversal of inhibition in sera by coagulase (Myrwik 1956, Wlodarsak and Jeljaszewicz 1959).

The source of antistaphylococcal factor in the serum is not definitely known. Spink & Vivino (1942) found that the antistaphylococcal power of citrated human blood was due to the serum fraction and not to the leucocytes. Hirsch (1960a) found that serum obtained from blood drawn into siliconized tubes and centrifuged before clotting showed a much reduced antistaphylococcal activity as compared with serum obtained following normal clotting, and suggested that this might be due to the liberation of an agent during the breakdown of platelets.

Although lysozyme, histone and other tissue basic polypeptides have been shown to possess bactericidal activity against staphylococci in vitro (Skarnes and Watson 1957, Hirsch 1960b), their role in vivo infection is not clearly defined.

One of the main difficulties in the study of non-specific humoral factors to Staphylococcus aureus is their widespread occurrence, so that no animal species under normal conditions escapes repeated exposure. Recently, germ-free mice have been found to survive an acute staphylococcal infection as well as conventional animals, although they appeared to succumb to a chronic infection in slightly greater numbers (Ekstedt 1965). The presence of antibodies to staphylococci has also been reported in the sera of germ-free mice (Oehen et al. 1963). Bloods from colostrum-deprived and colostrum fed piglets have also been

compared for their ability to phagocytose strains of staphylococci. It was found that even with the markedly hypogammaglobulinemic colostrum-deprived piglet blood, phagocytosis was rapid and efficient with coagulase negative, Smith "diffuse" and Smith "compact" strains (Ekstedt 1965). However a comparison of bactericidal activity of sow and colostrum deprived piglet blood revealed that the latter was less bactericidal for all the above mentioned strains particularly Smith diffuse strain. It can therefore be concluded that the innate defence mechanisms are active against staphylococci.

(B) Specific humoral immunity

The extent to which acquired immunity participates in protection against staphylococcal infection is also not well defined. Despite the presence of antistaphylococcal antibodies to both extracellular and structural antigens in the sera of most adult human beings (Rogers and Nelly 1965, Ekstedt 1965) repeated staphylococcal infections are not uncommon. Most adults possess antibodies against alpha toxin (Lack 1957, Rogers and Nelly 1961, Quie and Wannemaker 1964), leucocidin (Mudd et al. 1962), coagulase (Rammelkamp and Lebovitz 1956), Jensen's antigen A (Jensen 1958), teichoic acid (Torri, Kabat and Besser 1964) and an opsonin promoting phagocytosis of encapsulated Smith strain of Staphylococcus aureus (Rogers and Nelly 1962).

No correlation between the titre of a particular antibody and resistance to staphylococcal disease has been discovered, with the possible exception of antihuman leucocidin (Butler and Valentine 1943, Gladstone et al. 1962). It is noteworthy that patients with active staphylococcal infection often do not have higher titres of a variety of antistaphylococcal antibodies than do normal adults (Lack 1957, Lack and Towers 1962, Nadoff and Weinstein 1962, Yoshida and Haden 1962, Lenhart et al. 1963, Ekstedt 1965); in patients with osteomyelitis very high titres of alpha antihemolysin may be obtained (Hite, Banks and Dack 1958).

(1) Antitoxic immunity

Although much work on staphylococcal immunity has been done on animals infected intravenously or intraperitoneally, comments will be confined to experiments where animals were infected by the intradermal or subcutaneous route.

Antibodies directed against alpha toxin are protective only in certain experimental models. For example, rabbits immunised with alpha toxoid are protected against lesion formation in necrotic tissue but not in normal skin (Goshi, Chuff and Johnson 1961). Goshi, Chuff and Norman (1965) present evidence to indicate that alpha antitoxin immunity facilitates bacterial phagocytosis, inhibits bacterial multiplication and prevents the necrotising effects of experimentally induced staphylococcal infection.

Noble (1964) has also demonstrated partial protection of mice by passive immunisation with staphylococcal antitoxin. The protection was mainly directed against the skin necrosis that characterizes the mature lesion and had no effect on abscess formation. On the other hand Taubler et al. (1963) could not find any specific protection by alpha antitoxin against lesion formation in mice.

Immunity to alpha toxin may be of less importance in man than in rabbits and mice for as Elek (1959) points out, alpha toxin destroys rabbit and mouse leucocytes but does not affect human leucocytes. In human beings there is no clear relationship between circulating antitoxin and clinical protection (Parish and Cannon 1960).

The role of antileucocidin in immunity was suggested by the observation that the incidence of staphylococcal disease in newborn children was inversely correlated with the antileucocidin titre of cord-blood (Johanovsky 1958a). Immunization with leucocidin toxoid seemed to reduce the incidence of disease (Sebek, Schubert and Johanovsky 1959). There are, however, no subsequent confirmatory reports.

Besides the well known extracellular factors of Staphylococcus aureus, a non-toxic mouse protective antigen has been described in the supernatant fluids of strain Wood 46 cultures (Robson and Fisher 1965). It gave an absorption spectrum suggesting the presence of nucleic acid and was heat labile.

(2) Antibacterial immunity

The present evidence indicates that both the protein and polysaccharide components of staphylococcal cell wall may act as antigens, inducing the formation of protective antibodies.

Mudd et al. (1965) have shown that the cell wall teichoic acid of Staphylococcus aureus will absorb the phagocytosis promoting action of human sera. In the case of mucoid strains such as Smith strain of Staphylococcus aureus, the surface antigen, which contains 70% carbohydrate, has an important role to play in the phagocytosis of such strains (Horse 1962a, Koenig et al. 1962b, Fisher, Devlin and Erlanson 1963). Absorption of antiserum by the Smith surface antigen removes opsonizing antibodies and immunization of mice with this antigen gives protection against an intraperitoneal challenge with the Smith strain suspended in mucin. Other studies show that a protein antigen is also involved in the protection of mice against the Smith strain (Ekstedt 1963b, 1965). The importance of antibacterial immunity was emphasized by Greenberg and Cooper (1960) who claimed that polyvalent vaccines prepared by dissolving suitably selected strains of staphylococci with dornase, protected hamsters and rabbits against challenge with strains of most types isolated from patients. The studies of Lam et al. (1965) also suggest that antibacterial antibodies are required for protection against abscess formation.

The relative importance of antibacterial and antitoxic immunity in staphylococcal infection is not clear. Koenig, Nelly and Rogers (1962a) point out that in mice challenged intraperitoneally with the Smith diffuse strain of Staphylococcus aureus, antibacterial immunity is of greater importance than antitoxic immunity. Animals immunized with heat-killed whole-cell vaccines were significantly more resistant to intraperitoneal infection than those immunized with toxoid.

(C) Phagocytosis

The ability of the host's leucocytes to ingest and destroy staphylococci seems to play an important role in determining the final outcome of staphylococcal infection (Cohn 1962a). Several studies indicate that both heat-labile and heat stable serum factors are needed for efficient phagocytosis and eventual destruction of ingested Staphylococcus aureus (Cohn and Morse 1959, Rogers and Nelly 1960, Hirsch and Straus 1964, Li and Mudd 1965). The heat stable serum factor seems to be the specific antibody directed against antiphagocytic surface components of staphylococci, such as capsule or ribitol teichoic acid depending on whether or not the strain is capsulated (Morse 1962a, Mudd et al. 1963). Li and Mudd (1965) present evidence to suggest that the heat-labile component of serum corresponds to complement. However, besides complement, heat labile serum opsonins for staphylococci have been demonstrated in guinea-pig and rabbit serum (Hirsch and

Strauss 1964, Hirsch 1964). The heat-labile serum factors are required not only for rapid phagocytosis but also for intracellular killing of Staphylococcus aureus (Id, Mudd and Kapral 1963, Id and Mudd 1965).

There are conflicting reports about the immediate fate of Staphylococcus aureus within phagocytes. Some studies have indicated that after ingestion both non-pathogenic staphylococci and Staphylococcus aureus cells are rapidly killed within human and rabbit leucocytes in vitro and within mouse leucocytes in vivo (Cohn and Morse 1959, Mackaness 1960, Cohn 1962a). On the other hand there are reports that pathogenic strains of staphylococci survive for longer periods within the phagocytic cells of man and rabbit than non-pathogenic strains (Rogers and Melly 1960, Melly, Thomson and Rogers 1960, Shayegani and Kapral 1962). These discrepancies in the results of various workers may be due to a difference in the technique of studies and variations in the opsonic activity of the sera.

(D) Hypersensitivity

The possible role of hypersensitivity in staphylococcal infection was suggested by Panten and Valentine (1929) who demonstrated that repeated staphylococcal infection of rabbit skin was characterised by increasing resistance to large bacterial inocula but increasing susceptibility to small bacterial inocula. The co-existence of immunity and hypersensitivity after staphylococcal

infection has since been confirmed (Jochanovsky 1958b, Johnson, Cluff and Goshi 1961). Whether bacterial hypersensitivity enhances the infectivity of Staphylococcus aureus in human beings as it does in experimental rabbits, however, remains to be determined.

Staphylococcal antigens incriminated in the hypersensitivity reaction include alpha toxin (Goshi, Smith, Cluff and Norman 1963), polysaccharide A and a protein antigen common to both pathogenic and non-pathogenic strains of staphylococci (Julianelle and Wieghard 1955b, Julianelle and Hartmann 1956).

(E) Factors that alter host resistance to infection

To produce an experimental staphylococcal infection in man or animals an inoculum of more than a million cocci is needed. It is very likely, however, as Elek (1956) has suggested, that in the initiation of most natural infections, only a few cocci are involved. Therefore the circumstances favourable to the growth of cocci at the site of infection are quite important.

(1) Local trauma and foreign bodies

In staphylococcal disease it is usual to observe infection in areas of tissue injury such as wounds, abrasions, burns or obstructed hair follicles (Elek 1956, Goshi, Cluff, Johnson and Conti 1961, Goshi, Cluff and Johnson 1961). The devitalized tissue temporarily protects the invading staphylococci from the body's defences. There is a suggestion that trauma of tissues results in acute oedema and increased interstitial tissue pressure which leads to lowering of resistance to bacterial infection. Andriole and Lytton (1965) demonstrated that under conditions of increased tissue pressure there is an

increased susceptibility of guinea-pigs to intradermal injection of staphylococci. The increased tissue pressure resulted in a decreased capillary blood flow which accounted for a delay in the emigration of leucocytes and possibly other antibacterial agents necessary for adequate host defences.

Staphylococcal infection can also be enhanced by the presence of a foreign body in the tissue, for example a suture or cotton dust (Elek 1956, Elek and Conen 1957, James and MacLeod 1961, Noble 1964). The mechanism by which the foreign body enhances the infection is not properly understood. Elek (1956) suggests that there is a slight delay in the defence system which allows the requisite multiplication of cocci. However no experimental evidence was put forward to support this hypothesis.

(2) Hormones and host resistance

Hormones secreted by endocrine glands exert an influence on host resistance (see Kass 1960). Hyperthyroidism lowers the resistance of mice to experimental staphylococcal infection (Smith and Dubos 1956c) and adrenal glucocorticoids, in large doses, increase susceptibility in experimental animals (Dougherty and Schneebeli 1950, Cohn 1962b). The possible mode of action of adrenal glucocorticoids is discussed in the chapter on anti-inflammatory drugs.

(3) The influence of various disease processes

Various disease processes such as malignancy,

leukaemia, diabetes, agammaglobulinaemia, aplastic anaemia or agranulocytosis and viral influenza are associated with increased susceptibility of man to staphylococcal infection (Mahmias and Eichhoff 1961). There are conflicting reports of the effect of alloxan diabetes on experimental staphylococcal infection in animals (Cruikshank 1954, Cohn 1962b, Bohr 1965); Dubos (1954) has suggested that the resistance of the host is lowered only if the diabetes is uncontrolled.

(4) Nutrition

Imbalance in the diet of mice, especially shortage of adequate protein nutrients leads to increased susceptibility to staphylococcal infection (Dubos and Schaedler 1959, Schaedler and Dubos 1959). The quantity as well as the quality of amino acid nutrition were shown to be of importance. Relatively short periods of starvation enhance the susceptibility of mice to staphylococcal infection (Smith and Dubos 1956b). The precise mechanism whereby nutritional disturbances alter the resistance is unknown; the effect may be a direct one or it may operate through alteration of the intestinal flora (Dubos and Schaedler 1962).

(5) Inflammation and its modification

Among the early responses to injury, the inflammatory reaction is of paramount importance. The fate of the invading

microorganisms is to some extent determined by the inflammatory response of the tissues.

During recent years the inflammation produced by staphylococcal infection in experimental animals has been investigated (Higdon 1941, Burke and Miles 1958) by inoculating small doses of bacteria into the skin. Increased vascular permeability was measured by the exudation into the skin of a vital dye introduced into the circulation. The vital dyes like Evans blue and pontamine sky blue 6BK behave as if they are bound to plasma albumins (Rawson 1943), and therefore their exudation indicates movement of plasma proteins from blood vessels. It is also possible that free dye exudes but its molecular weight is more than 20,000 and hence the exudation probably indicates increased permeability to large molecules including proteins (Miles and Wilhelm 1960).

In the experiments of Higdon (1941) and of Burke and Miles (1958) the increased vascular permeability occurred in two phases. The first phase was immediate and transient usually lasting a few minutes after which permeability returned to normal within 30 min. This was followed by a delayed phase of exudation which came to a maximum several hours later depending on the dose of bacteria. The degree of increased vascular permeability was found to correlate directly with the extent of persistent induration and necrosis at the inoculation site (Burke and Miles 1958). The immediate response could be largely eliminated

by prolonged washing of the bacterial suspension used for inoculation (Burke and Miles 1958). The immediate phase also seemed to depend on the species of animal used in the experiment, being lacking in the rat (Miles 1961).

The second component of the inflammatory response - namely infiltration of the inoculated tissue by leucocytes was first observed at 1 hour, and progressed steadily after that (Burke and Miles 1958).

The type of blood vessels affected during acute inflammation has been studied by carbon labelling (Majno, Palade and Schoefl 1961). The increased vascular permeability in response to chemical and thermal injury has been shown to be mainly due to venular damage (Hurley 1964a, Ham and Hurley 1965, Spector, Walters and Willoughby 1965). However, Wells & Miles (1963) report that though, in the immediate phase, the damage is mainly venular, in the delayed phase it is capillary.

There is now a great deal of evidence to indicate that increased vascular permeability is due to chemical mediators (Spector and Willoughby 1963) but these will not be discussed here. To what extent the emigration of leucocytes is under the influence of chemical mediators remains uncertain.

Chemotaxis

The exact mechanism of leucocyte emigration remains unknown. Harris (1953, 1960) tested the chemotactic ability of tissue

extracts and various bacteria for granulocytes in vitro. While living Staphylococcus albus cells were found to be chemotactic to granulocytes, Staphylococcus aureus cells were not chemotactic and exerted a toxic effect. Harris found that tissue extracts were not capable of exerting any chemotactic effect on granulocytes, but in vivo a contrary result has been reported (Hurley 1964b, Spector and Willoughby 1964a).

The analysis of leucocyte emigration in vivo following staphylococcal infection seems to support the in vitro findings of Harris. There is a relative delay in the influx of polymorphonuclear leucocytes in the lesion site with Staphylococcus aureus infection as compared to Staphylococcus albus (Delaunay 1942, Cohn 1962a).

Recently Boyden (1962) has shown that mixtures of antibody and antigen have strong chemotactic effect on polymorphonuclear leucocytes in vitro; the chemotactic substance is probably produced as a result of interaction between antigen - antibody complex and a heat labile substance of serum. Staphylococcal infection in rabbits possessing some immunity to alpha haemolysin has been shown to be associated with a more rapid infiltration of lesion sites with polymorphonuclears than in normal control rabbits (Goshi, Cluff and Norman 1963). The rapid leucocyte emigration in immune rabbits may be partly due to the chemotactic influence of antigen - antibody mixture and partly due to the neutralisation of the leucotoxic effect of alpha haemolysin.

Anti-inflammatory agents

The severity of staphylococcal lesions in experimental animals can be considerably increased by the use of anti-inflammatory agents.

(a) Adrenaline

Miles and co-workers (Evans, Miles and Niven 1948) demonstrated that the local injection of adrenaline in guinea-pigs rendered the treated tissue more susceptible to Staphylococcus aureus. The infection was enhanced 10 to 100 fold. The enhancement of infection was attributed to local ischaemia which led to an inhibition of leucocytic emigration and exudation of fluids from blood vessels

(b) Native levans

Native levans and dextrans have been found to possess marked infection-promoting activity in various types of experimental infection including Staphylococcus aureus (Shilo 1964, Bonventre and Black-Schaffer 1965). There is evidence to indicate that these substances produce their infection-enhancing activity by hindering the diapedesis of leucocytes and extravasation of fluid from blood vessels into loci of infection (Shilo 1962). However Bonventre and Black-Schaffer (1965) put forward the hypothesis that the infection promoting activity of high molecular weight dextran is due to reticulo-endothelial system blockade.

(c) Antihistaminics

In experimentally induced inflammation antihistaminic drugs usually fail to influence the course of events (Spector and Willoughby 1964b). In staphylococcal infection in rabbit skin only the short lived immediate vascular phase is suppressed by antihistaminic drugs; the delayed phase remains unaffected (Wilhelm 1962). Furthermore, Smith and Miles (1960) studying experimental bacterial peritonitis in rat, failed to modify the inflammatory response by anti-histamine drugs.

(d) Endotoxin

The administration of bacterial endotoxin transiently increases the susceptibility of animals to staphylococcal infection (Miles and Miven 1950, Conti, Cluff and Scheder 1961, Cohn 1962b and Sultzser and Freedman 1965). Staphylococcal lesions enhanced by endotoxin are characterised by extensive haemorrhagic dermonecrosis, accelerated bacterial multiplication and liberation of large amounts of alpha haemolysin (Conti, Cluff and Scheder 1961).

The infection-promoting activity of endotoxin has been attributed to delay in the emigration of leucocytes into the infected site (Cohn 1962b, Conti et al. 1961) perhaps due to vasoconstriction; it can be prevented by the administration of adrenergic blocking agents (Sultzser and Freedman 1965). On the other hand delayed leucocyte emigration has also been attributed

to leucopenia (Mulholland and Cluff 1964) and to hypotension (Niles and Niven 1950).

Endotoxin has a biphasic effect upon susceptibility of experimental animals to infection. The increased susceptibility lasts for a few hours only and by 24 hours a state of non-specific resistance to infection, including Staphylococcus aureus, is established which lasts for 7 - 14 days (Cohn 1962b, Mulholland and Cluff 1964). The enhanced resistance may be due to RES stimulation (Bisetti et al. 1955), to an increased number of phagocytes at the lesion site, or to an enhanced efficiency of phagocytes (Cohn 1962b).

(a) Corticosteroids

Cortisone in large doses is capable of enhancing the susceptibility of experimental animals to staphylococcal infection (Bayer and Lemmens 1961, Cohn 1962b) and probably represents a summation of hormone action on the host defence mechanism at several levels (see Gernuth 1956, Allison 1965). The most striking effect is the suppression of inflammation, particularly the diapedesis of leucocytes (Allison et al. 1955 a,b, Northover 1964b) probably by interfering with the margination and sticking of leucocytes to vascular endothelium. Wilhelm (1962) demonstrated that cortisone inhibits both fluid exudation and diapedesis of leucocytes in inflammation. Recent studies indicate that at least a part of steroid effect in suppressing inflammation

may be due to a stabilizing action on the membranes of intracellular lysosomes (Weissmann & Thomas 1965).

Corticosteroids have also been shown to suppress antibody formation (Weiser et al 1941) and alter the functional capacity of phagocytes (Allison and Adcock 1964). However Hirsch and Church (1961) failed to find any deficiency in the functional capacity of phagocytes.

(f) Sodium salicylate

Many workers have demonstrated an anti-inflammatory effect of Sodium salicylate (Northover 1964a, b, Spector 1958, Wilhelm 1962). It is suggested that this drug suppresses fluid exudation without altering the emigration of leucocytes (Northover 1964 a,b). Its mode of action is not known; amongst the possible explanations for its activity include inhibitory effects on enzyme systems (Spector and Willoughby 1960) and adrenal stimulation (Bacchus and Bacchus 1955). However it is also suggested that its activity is not mediated through adrenal glands but is a peripheral action at the site of inflammation (Randall 1963).

(g) Phenylbutazone

Like sodium salicylate, phenylbutazone has also been shown to suppress the fluid exudation without altering the diapedesis of leucocytes (Northover 1964b). Its exact mode of action remains unknown, but it is supposed to be due to a peripheral

action at the site of inflammation (Randall 1963).

(h) Colchicine

Colchicine has been shown to possess anti-inflammatory activity, particularly suppressing the diapedesis of leucocytes (Northover 1964b, Boland 1964). The anti-inflammatory effect of colchicine has been attributed to decreased protoplasmic viscosity of cells (Malawista 1965) which possibly would interfere with amoeboid activities of phagocytes. But its action has also been suggested to be due to peripheral vasoconstriction (Ferguson 1952).

(i) Indomethacin

Indomethacin has been shown to be an effective anti-inflammatory agent in rats when tested against cotton-wool granulomas and thermal injury (Winter et al 1963, Walters & Willoughby 1965). However it failed to suppress the inflammatory reaction elicited by staphylococcal alpha haemolysin toxoid (Walters and Willoughby 1965). When indomethacin was examined for its effect on immune inflammation it was found to suppress the erythema of the tuberculin reaction without affecting the leucocytic emigration. Walters and Willoughby (1965) attribute the anti-inflammatory activity of indomethacin in delayed hypersensitivity to suppression of lymph node permeability factor (LNP). In non-immune inflammation its activity has been attributed to suppression of kinin formation.

(j) X-irradiation

Total body irradiation (Cohn 1962b) or focal x-irradiation (Kahn 1962) have been shown to enhance the susceptibility of experimental animals to staphylococcal infection. X-irradiation leads to a reduction in number of circulating leucocytes and the subsequent influx of phagocytes in the area of the lesion.

IV AIMS OF THE PRESENT INVESTIGATION

Since the dynamics of infection involves two major processes namely growth of bacteria and the mobilization of body defences to sterilize the inoculum, I decided to study the inflammatory response of mice and the changes in the bacterial population in the lesions at intervals after the subcutaneous inoculation of staphylococci. The interest in the inflammatory response during the first few hours of injection stemmed from the considerable body of evidence indicating the importance of the period immediately following infection in deciding the final outcome of the infection.

It is well established that the presence of a foreign body like cotton dust or a suture in the tissue enhances staphylococcal infection. It seemed of interest to measure the inflammatory response and the bacterial population changes in the lesion in the presence and absence of cotton dust with a view to correlating these factors with the severity of the lesion that finally developed.

A comparison of the reaction of the host to strains of staphylococci and micrococci differing in their 'virulence' might serve to elucidate the significance of inflammatory response in the pathogenesis of staphylococcal lesions. Therefore it was decided to see whether there was any correlation between the severity of final lesion and the type of inflammatory response and growth pattern of cocci during the first few hours of infection.

In view of the fact that animals having past experience of staphylococcal infection develop immunity as well as hypersensitivity to reinfection, it was thought to be important to examine in detail the inflammatory reaction as well as growth of cocci in the lesions of these mice. It was also decided to attempt the transfer of the altered reactivity of 'previously-infected' mice for staphylococci by injection of serum and lymphoid cells from 'previously-infected' to normal mice.

In the studies reported the effect of anti-inflammatory agents on the two components of staphylococcal inflammation and lesion production has been examined. The use of these drugs might serve to define the relative importance of humoral and cellular factors in the defence of host against staphylococcal infection.

V MATERIALS AND METHODS

Bacterial Strains

The strains of staphylococci and micrococci used in the experimental work are shown in Table 1.

Media

Media used in the experimental work are described in the appendix.

Mice

The Wright-Fleming Institute strain of mice were used. They are derived from a cross of 'P strain' and 'strong A'. Male and female mice weighing 20 - 25 gms. and 9 to 12 weeks old were used. Mice were housed in groups of up to 12 in cages 75 square inch in area and were fed 'Diet 41B' and water throughout the experiment without any restrictions.

Method of Producing Subcutaneous Abscess

Acute staphylococcal infection was produced in mice, using the method described by Noble (1965). The hair from the back of each mouse was removed first by means of electric clippers and later by applying barium sulphide depilating cream (see appendix) on to the clipped area. After 3 - 4 minutes, the paste and hairs were washed away with warm water. The animals were dried by keeping the cage in front of a heater. In one to two hours the mice had completely dried and were ready for injection.

Table 1. Bacterial strains used in the experimental work

Strain	Source	Haemolysins			Phage type	Antibiotic* sensitivity
		alpha	beta	delta		
<u>Staphylococcus aureus</u>						
PS80	NCTC 9789	+	-	+	80/81	R to P
Wood 46	NCTC 7121	+	-	+	47+	S to all
Orbach	Sputum-case of bronchopneumonia	+	-	+	83A	R to P & T
Smith	"Diffuse" variant of Hunt & Moses + (1958)		-	+	Untypable	S to all
PS 83A	NCTC 10039	+	-	+	6/7/53/83A	S to all
P66/7	Tropical myositis in Uganda	-	-	+	3B/3C/4	R to P
Ward	Boils	+	-	+	52A	S to all
1531	Human meningitis post-mortem at Melbourne (Fisher 1965)	+	-	+	29+	R to P
<u>Coagulase negative Staphylococci</u>						
N.hyalinus	NCTC 10350 Contagious impetigo of swine	-	-	-	Untypable	S to all
NCTC 7292		-	-	-	Untypable	S to all
<u>Micrococcus</u>						
278	Peritoneal dialysis fluid of a kidney graft patient	-	-	-	Untypable	R to P & St.
<u>Key:</u> + = Lysin produced - = Lysin not produced * = Antibiotics used for sensitivity test - Penicillin, Streptomycin, Tetracycline, Chloramphenicol, Erythromycin, Neomycin, Novobiocin and Cloxacillin. R = Resistant, S = Sensitive P = Penicillin T = Tetracycline St = Streptomycin S to all = Sensitive to all the antibiotics used for sensitivity test						

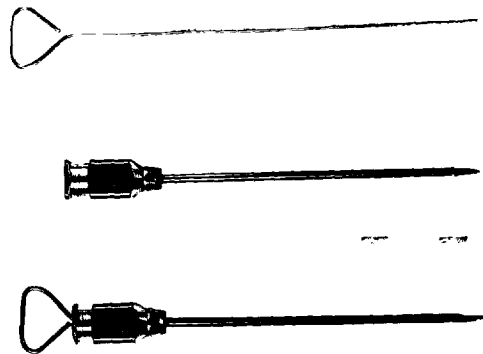


Figure 1: Needles and wire plunger used for the injection of cocci mixed with cotton-dust. Two cotton-plugs are also shown.

Gauge sixteen transfusion needles were used for subcutaneous injection. The needles are fitted with a wire plunger to expel the cotton dust (Figure 1). The plunger was marked so that it could be withdrawn up the barrel of needle for a distance of 3 mm. and the lumen of needle between its tip and the wire plunger was packed with cotton dust. About 2 mg. of cotton dust was used for each plug.

Cotton dust: The cotton was C_4 white cotton flock which is pure cellulose and corresponds to the cotton in bleached cotton fabric. I am grateful to Shirley Institute, Didsbury, Manchester and to Messrs. Hutchinson (Ramsay) Ltd. for this material.

The cotton plug was inoculated with $5\mu\text{l}$ of an eighteen hours broth culture of microorganisms. The inoculation was done by means of GNE ultramicro pipette (E - MII). The number of viable cocci on the cotton plug was determined by suspending the plug in $\frac{1}{4}$ strength Ringer's solution and doing the viable count on 5% serum agar plates by the Miles and Misra (1938) method.

Mice were injected subcutaneously in the depilated area with the cotton plug containing cocci. The skin of the back was pinched up between the fingers, the needle inserted at the base of the fold and the plug expelled by means of wire plunger. The reaction was read daily. In the absence of cotton, 0.05 ml of a suitable dilution of culture was injected and the dose determined by doing the viable count (Miles and Misra 1938).

Method of scoring lesion severity

The lesions were graded according to severity as follows:

- 0 = (No Reaction) Complete absence of pus visible at the skin surface and no erythema except in the skin immediately above the cotton plug.
- 1 = (Slight Reaction) Minimal pus visible through the skin immediately above the plug and erythema in the skin around the plug.
- 2 = (Moderate Reaction) An area of necrosis or a white zone not more than $\frac{1}{2}$ cm. in diameter. The white zones always progressed to necrosis within 24 hours.
- 3 = (Severe Reaction) The zone of necrosis or white area greater than $\frac{1}{2}$ cm.

The diameter of elliptical lesions were recorded as $\sqrt{Dd}$, where D and d are the lengths of the major and minor axes.

The results are usually quoted as the sum of scores for the first two days following challenge; thus a mouse showing a slight reaction on first day and a moderate reaction on second day would score $1 + 2 = 3$ marks. The maximum score for any mouse would be $3 + 3 = 6$ marks.

Within any one test, mice of only one sex were used.

Table 2

The reproducibility of scoring the lesion severity

	House Number												Total Score	Mean Score
	1	2	3	4	5	6	7	8	9	10	11	12		
First Assessment	At 24 hours												48	4.0
	2	2	2	1	2	2	1	1	1	2	3	3		
Second Assessment	At 48 hours												49	4.08
	2	2	2	2	2	2	3	2	2	2	3	2		

Mice were found to vary in the severity of the lesion for a given dose of cocci as emphasized by Noble (1964). As the scoring of lesion severity is subjective an investigation was carried out to estimate the personal error in assessing the lesion. Twelve mice were marked on the abdominal surface, so that the markings were not visible while scoring the lesion. The mice were inoculated subcutaneously and the lesions were scored 24 and 48 hours after challenge. Mice were taken out of the cage at random by an assistant who kept the record of the order in which mice were given for assessment. In this way the assessment was done twice on each day. The average lesion score per mouse was approximately similar in both assessments; it was 4.0 and 4.08 respectively (Table 2). On three occasions the scoring differed in the two assessments when the lesions were 24 hours old. At 48 hours there was no difference in the assessment of lesion score in the two instances.

Study of acute inflammatory response

The acute inflammatory response to the subcutaneous injection of cotton and cocci was studied by the following methods.

1. Increased vascular permeability

This was measured by two methods. (a) Immediately after the subcutaneous injection of mice with microorganisms, the animals were injected intravenously with 0.2 ml. of 1% solution of Evans blue (Boots Pure Drug Co. Ltd.) The dye becomes fixed on the serum



Figure 2: Punch used to excise the skin
and subcutaneous tissue.

albumin (Rawson 1943) and in areas of increased vascular permeability it shows up in the tissues as clearly visible blue spots. The injections were made in the lateral tail vein and the animals were subsequently killed by chloroform after various intervals; the flap of skin and subcutaneous tissue bearing the lesion was reflected and the blue areas examined on the inner aspect of skin flap. The degree of fluid exudation was estimated by measuring the diameter of the blue staining of tissue around the lesion. The blue area was usually circular; when elliptical, the diameter of bluing was recorded as $\sqrt{Dd}$, where D and d are the lengths of the major and minor axes.

(b) After measuring the diameter of the blue areas, the weight of the edema fluid in the inflamed tissue was estimated. This was done by excising the area of skin and subcutaneous tissue containing the lesion with a 2 cm. diameter punch (Figure 2) and weighing this piece of tissue. Edema weight was calculated by subtracting the expected weight of this piece of tissue when not inflamed from the observed weight. The expected weight of tissue when not inflamed was calculated in the following way. When two pieces of tissue of same size were excised from the back of each of a number of mice, it was found that, in general, the weight of the tissue excised from the lower half of the back of mouse was more than the weight of the tissue

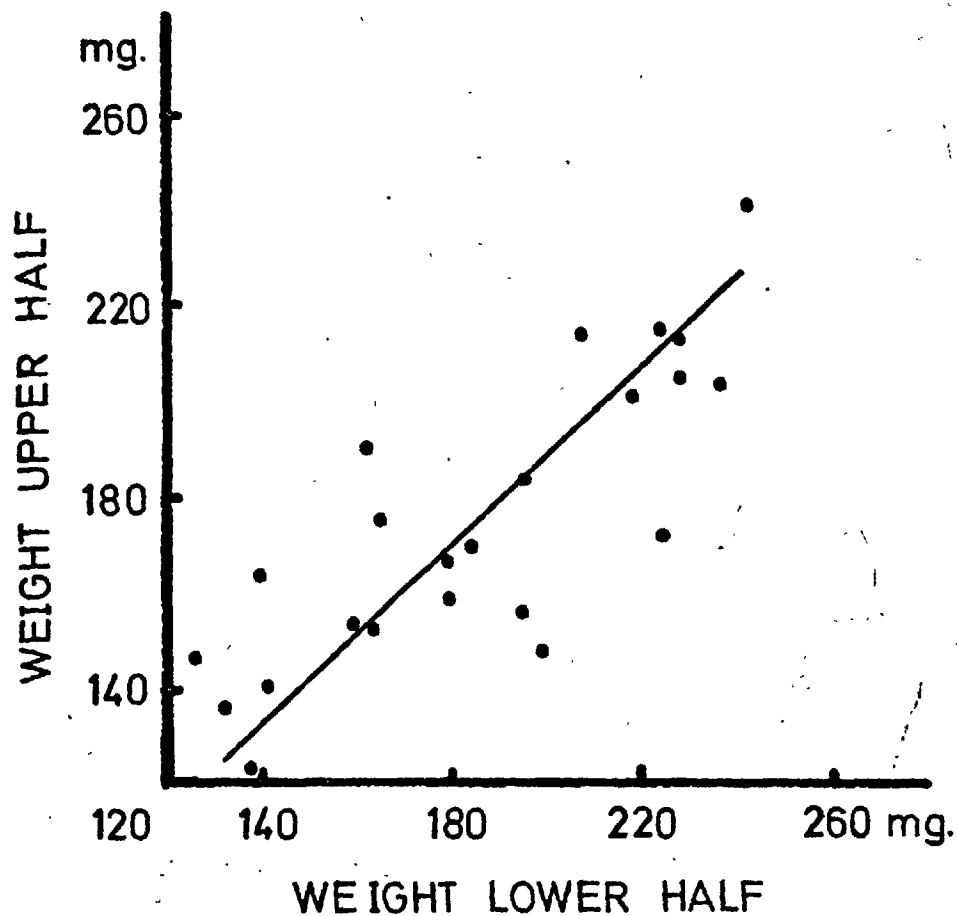


Figure 3. Regression curve showing the relation of the weight of the tissue excised from the lower half and upper half of the back of the mouse.

from the upper half of the back of mouse. The weights from a number of mice were plotted on a graph (Figure 3), and the points, though scattered, tend to fall around a straight line; the correlation coefficient was + 0.676. The probability of this being due to chance is 0.01. The regression equation calculated on the basis of these figures was: $x = 28.91 + 0.876 y$

x: The expected weight of tissue from lower half of the back.

y: The weight of tissue from upper half of the back

In all experiments, cocci mixed with cotton dust were injected into the subcutaneous tissue of the lower half of the back of mouse. After killing the animal, a control piece of tissue from the upper half of the back of mouse was also excised as well as the tissue bearing the lesion. Both the pieces were weighed and the expected weight of the tissue from the lower half of the back of mouse when not inflamed was determined from the regression equation.

2. Leucocyte emigration

The leucocyte emigration in the inflamed area was estimated by examining a histological preparation of the area of the lesion (Pas and Spector 1962). After weighing, the inflamed tissue was fixed in 10% neutral formal-saline for 18 to 24 hours.

After fixation the tissue was cut through the middle of the lesion and processed as follows:

1. The tissue was treated with 4% phenol (Lundrum's method) for 3 to 4 days. This procedure softens the tissue.
2. Dehydration and clearing of the tissue was carried out by means of an automatic tissue processor in the usual way.
3. After dehydration and clearing the tissues were embedded in paraffin wax.

Sections of 6/ μ thickness were cut by means of a Cambridge rocking microtome and stained with hematoxylin and eosin or 1% aqueous toluidine blue. The degree of leucocyte emigration was determined by counting the number of leucocytes in standard areas. This was achieved by using a graticule 7 x 7 mm divided into 100 squares. A 4 mm. objective was used for counting. The counts were done in as many areas as possible, a mean value calculated and results expressed as the total number of cells per 4 mm field. The presence of more than 100 leucocytes/H.P.F. was indicative of leucocyte emigration, as the normal subcutaneous tissue of mouse was found to contain up to a maximum of 100 leucocytes/H.P.F.

3. Analysis of Vascular involvement

The damaged blood vessels were demonstrated by injecting a colloidal suspension of carbon black intravenously into the mice. The carbon black was Pelikan ink prepared by the

Oesither-Wagner Pelikan-Werke, Hannover, Germany (Batch No. 611/1431a). Before use it was centrifuged at 5000 r.p.m. for 15 minutes to remove heavier particles. The supernatant contained 14.2 mgs. of carbon per ml. The ink was suitably diluted in double distilled water.

Pelikan ink (0.2 ml = 2.8 mg.) was injected intravenously in lateral tail vein of mouse. Half an hour after injection when all carbon had been cleared from circulation, the mice were killed. The injections were given at intervals varying from 30 minutes to 24 hours after the subcutaneous injection of cocci mixed with cotton dust. A piece of skin and subcutaneous tissue 1.5 in. square containing the lesion was excised. The four corners were pinned on to a piece of cork in order to keep the tissue stretched. After formalin fixation for 48 hours, the tissue was briefly rinsed in water and then cleared in two changes of glycerine for 48 and 24 hours. Finally the preparation was trimmed, floated in two changes of warm glycerine jelly for a few minutes and mounted under a cover slip. In this state the preparation is permanent. The formula for glycerine jelly is given in appendix.

The cleared specimen was first examined at a low magnification of 8 to 10 times. At this magnification only large blood vessels (presumably venules) are seen and gives an idea of the extent of vascular labelling with carbon. Next the preparation

was examined under higher magnification to determine the type of blood vessels affected and the leakage of carbon particles from blood vessels.

To demonstrate the vascular pattern of normal subcutaneous tissue and skin of the mouse, the whole vascular system of a normal mouse was filled with a mixture of 5% gelatine and colloidal carbon (see appendix).

Immediately after the mouse was killed, its thorax was opened and the left ventricle was cannulated with a fine polythene tube whose other end was attached to a syringe and needle. The inferior vena cava was cut open and the animal was perfused through the cannula with approximately 10 ml. of warm saline followed by 6 ml. of ink-gelatine mixture. Finally the tissue was cleared and mounted as described above.

Bacterial counts in the lesion

At intervals after the subcutaneous inoculation of cocci with or without a cotton plug, pairs of mice selected at random were killed and the lesion containing the cotton dust was excised. When cocci were injected without cotton dust, the area of inoculum was made visible by intravenous injection of Evans blue which exudes out in the areas of inflammation. The excised tissue was homogenized in a tissue grinder with 2 ml. of $\frac{1}{2}$ strength Ringer's solution and a small quantity of sterile sand. Ten-fold dilutions of the homogenized suspension were made in $\frac{1}{2}$ strength

Ringer's solution and the viable counts of cocci done on 5% serum agar plates by the method of Miles and Misra (1958). In all cases the zero hour counts were made by killing the animal immediately after the inoculation of cocci.

Anti-inflammatory agents

A number of anti-inflammatory drugs were used. All the drugs were dissolved in sterile double distilled water, except Indomethacin which is not soluble in distilled water, and 0.1 ml. injected as follows:-

<u>DRUG</u>	<u>DOSE *</u>	<u>ROUTE</u>	<u>NO. OF INJECTIONS</u>
Colchicine	1 μ g/g	S.C.	S
Sodium salicylate	400 μ g/g	S.C.	S
Phenylbutazone sodium ("Butasolidin", Geigy)	240 μ g/g	S.C.	S
Chlorpromazine hydrochloride ("Largactil", M & B)	1 μ g/g	S.C.	S
Quinine hydrochloride	160 μ g/g	S.C.	S
Indomethacin ("Indocid", Merck Sharp and Dohme)	10 μ g/g	I.V.	S +
Cortisone acetate ("Cortisyl", Roussel Lab.Ltd.)	125 μ g/g	I.M.	4D
Prednisolone disodium phosphate ("Predsol", Glaxo)	100 μ g/g	I.P.	S
Dexamethasone-21-phosphate disodium salt ("Decadron", Merck Sharp and Dohme Ltd.)	2 μ g/g	I.M.	4D
Betamethasone disodium phosphate salt ("Betnesol", Glaxo)	8 μ g/g	I.M.	4D

Key: * = The dose of each drug is given in
 μ g per g body weight.

S.C. = subcutaneous

I.V. = Intravenous

I.M. = Intramuscular

I.P. = Intraperitoneal

S = Single dose given $\frac{1}{2}$ hour before the injection
of cocci.

S+ = Single dose given immediately before the
inoculation of cocci.

4D = 4 daily doses, the last dose given 2 hours
before the injection of cocci.

Preparation of Indomethacin solution

I am grateful to Merck Sharp & Dohme for supplying Indomethacin powder. As it is insoluble in distilled water, the following procedure was used to obtain a solution. One percent solution of sodium bicarbonate was added drop by drop till the powder dissolved. Finally the requisite concentration of drug was made up by adding sterile double distilled water. The pH was checked and was kept between 8 and 9. The powder precipitates out when the solution has a pH value below 8.

Endotoxin treatment

Bacto Lipopolysaccharide from Escherichia coli

O: 128: B12 (Difco Lab.) was used. A stock solution containing 1 mg of lipopolysaccharide powder per ml. of

sterile saline (0.9%) was prepared and stored at -20°C . Suitable dilutions were made in saline for injection. The dose of endotoxin was $0.5 \mu\text{g/g}$ body weight and it was administered by the I.V. route. Immediately afterwards mice were challenged with the subcutaneous injection of cocci mixed with cotton.

Passive transfer of immunity and hypersensitivity

Serum from mice previously infected with homologous strain of Staphylococcus aureus was collected and 0.25 ml. was injected intravenously into normal mice. The injection was given immediately before the subcutaneous inoculation of mice with Staphylococcus aureus.

Lymph node cells for passive transfer were prepared from mice previously infected with Staphylococcus aureus. After killing the mouse, the lymph nodes were removed aseptically from cervical, axillary, inguinal, anterior abdominal wall and mesenteric areas. The lymph nodes were freed of fat and put in cold sterile Hank's solution (Oxoid) containing 1% lactalbumin hydrolysate.

The lymph nodes were teased between two pairs of forceps. The suspension was freed of cell debris by passage through fine nylon and the cells collected by centrifugation in a MSE angle centrifuge at 2000 R.P.M. for few minutes. The pad of cells were resuspended in an appropriate volume of Hank's solution and the total number of cells counted in a counting chamber. In practice

all the lymph node cells of one mouse were injected into a recipient mouse. The number of cells injected varied between 70 to 120 million cells per mouse. The interval between killing the lymph node donor mice and injecting their cells into recipient was never more than 4 hours. The lymph node cells were injected intravenously into the recipient mice. Eighteen hours later the recipient mice were injected subcutaneously with Staphylococcus aureus and cotton dust.

VI EXPERIMENTAL RESULTS

Although it is well established that in the presence of a foreign body, a small number of staphylococci can establish an infection, the mechanism by which a foreign body promotes the staphylococcal infection is not clearly understood. I therefore, decided to study the correlation of the severity of staphylococcal infections in mice in the presence and absence of cotton with the degree of fluid exudation and leucocyte emigration as well as changes in the bacterial population at the site of infection.

1. The infection-enhancing effect of cotton

(a) Severity of lesion

When 10^6 viable units of Staphylococcus aureus strain PS 80 were injected without cotton, the lesions produced were mild; only erythema or slight pus formation was noticed up to 2 days after infection and there was rarely any necrosis or white zone. The average lesion score (see page 55) was 1.3. On the other hand, a similar dose of PS 80 in the presence of cotton led to severe necropurulent lesion, with a necrotic or white zone more than 5 mm. in diameter in most cases; the average lesion score was 5.8. These mean lesion scores are based on the observations on 24 mice in each case. Although mice vary in the severity of the lesion for a given dose of cocci, with cotton all

MEAN OEDEMA
WEIGHT (mg.)

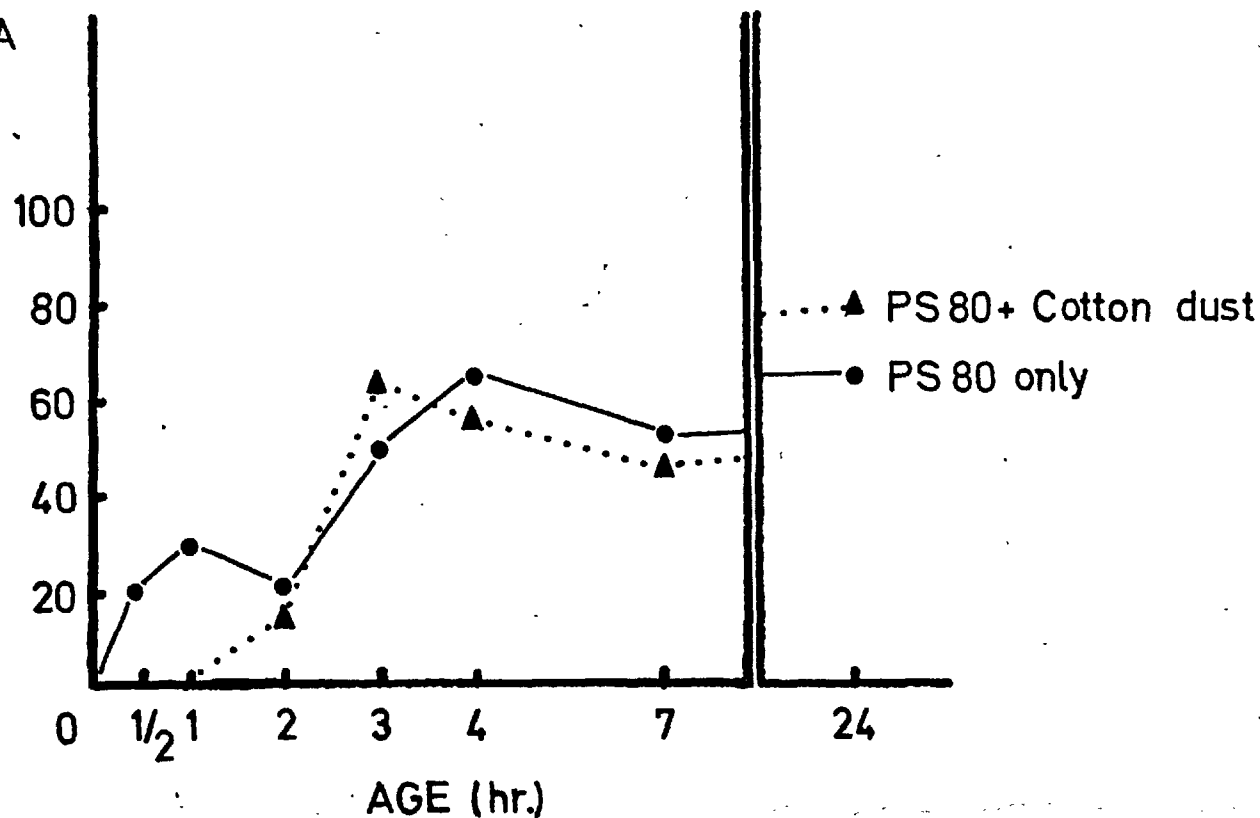


Figure 4: Comparison of mean oedema weight of tissue to the subcutaneous injection of 10^6 Staphylococcus aureus PS 80, with and without cotton-dust.

the mice had an area of necrosis more than 5 mm in diameter and in the absence of cotton all the mice showed mild lesions.

(b) Fluid exudation

When PS 80 (dose 10^6) was injected without cotton, an increase in vascular permeability, as judged by the weight of oedema fluid in the lesion, was evident half an hour after injection (Figure 4). Each point on this graph represents the mean of the results from four mice. As in the case of lesion severity, the mice varied in the amount of fluid exudation at the site of inflammation for a given dose of cocci; however 3 out of 4 mice at half an hour and all the mice at 1 and 2 hour period showed an increase in the tissue weight, indicating exudation of fluid. Therefore, in spite of variability in the response of mice, there was definitely an early exudation of fluid.

On the other hand, a similar dose of PS 80 injected with cotton dust elicited the exudation of fluid only after an initial delay (Figure 4). The fluid exudation commenced between one and two hours after injection. Three out of four mice showed little or no exudation of fluid up to one hour. After 2 hours there was not much difference in the exudation of fluid under two conditions. The above results were reproducible; in the repeat experiments there was always an initial delay in the exudation of fluid after injection of PS 80 in cotton dust. However some other strains of staphylococci, even in cotton dust, produced more rapid exudation

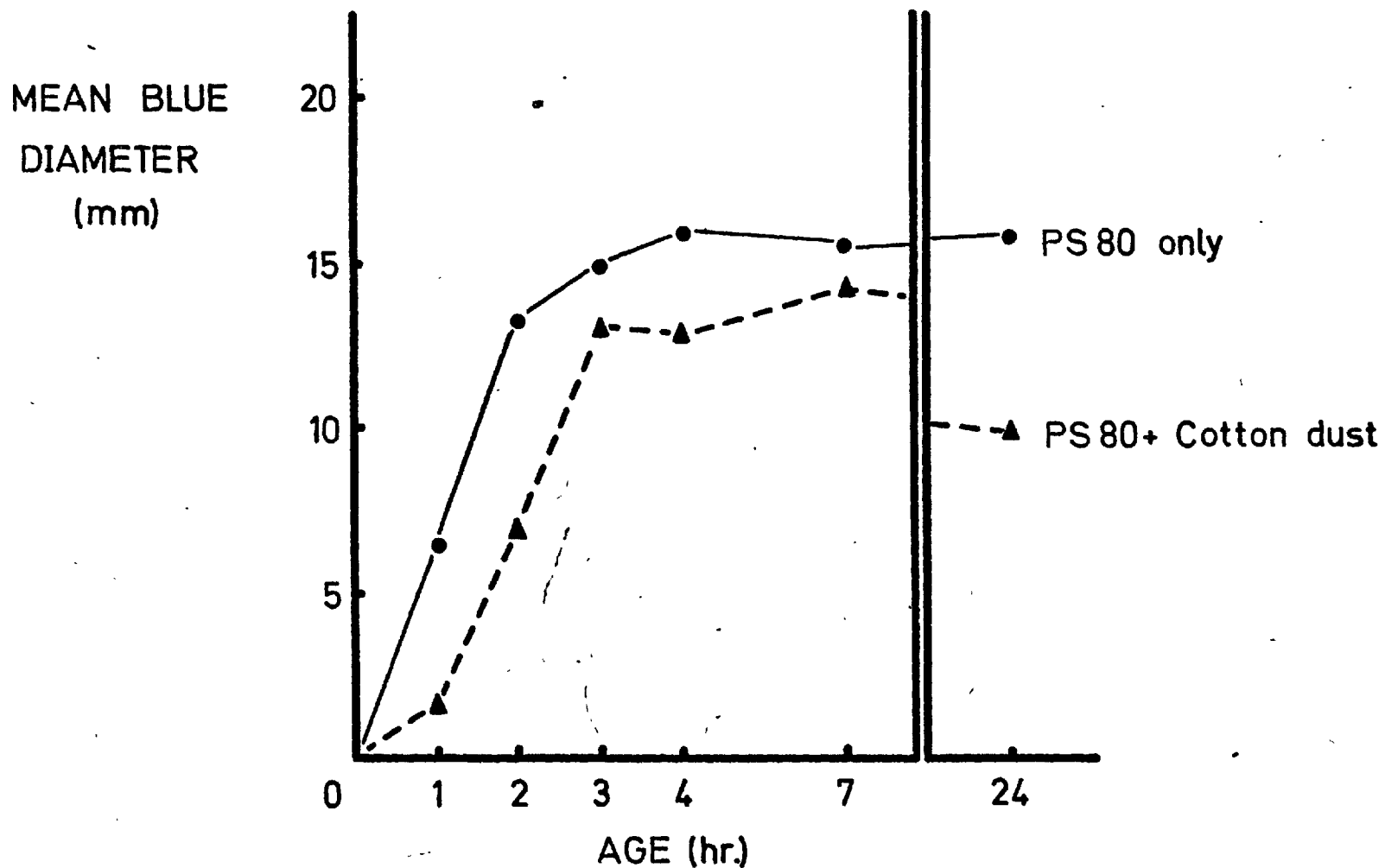


Figure 5: Increased vascular permeability, expressed as diameter of area of exudation of dye-stained plasma protein, to the subcutaneous injection of 10^6 Staphylococcus aureus PS80, with and without cotton-dust.

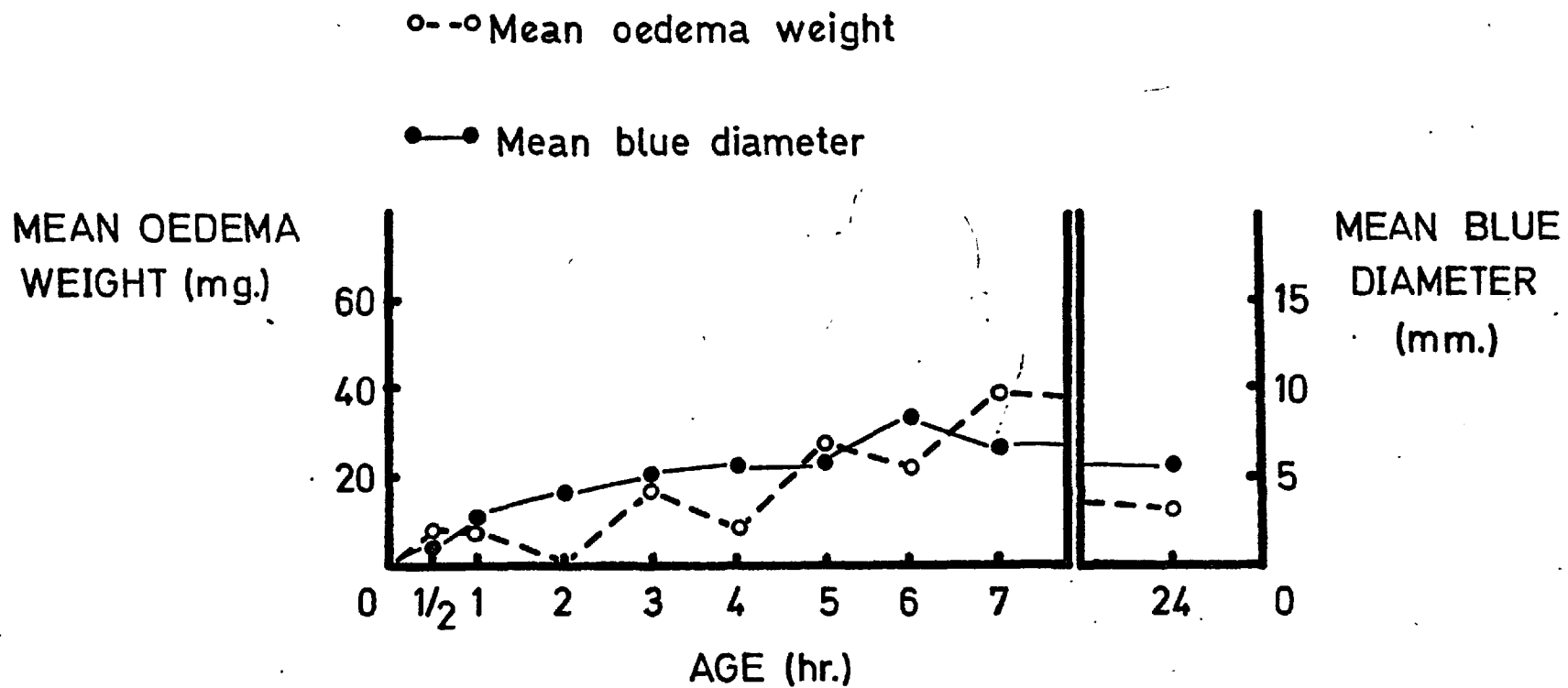


Figure 6: Increased vascular permeability to sterile cotton-dust and nutrient broth.

Leucocytes
per H.P.F.

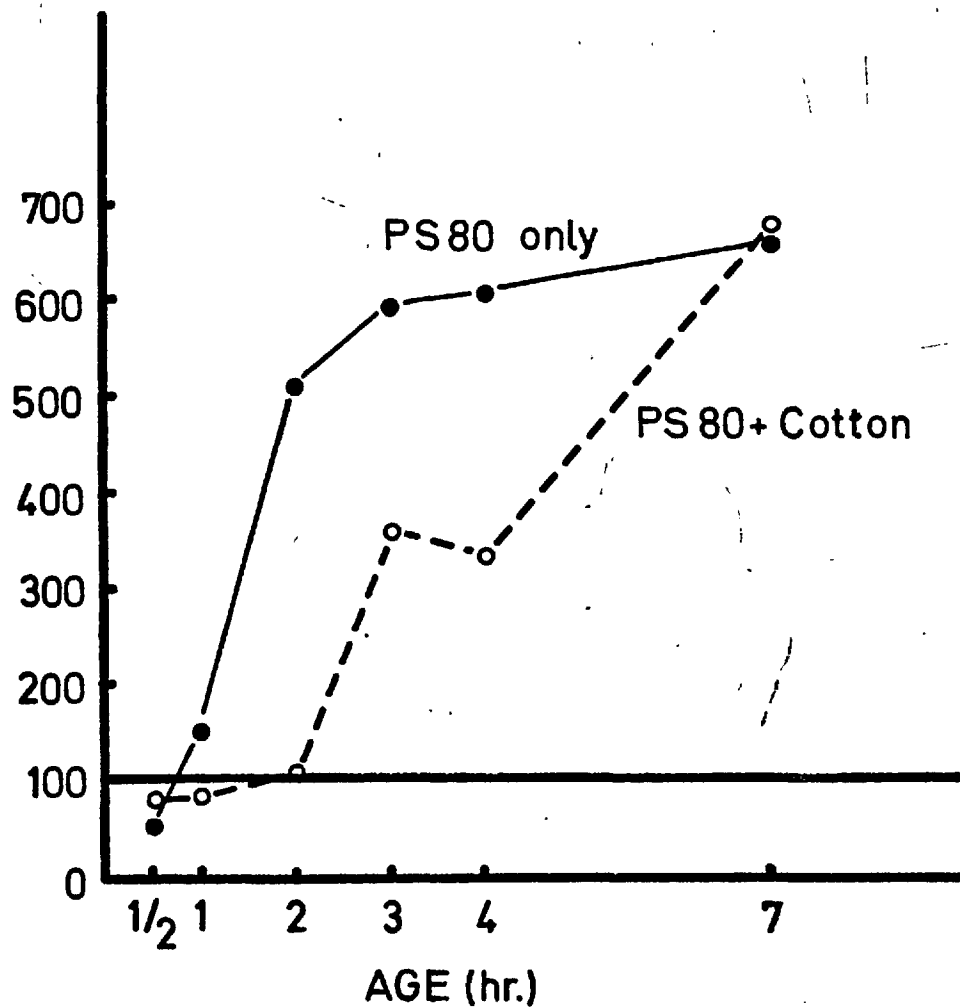


Figure 7: Comparison of leucocyte emigration at the site of subcutaneous injection of 10^6 Staphylococcus aureus PS80 with and without cotton-dust.

as described in the next section. Similar findings were noted when increased vascular permeability was judged by the exudation of protein-bound dye, Evans blue, at the site of injection (Figure 5).

In order to find out the effect of cotton dust alone in eliciting the exudation of fluid, sterile cotton dust with 5/1 of sterile nutrient broth was inoculated subcutaneously into a number of mice. There was little exudation of fluid in the first 2 hours of injection (Figure 6). The main phase of vascular permeability was after that period and it reached its maximum 7 hours after inoculation.

(c) Leucocyte emigration

The leucocyte response to the inoculation of Staphylococcus aureus strain PS 80 with and without cotton dust is shown in figure 7. About 100 leucocytes/H.P.F. was the upper limit of normal in the mouse tissue sections with the method of examination used.

In the absence of cotton the mean count was 500 cells per H.P.F. after 2 hours; in the presence of cotton there was no notable emigration until after 2 hours and it was 7 hours before there was any significant infiltration of the cotton dust itself.

The histological sections showed that in the absence of cotton dust, large numbers of leucocytes were present at the site of injection of cocci at 2 hours (Figure 8 a,b); by 24 hours, a well

(a)



(b)

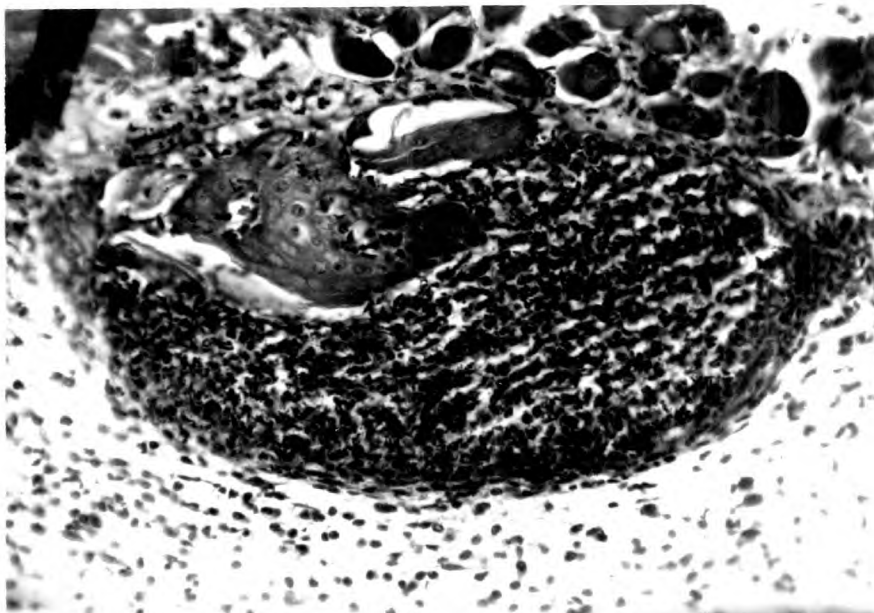


Figure 8: Two hour old lesion in mice after the subcutaneous inoculation of 10^6 Staphylococcus aureus PS 80.
(a) Large collection of leucocytes at the site of injection. X40. (b) Same as above. X160.

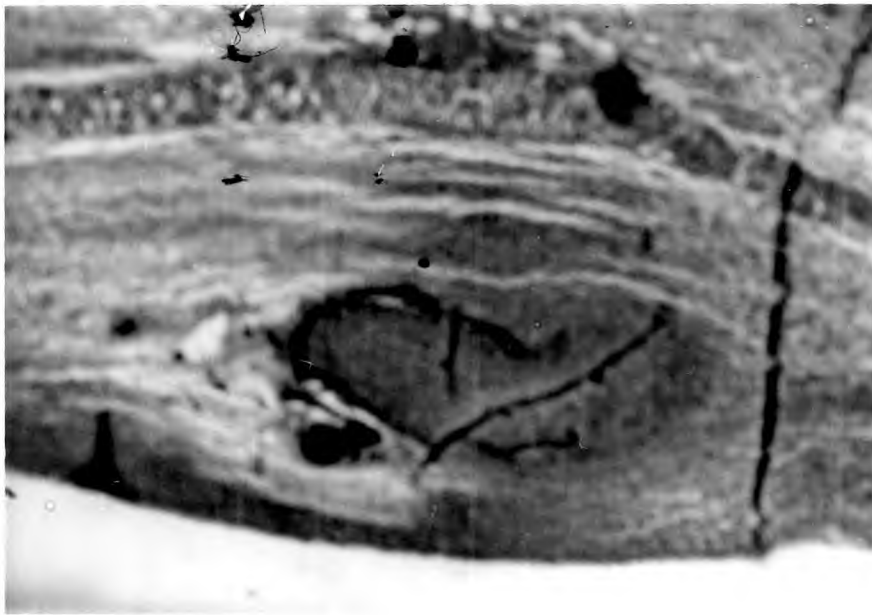


Figure 9: Abscess at 24 hours after the subcutaneous injection of 10^6 Staphylococcus aureus PS 80 X40.

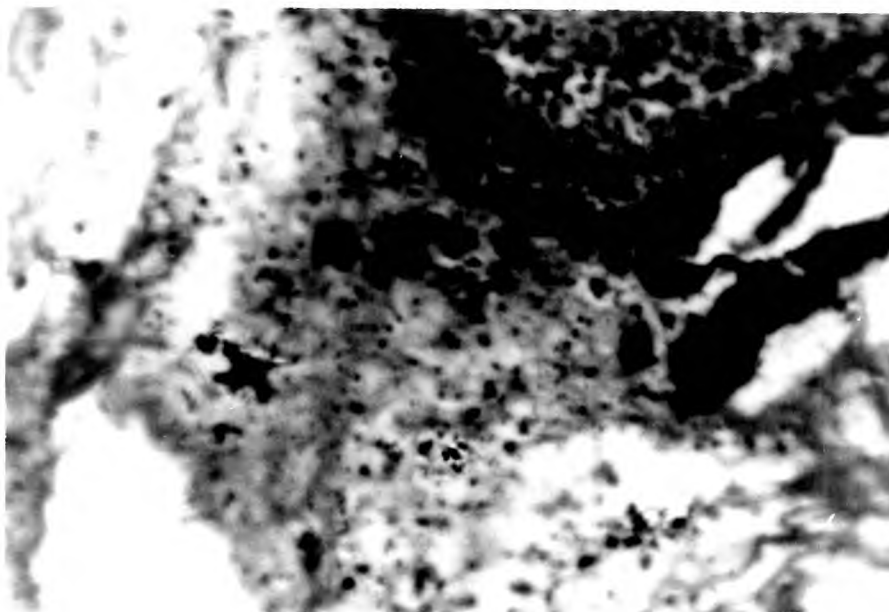


Figure 10: Abscess at 24 hours after the subcutaneous injection of 10^6 Staphylococcus aureus PS 80. Note the distortion of leucocytes in the area adjacent to cocci. X250.

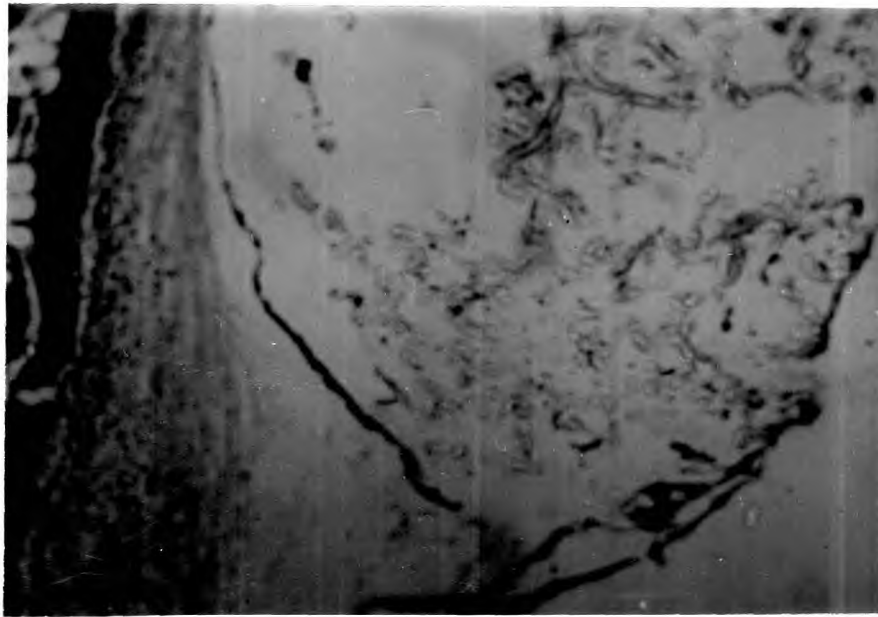


Figure 11: Four hour old lesion in mice after the subcutaneous inoculation of 10^6 Staphylococcus aureus PS 80 mixed with cotton-dust. Leucocytes are at some distance from the periphery of the cotton-dust which contains cocci. X40.

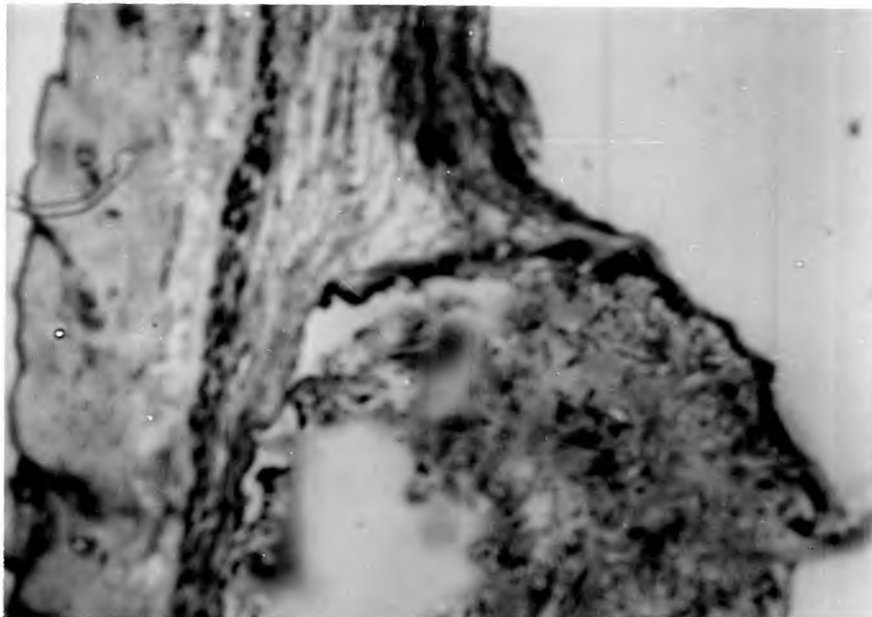


Figure 12: Twenty-four hours after the subcutaneous injection of 10^6 Staphylococcus aureus PS 80 mixed with cotton-dust. Leucocytes have not fully infiltrated the cotton-dust. X40.

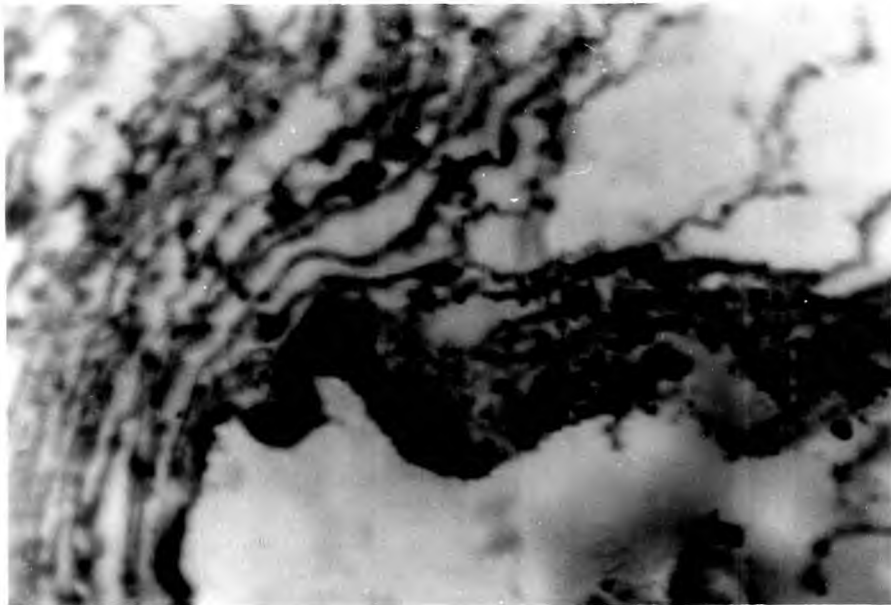


Figure 13: Same as figure 12 showing the presence of masses of cocci in the cotton-dust but an absence of leucocytes. X250.

defined abscess was formed (Figure 9). The high power view of the 24 hour lesion showed that the leucocytes were distorted in the immediate vicinity of cocci (Figure 10).

In contrast, in the presence of cotton the histological sections showed no cellular response for the first two hours. Even four hours after injection, leucocytes had not infiltrated the cotton, and in fact in most cases they remained at some distance from the periphery of cotton dust (Figure 11). Though the infiltration of cotton by leucocytes started at 7 hours, the entire area of cotton dust, particularly the central parts, was not infiltrated even at 24 hours (Figure 12, 13). Thus the cotton dust contained masses of cocci without the infiltration by phagocytic cells.

When sterile cotton dust was injected without staphylococci, leucocyte emigration started 1 hour after the introduction of cotton. The cotton dust was infiltrated by leucocytes at 3 hours; at 7-8 hours large numbers of leucocytes had infiltrated the entire cotton dust.

(d) Bacterial counts in the lesion

The viable counts of cocci in the lesion are shown in figure 14. In the absence of cotton, little or no increase in the numbers of viable cocci was observed; 24 hours after injection the bacterial count in the lesion was less than the dose inoculated. On the other hand, in the presence of cotton dust, after an initial lag period there was a rapid increase in the number of organisms

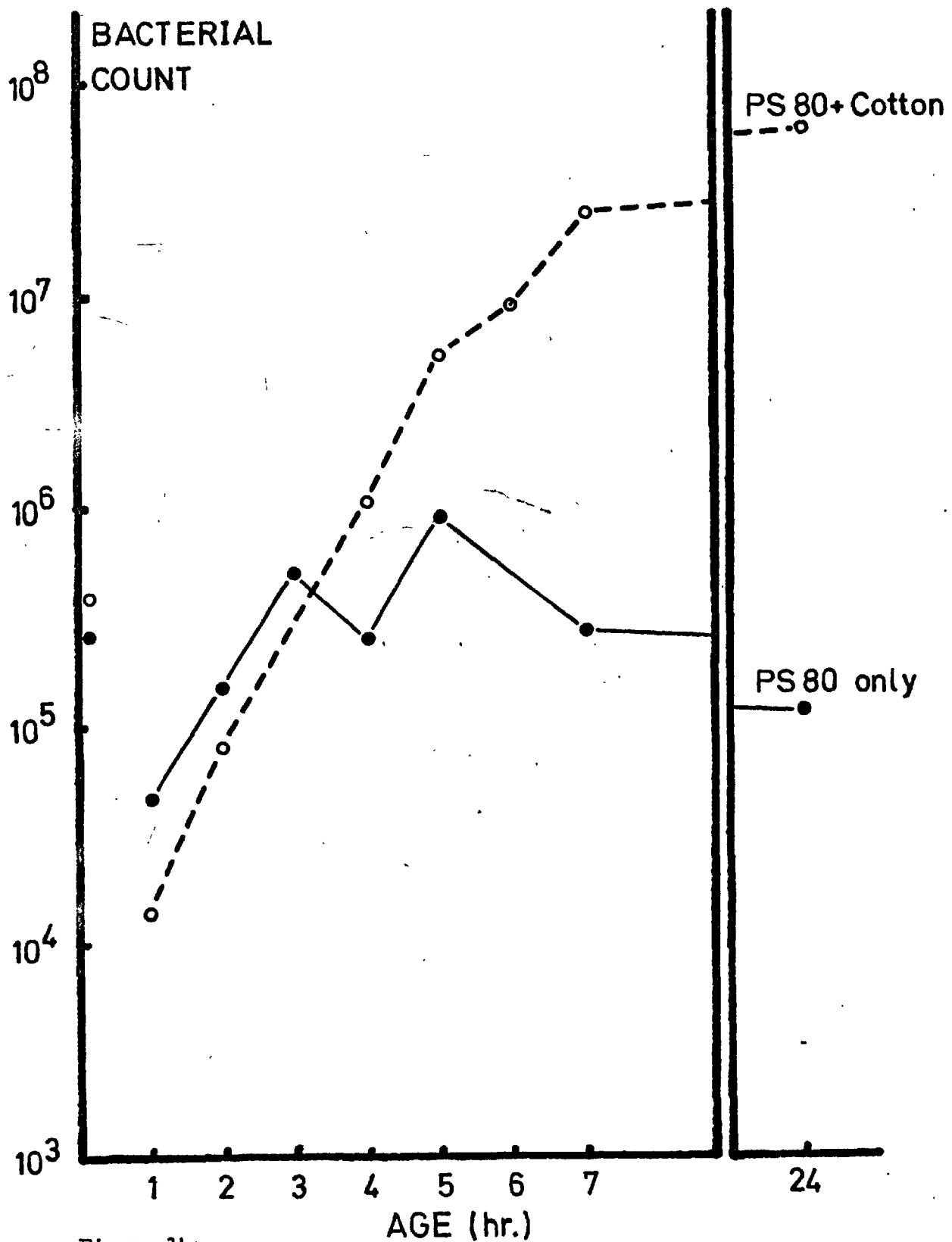


Figure 11:

Bacterial counts at the site of subcutaneous injection of Staphylococcus aureus PS80 with and without cotton-dust.

recovered from the lesion. Twenty four hours after injection, bacterial counts increased more than 100 fold over the initial dose inoculated.

(e) Further investigation into the mechanism of action of cotton dust.

Other possible mechanisms of action of cotton dust were also investigated. For example the infection enhancing activity of the cotton-dust could be due to its specific toxicity, or due to the retention of cocci at the site of injection. Moreover, in the above experiments when PS 80 was injected without cotton, the volume of broth culture injected was 0.05 ml, but with the cotton 0.005 ml ($5/\mu$ l) of broth culture was used. Therefore there was a possibility that greater early inflammatory reaction in the absence of cotton might be due to a larger volume of nutrient broth which is known to elicit an exudation of fluid (Burke and Miles 1958). To investigate this possibility 10^6 viable units of Staphylococcus aureus strain PS 80 were injected in a volume of $5/\mu$ l broth culture by means of a microsyringe; the response was similar to that elicited by the injection of 0.05 ml. of culture fluid. Therefore the earlier exudation of fluid and emigration of leucocytes in the absence of cotton was not due to the greater volume of broth injected.

The exact mechanism by which cotton dust causes the initial delay in the onset of the inflammatory reaction is unknown. Nicholls (1961) showed that extracts of cotton dust from the cardrooms of English and Dutch cotton mills caused contraction of

the isolated ileum of the guinea-pig and vasoconstriction in the perfused hind quarter of rats. Extracts of the cotton dust used in the present studies were prepared by grinding 5 g. of the material with 20 ml. of saline in a mortar; this extract was injected along with PS 80. The lesions produced were similar to those caused by the injection of PS 80 alone.

Mice that were injected with PS 80 in the presence or absence of cotton-dust were killed. Both kidneys, liver, spleen and heart blood were removed aseptically and cultured for the presence of staphylococci. All the cultures were sterile. However there was a possibility that the presence of small numbers of cocci in the viscera could have been missed. Therefore the possibility that in the absence of cotton dust, the cocci were quickly washed away from the local site could not be discounted. But there was no widespread dissemination of cocci as indicated by the results of culture.

It was found that the cotton plug was penetrated within minutes of intravenous injection of Evans blue. This suggested that the material present in the blood especially the humoral factors were able to penetrate the cotton plug.

2. The effect of varying dosage of *Staphylococcus aureus* PS 80 with cotton dust.

In the previous section, the infection enhancing effect of cotton dust was described. This section deals with the response observed by injecting different doses of staphylococcus PS 80 with

MEAN OEDEMA
WEIGHT (mg.)

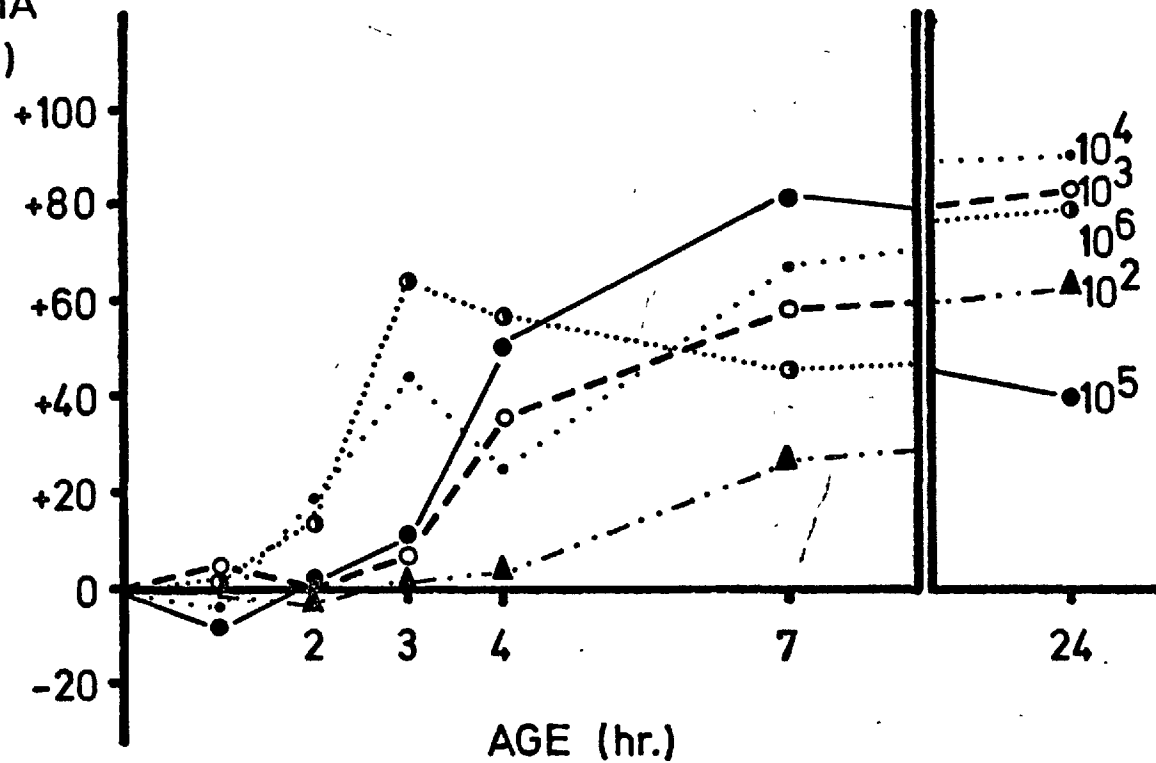


Figure 15: Mean oedema weight of tissue to the subcutaneous injection of varying doses of Staphylococcus aureus PS80 mixed with cotton-dust.

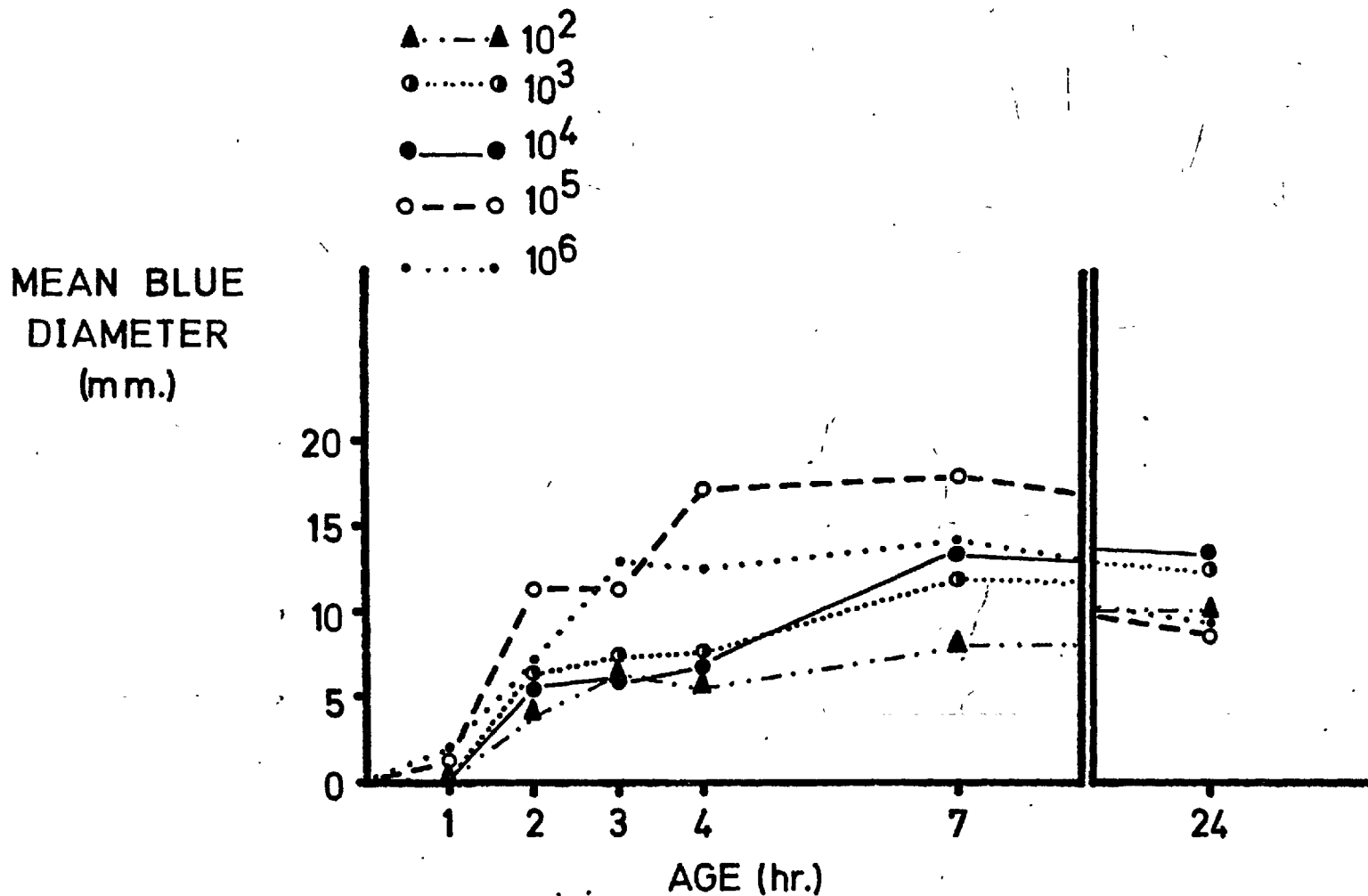


Figure 16: Increased vascular permeability, expressed as diameter of area of exudation of dye-stained plasma protein, to the subcutaneous injection of varying doses of Staphylococcus aureus PS80 mixed with cotton-dust.

a constant amount of cotton dust.

Mice were injected with varying doses of staphylococci of strain PS 80 in cotton dust and examined after 1, 2, 3, 4, 7 and 24 hours (figures 15 to 19). The experiments with varying dosage of PS 80 were not all done simultaneously. However, 10^5 PS 80 were injected into mice on two different occasions and similar results were obtained. While with 10^5 dose each point on the graph represents the mean from 8 mice, with the rest each point is a mean from 4 mice.

(a) Fluid exudation

The relation of fluid exudation to dose is shown in figures 15 and 16. According to the tissue weight determinations (figure 15), although the time of onset of exudation tended to be earlier with the larger doses, there was never evidence of exudation at 1 hour and rather little at 2 hours after injection. The fact that in some mice there appeared to be a diminution in tissue weight may represent the natural variability in the determinations or may perhaps result from actual constriction of blood vessels. However the fact, that in most cases at least three out of four mice did not show any exudation up to the first hour, means that there was truly a delay in the onset of fluid exudation.

Measurement of the "blue diameter", after injection of Evans blue (figure 16) gave very similar results to the weight estimations, though suggesting that the fluid exudation commenced slightly earlier.

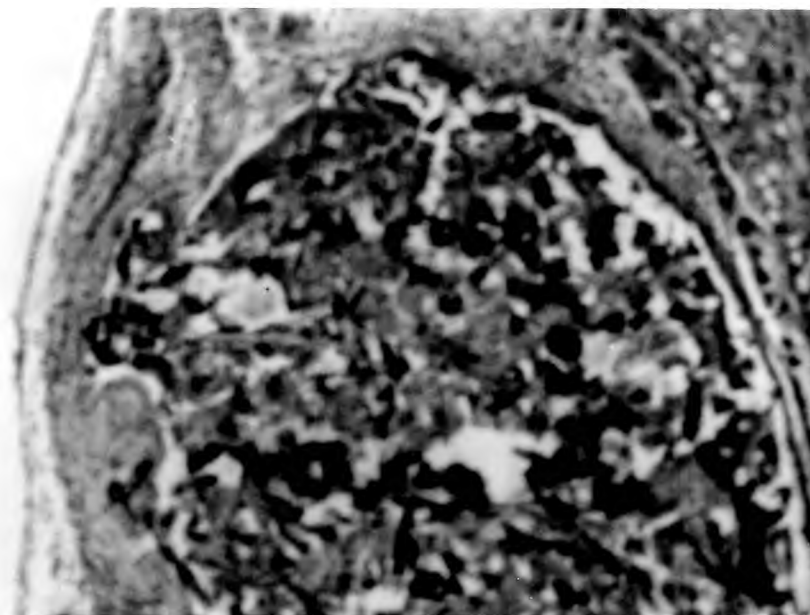


Figure 17: Twenty-four hours after the subcutaneous injection of 10^4 Staphylococcus aureus PS 80 mixed with cotton-dust. Leucocytes have infiltrated throughout the cotton-dust. X40.

This might mean that the Evans blue method is the more sensitive, but Spector (1958) considered that the tissue weight offered the better method for determination of exudation in subcutaneous tissue.

(b) Leucocyte emigration

With a high dose of PS 80 (10^6 to 10^5) the emigration of leucocytes was not noticeable until after 2 hours and by 4 hours, although the leucocytes had come out of blood vessels, they remained at some distance from the edge of the cotton. It was 7 hours before any significant infiltration of the cotton dust was seen. With the 10^6 dose the leucocytes had not completely penetrated to the central part even at 24 hours; however with 10^5 cocci there was heavy and complete infiltration of cotton at 24 hours. With lower doses of cocci, although the emigration was found to start at 2 hours, the infiltration of cotton which contains cocci was noticeable 3 - 4 hours after the challenge. At 24 hours there was a heavy infiltration of entire cotton dust (figure 17).

(c) Bacterial counts in the lesion

Counts were made of the numbers of cocci inoculated ("inoculum") and of the numbers recovered from the tissue of mice killed immediately after inoculation ('zero-hour count'), as well as of the numbers recovered at intervals subsequently. The results are shown in figure 18. Each point on the graph represents the mean

BACTERIAL COUNT

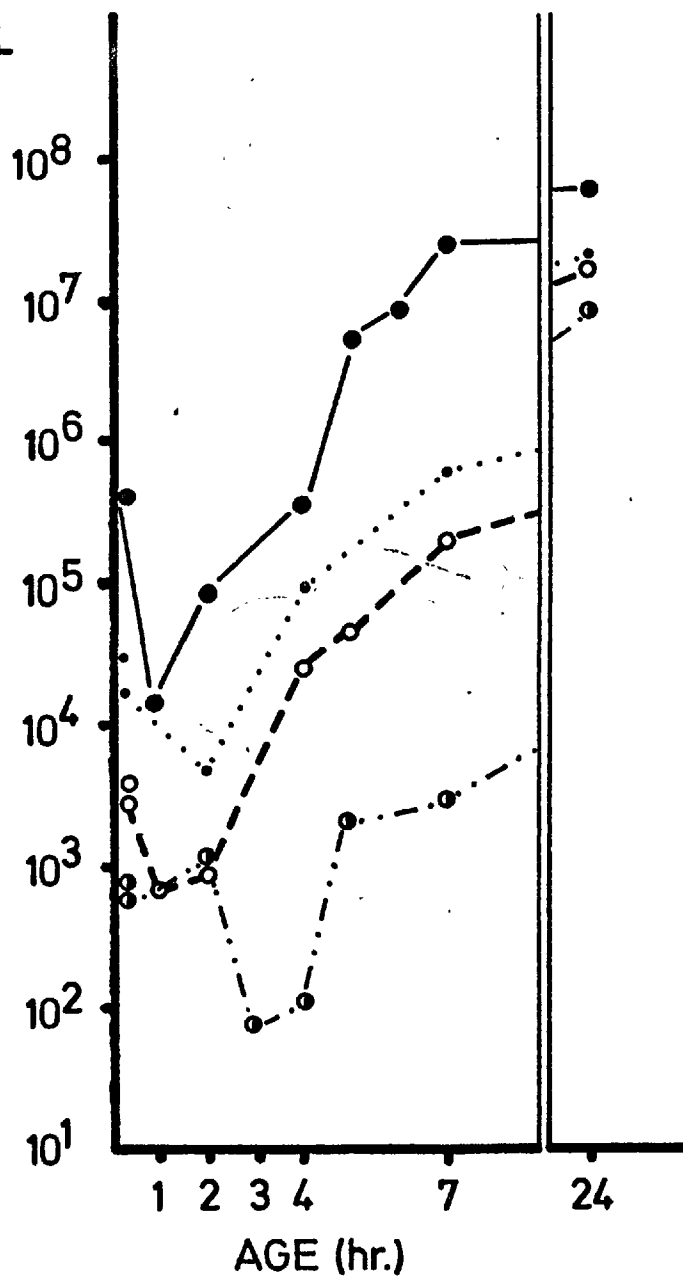


Figure 18: Bacterial counts in the lesion after the subcutaneous injection of varying doses of Staphylococcus aureus PS80 mixed with cotton-dust.

of the values from two mice. With the 10^5 inoculum, a zero-hour count was not done. The upper point on the left hand margin of the graph represents the inoculum and the lower, the zero-hour count.

The zero-hour count was always lower than the inoculum, by 20 - 50 per cent. This indicates that it was difficult to recover all the cocci in the lesion, because it seems unlikely that such a high proportion of cocci could be killed within seconds of being introduced. Moreover, whether the original inoculating dose was high (10^4) or low (10^2), the reduction was proportional and in no case did the count drop to zero.

The immediate drop in count might be due to clumping of cocci, which has been reported during the course of staphylococcal peritonitis in mice (Kapral 1965). It was therefore decided to test the importance of clumping in the subcutaneous lesion. A 0.5% trypsin solution in $\frac{1}{2}$ Ringers was made and sterilized by seitz filtration; 0.1 ml of trypsin solution was added to 0.9 ml. of homogenized tissue. For control 0.1 ml. of $\frac{1}{2}$ Ringers was added to a similar volume of homogenized tissue. Both the test and control materials were left at room temperature for 30 minutes before doing the bacterial count. The viable counts of cocci from the lesion was not different in the two cases. It appears that clumping may not be playing an important role in altering the bacterial count in the subcutaneous lesion. As described in previous section, there was also no

TABLE 3

Bacterial counts in the lesions produced by varying dosage of PS 80.

Initial inoculum	Initial drop	Time of Lowest count in hour	Rate of growth k/hour	Count at	
				7 hours	24 hours
4×10^5	not tested	1	0.51	2.3×10^7	5.91×10^7
3×10^4	1.68×10^4	2	0.41	5.6×10^5	1.95×10^7
3.81×10^3	2.83×10^3	1	0.41	1.83×10^5	1.68×10^7
7.83×10^2	6.16×10^2	3	0.46	2.88×10^3	8.06×10^6

BACTERIAL COUNT

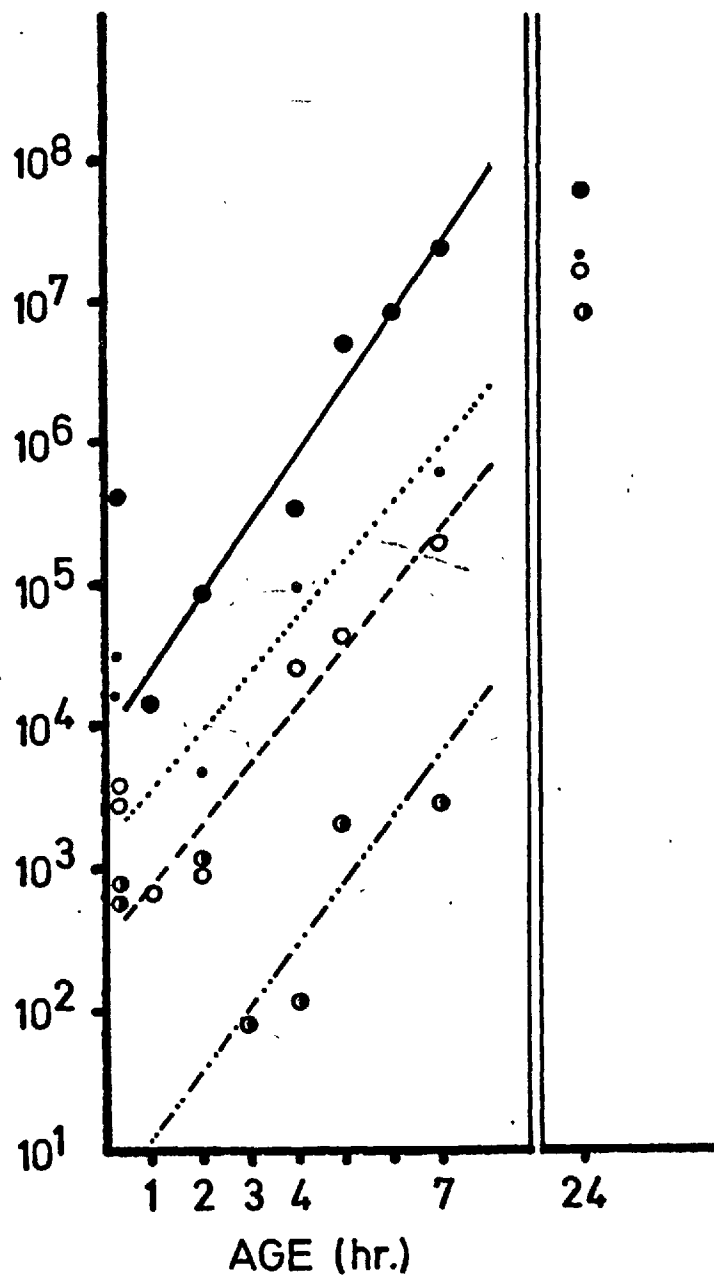


Figure 19: Growth curves of cocci in the lesion after the subcutaneous injection of varying doses of *Staphylococcus aureus* P880 mixed with cotton-dust.

evidence of dissemination of cocci to other parts of the body.

With all the inocula there was a further drop in the numbers of viable cocci recovered during the period after inoculation; the lowest count was observed during 1 to 3 hour sampling depending on the initial inoculum. The detailed results of bacterial counts are shown in Table 3. As there was no evidence of widespread dissemination of cocci from the site of injection or clumping of cocci in the lesion, it seemed that these low counts represented the actual destruction of cocci.

The rate of multiplication of the cocci after the drop in count is shown in Table 3. The growth rate was calculated according to the formula $k = \frac{\log nt - \log n}{t}$ (Keynell and Keynell 1965); where k is the growth rate constant, t is the time in hour and n is the number of bacteria. As this equation presupposes that the growth curve in the logarithmic phase is represented by a straight line, the bacterial counts at various intervals with different doses of PS 80 were plotted on a log paper and the straight lines closest to the points were drawn (Fig. 19). With 10^2 dose the regression line was drawn on the basis of a few points, therefore its slope may not have the same validity as in the case of 10^5 . The value of k was then calculated on the basis of these straight lines. The growth rate of 10^5 PS 80 was slightly greater than the rest. With the lower 3 doses, the

TABLE 4

Severity of Lesion produced by injecting varying
numbers of PS 80 with cotton dust.

Dose of PS 80 injected	No. of mice tested	Lesion score*	Diameter of necrotic zone
10^6	24	5.8	8.5 mm.
10^5	24	4.8	4.2 mm.
10^4	12	3.6	4.2 mm.
10^3	24	2.8	1.4 mm.
10^2	12	2.5	0.4 mm.

* The maximum possible lesion score is 6

(see page 55)

growth rates were very similar. The growth curves also indicated that with 10^5 dose the maximum bacterial population was reached in about 8 hours; with lower dosage it reached presumably at varying times later on.

At 24 hours the bacterial population was very similar for all four inocula. Although the number of viable units of cocci in the original inoculum differed by 4 log units, the bacterial count at 24 hours with the varying inocula differed only by 1 log unit.

(d) Lesion score

Although the viable counts of cocci in the lesion 24 hours after the injection of varying numbers of PS 80 did not show much difference, the lesions differed considerably in their severity. The average lesion score and the diameter of necrotic zone is shown in Table 4.

With the lowest dose (10^2), the lesions were mainly purulent and only rarely showed a 1-2 mm. zone of necrosis. With higher dosage the lesions were necropurulent and with 10^6 dose, there was hardly any swelling; almost all the mice had an area of necrosis more than 5 mm. in diameter.

3. Response to different strains of staphylococci and micrococci

Since there is evidence, based both on epidemiological studies and animal experiments, that strains of Staphylococcus aureus vary in their virulence (Williams 1963, Noble 1966), it seemed of interest to investigate the details of the inflammatory response

TABLE 5.

Average lesion score per mouse

Strain	Number of mice	Lesion score
1531	12	5.0
PS 80	24	4.8
Orbach	12	4.6
SSmith	12	2.8
P66/7	6	2.8
Wood 46	12	2.3
Ward	12	2.1
M. hyicus	12	2.0
PS 83A	12	2.0
NOTC 7292	12	1.6
278	12	1.6

elicited by strains of staphylococci that differ in their ability to produce subcutaneous lesions in mice.

In these experiments 10^5 viable units of different strains of staphylococci and micrococci were injected subcutaneously in mice with the cotton dust.

(a) Lesion score

The strains used in the experiment were graded according to the severity of the lesion produced after the inoculation of 10^5 cocci in the cotton dust (Table 5). In most cases the average lesion score is based on the observation on 12 mice, but with PS 80 the experiments were done several times and each time the lesions produced were severe. Of the strains tested, 278 is a micrococcus; M. lylis and NCTC 7292 are coagulase negative staphylococci and the rest of the strains are Staphylococcus aureus. Strains 1531, PS 80 and Orbach produced lesions of great severity, the rest of the strains produced lesions of moderate to mild severity. Strains having higher lesion scores produced big necrotic zones, while those with low lesion scores were characterized mainly by swelling and pus formation. It is significant that although Wood 46 produced large quantities of alpha haemolysin and delta haemolysin in vitro, it failed to produce a large necrotic lesion in the mouse.

(b) Fluid exudation

In these experiments fluid exudation was deduced by

MEAN OEDEMA
WEIGHT (mg.)

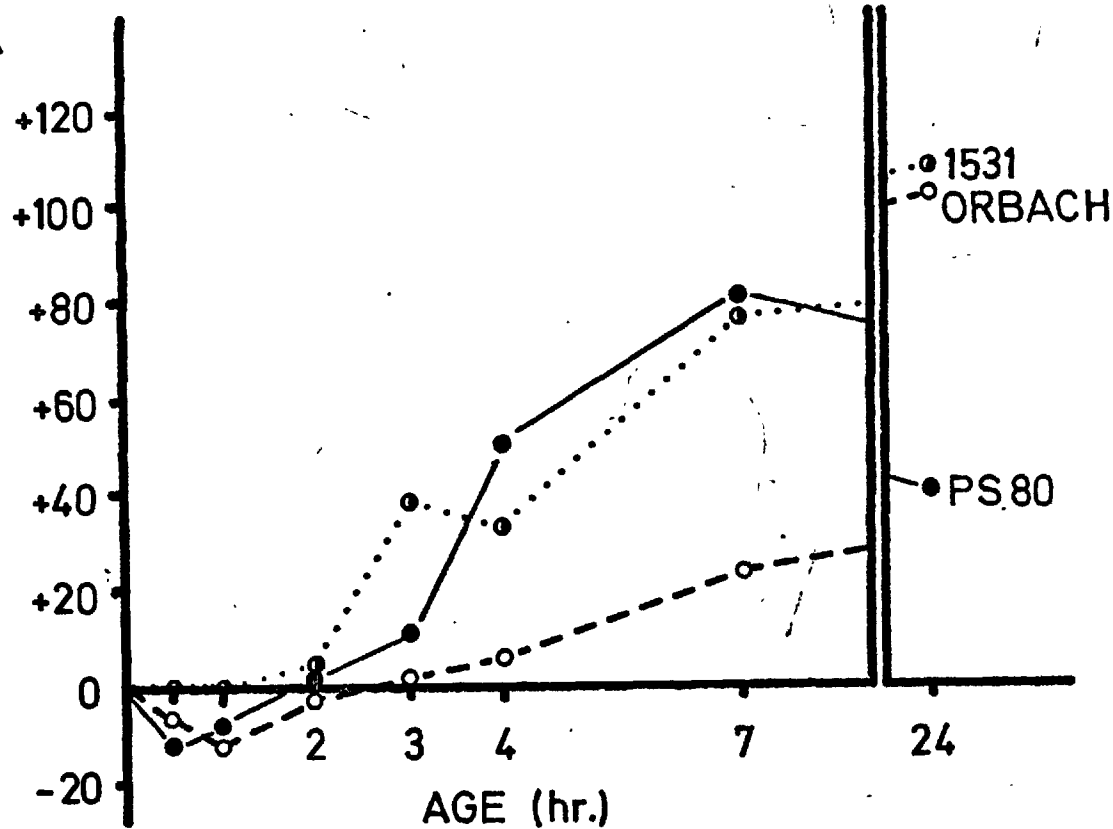


Figure 20: Mean oedema weight of tissue after the subcutaneous injection of 10^5 Staphylococcus aureus strains 1531, Orbach and PS80 mixed with cotton-dust.

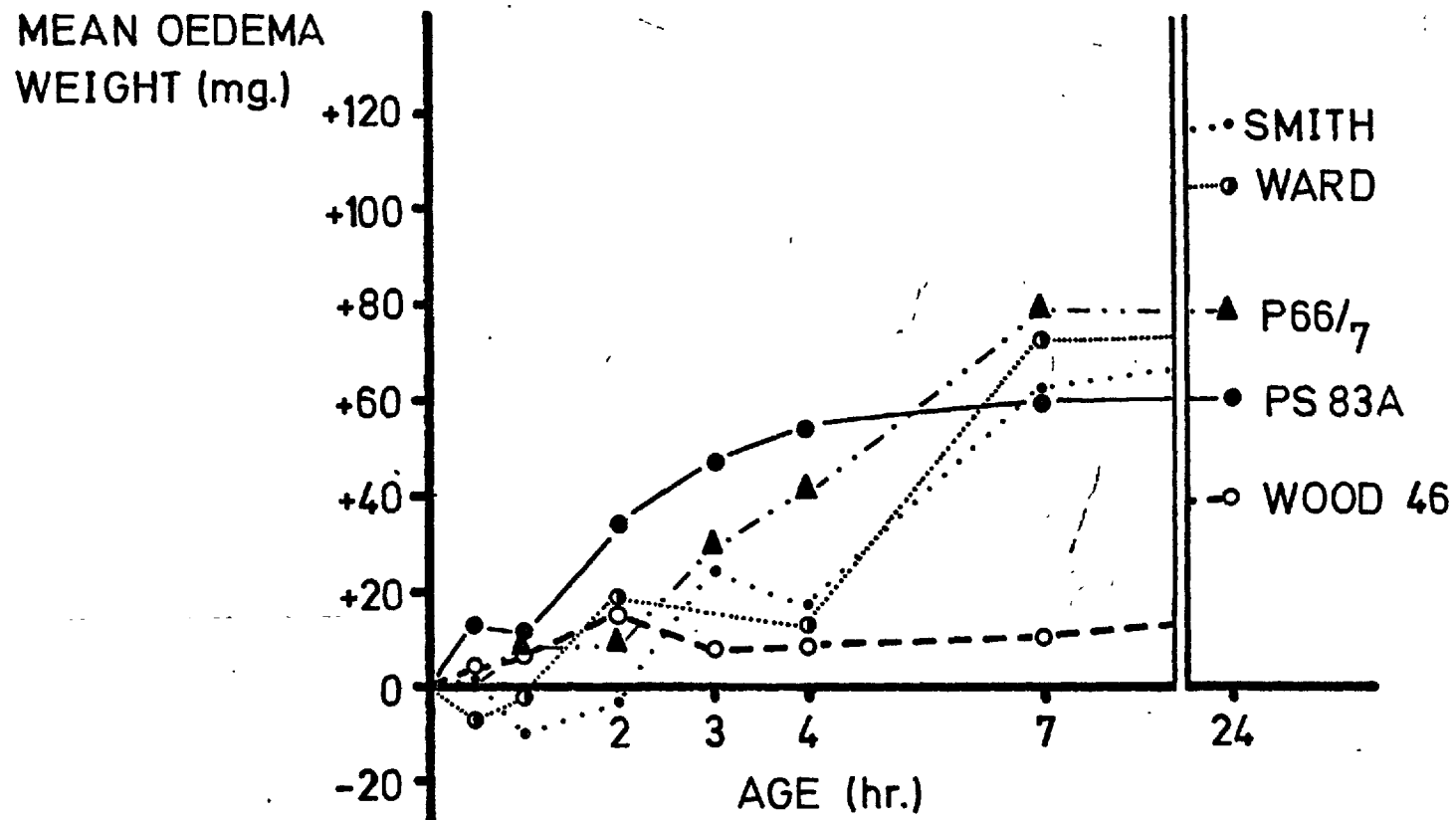


Figure 21: Mean oedema weight of tissue after the subcutaneous injection of 10^5 Staphylococcus aureus strains Smith, Ward, P66/7, PS 83A and Wood 46 mixed with cotton-dust.

Mean oedema weight (mg.)

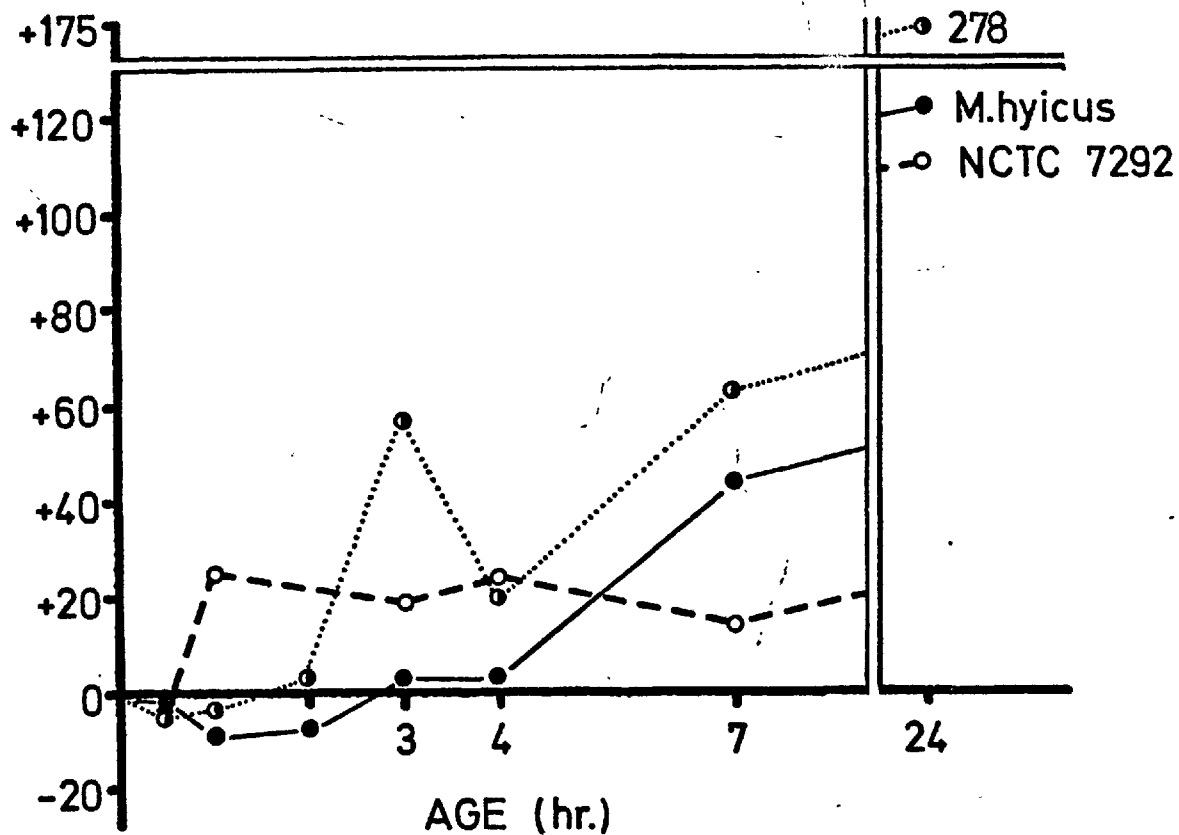


Figure 22: Mean oedema weight of tissue after the subcutaneous injection of 10^5 coagulase-negative staphylococci and micrococcus strain 278 in the presence of cotton-dust.

Leucocytes
per H.P.F.

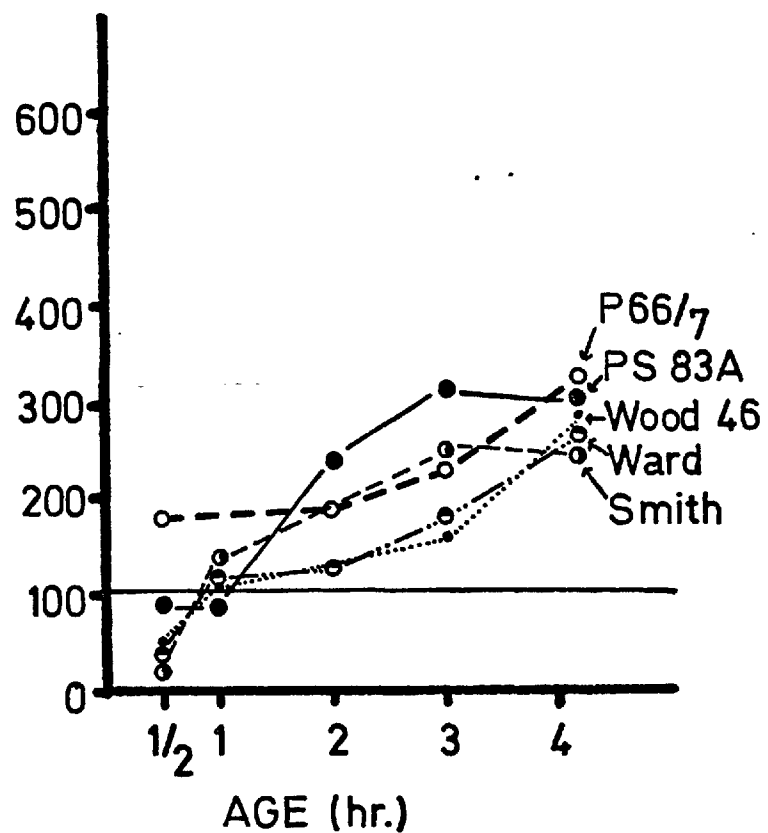
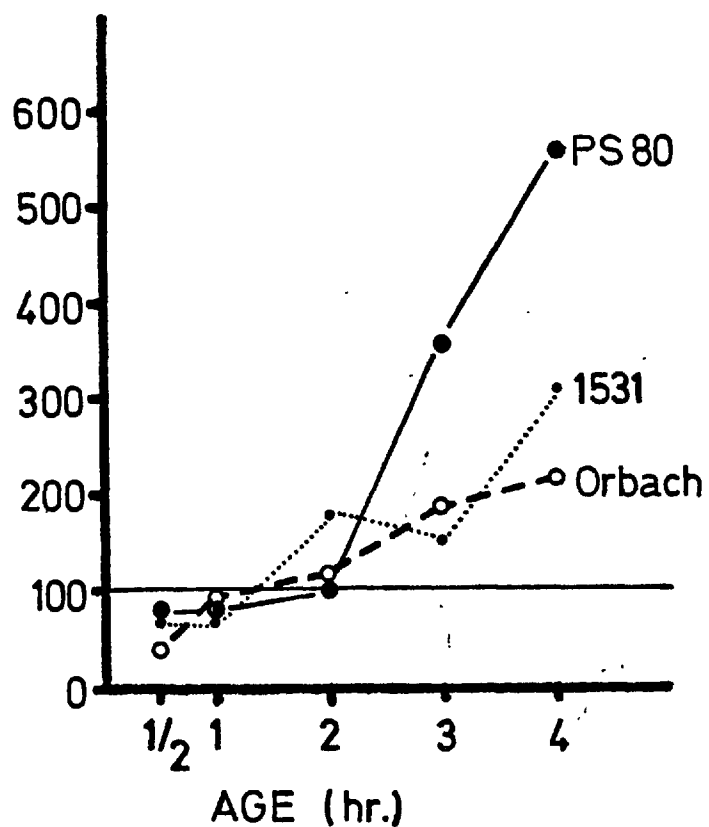


Figure 23: Leucocyte count in the tissue after the subcutaneous injection of 10^5 dose of different strains of Staphylococcus aureus mixed with cotton-dust.

measurements of tissue weight (figures 20 to 22). The three mouse-virulent strains - 1531, PS 80 and Orbach elicited the exudation of fluid only after a delay of 2 hours (figure 20). In the case of Orbach and PS 80 for the first two hours, the weight of the tissue containing the lesion was less than its expected normal weight. Of the remaining 5 strains of Staphylococcus aureus which produced lesions of moderate to mild severity, 4 produced an earlier exudation of oedema fluid than that produced by the 'virulent' strains. The exudation of fluid began between $\frac{1}{2}$ and 1 hour after inoculation (figure 21). The fifth strain, Smith, elicited the exudation of fluid with an initial delay of 2 hours like the 3 most virulent strains.

The fluid exudation elicited by strains of coagulase negative staphylococci and the micrococcal strain is shown in figure 22. Strain NOTC 7292 elicited the exudation of fluid 1 hour after the injection. Strain 278 and M. hyicus although producing lesions of only mild severity elicited the exudation of fluid with an initial delay of 2 to 3 hours like the 3 most virulent strains. At 24 hours these 3 strains produced more oedema than any of the other strains except Smith.

(c) Leucocyte emigration

The leucocyte counts in the tissue are shown in figure 23 and 24. With the three mouse-virulent strains the emigration of leucocytes occurred after an initial delay of 2 hours. On the other hand 6 out of 8 strains producing lesions of moderate to mild

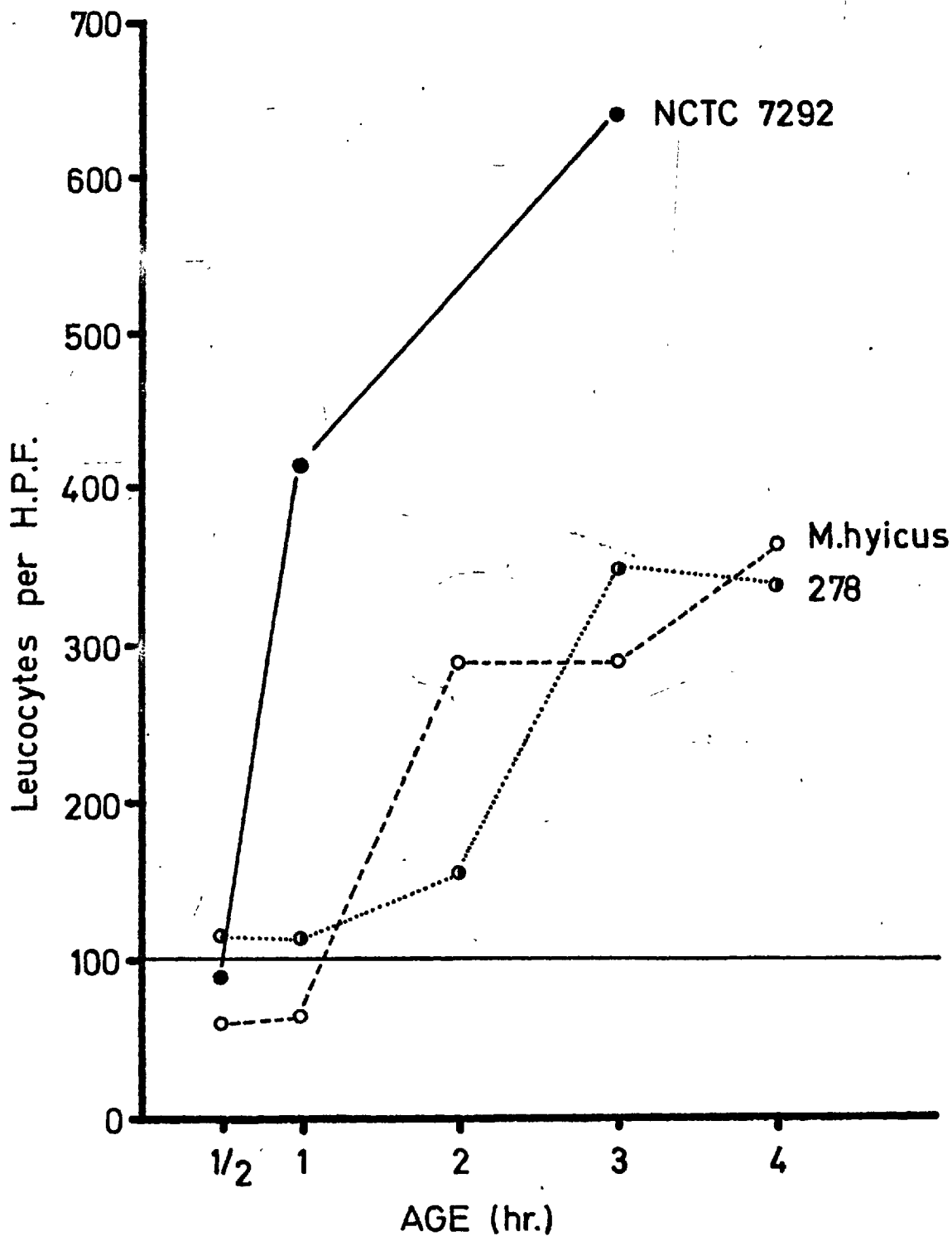


Figure 24: Leucocyte count in the tissue after the subcutaneous injection of 10^5 coagulase-negative staphylococci and micrococcus strain 278 in the presence of cotton-dust.



Figure 25: Seven hours after the subcutaneous injection of 10^5 Staphylococcus aureus strain 1531 mixed with cotton-dust; only the peripheral parts of cotton-dust being infiltrated by leucocytes. X40.

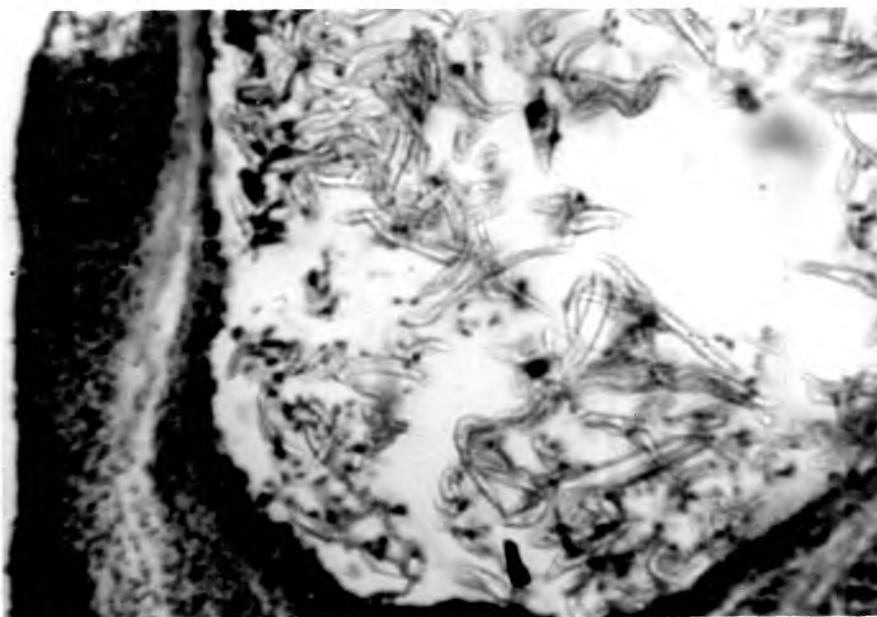


Figure 26: Twenty-four hour old lesion in mice produced by the subcutaneous injection of 10^5 Staphylococcus aureus strain 1531 mixed with cotton-dust. No infiltration of cotton-dust by leucocytes. X60.

severity gave rise to an early tissue leucocytosis. While with P66/7 and 278 infection leucocyte emigration was seen $\frac{1}{2}$ an hour after the challenge, with Smith, Ward, Wood 46 and NOTC 7292 it was apparent 1 hour after the inoculation. With PS 83A and M. hyicus which gave poor lesion scores, the emigration of leucocytes was delayed until 2 hours after the injection as was the case with the mouse-virulent strains.

In no case was the infiltration of cotton dust apparent before 3 to 4 hours and in the lesions produced by PS 80, Orbach and 1531 only the peripheral parts of the cotton were infiltrated even at 7 hours (Figure 25). With the other strains, the cotton was infiltrated throughout by leucocytes by 7 hours. A few histological preparations taken from the 24 hour old lesions of Orbach and 1531 showed no evidence of leucocyte infiltration (Figure 26). With the other strains there was a dense collection of leucocytes in the region of cotton dust by this time.

It was not possible to quantitate the cellular reaction at 24 hours with accuracy, because of the very dense collection of leucocytes in the region of cotton dust. Moreover the leucocytes were not uniformly distributed throughout the lesion. Lastly, in many sections, the leucocytes were broken up and distorted making accurate counts impossible.

The morphological changes produced in mouse leucocytes by the extracellular products of Staphylococcus aureus have been described

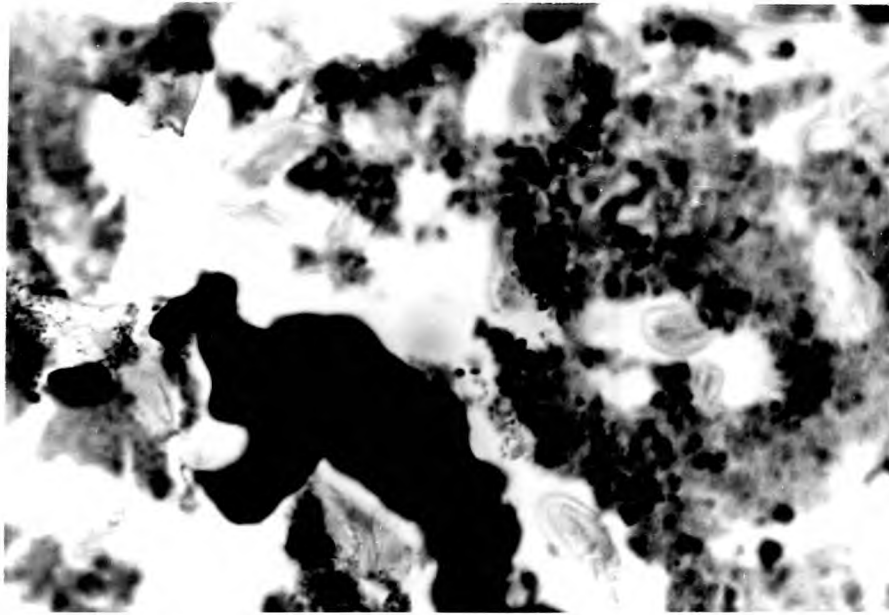


Figure 27: Twenty-four hour old lesion in mice produced by the subcutaneous injection of 10^5 Staphylococcus aureus strain 1531 mixed with cotton-dust. See the distorted leucocytes. X250.

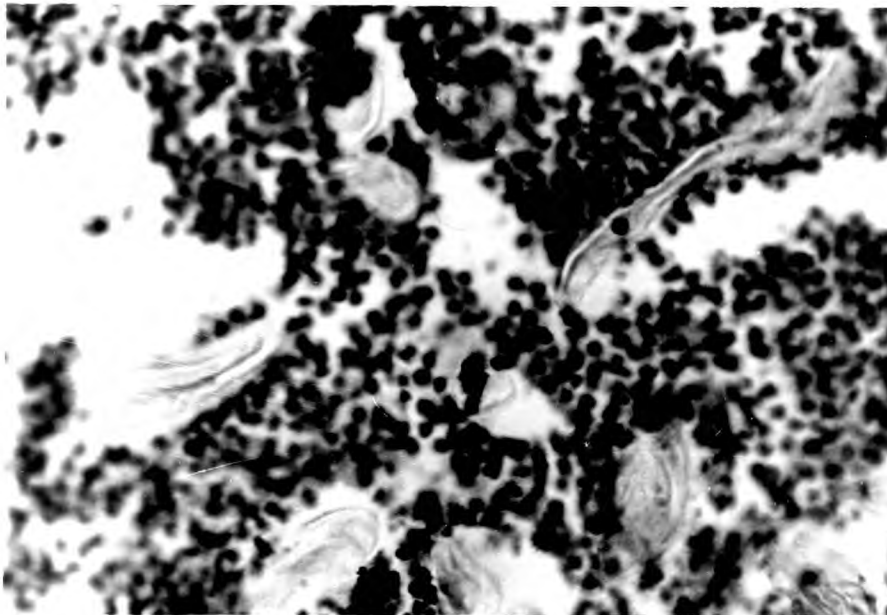


Figure 27a: Twenty-four hour old lesion in mice produced by the subcutaneous injection of 10^5 Staphylococcus aureus strain Wood 46 mixed with cotton-dust. Leucocytes better preserved than in fig. 27. X250.

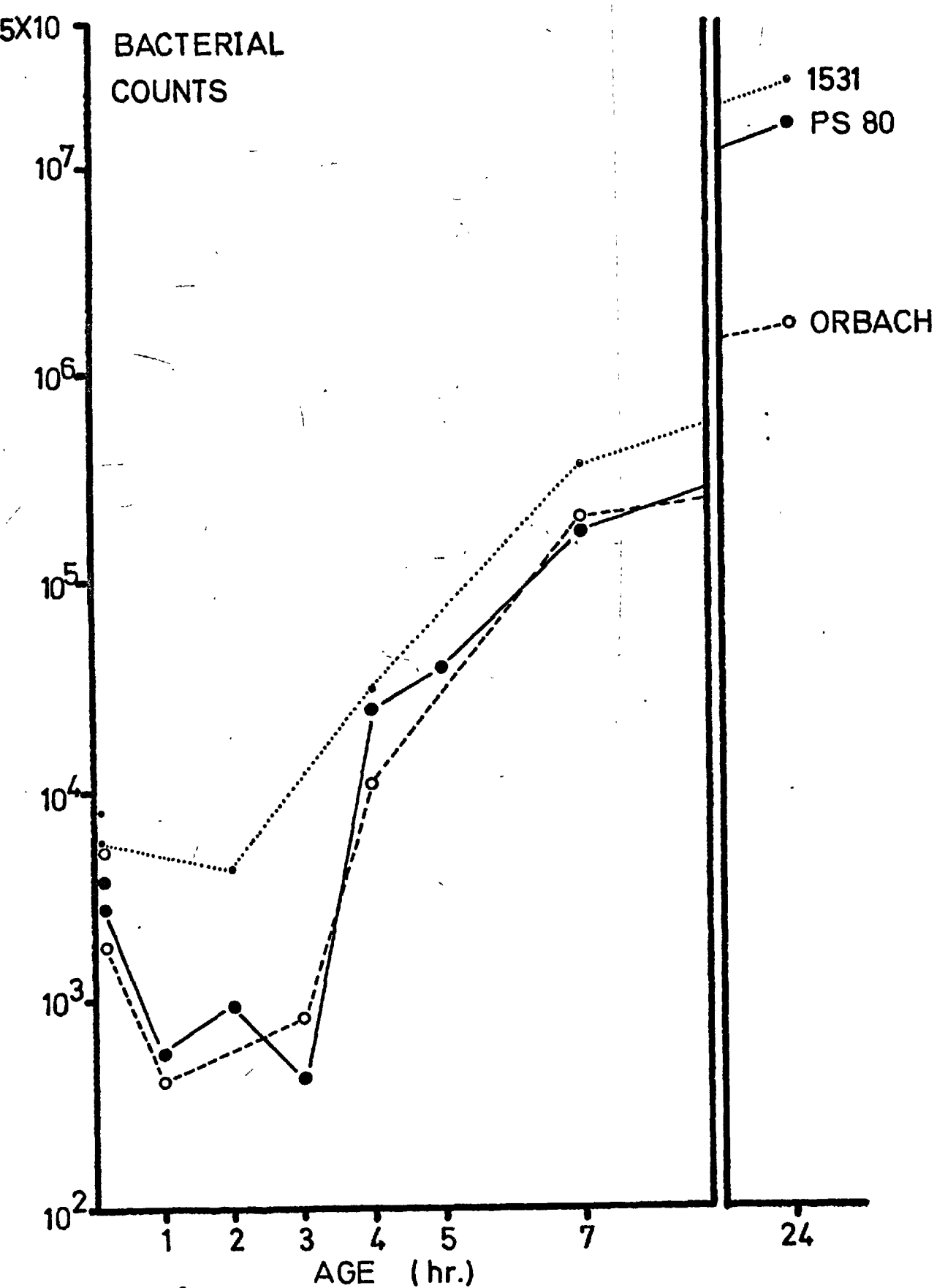


Figure 28:

Bacterial counts in the lesion after the subcutaneous injection of *Staphylococcus aureus* strains 1531, PS 80 and Orbach in the presence of t n-dust.

by Gladstone and van Heyningen (1957). Distortion of the leucocytes was apparent in the mature lesions. All the strains producing alpha and delta haemolysin in vitro showed the distortion of the leucocytes in the lesions (figure 27), except Wood 46. This strain produces delta haemolysin and large quantities of alpha haemolysin in vitro, yet the leucocytes were better preserved in the lesion (Figure 27a). In the lesions of strains 278, NCTC 7292 and M.hydans which produced neither delta or alpha haemolysin, the form of the leucocytes was also maintained.

(d) Bacterial counts in the lesion

While all the previous studies i.e., lesion score, increased vascular permeability and leucocyte emigration were carried out by injecting 10^5 cocci, for the bacterial counts in the lesion 10^3 cocci were introduced along with the cotton dust, because with a dose of 10^5 cocci, the finer differences in the growth patterns of different strains were not brought out.

With all the strains, 'zero-hour counts' were always lower than the initial inoculum, and during the period after injection there was a further drop in the numbers of viable cocci.

Figure 28 shows the result of bacterial counts in the lesions of the three mouse-virulent strains. There was a rapid rise in the number of recoverable viable units after the first 2 hours.

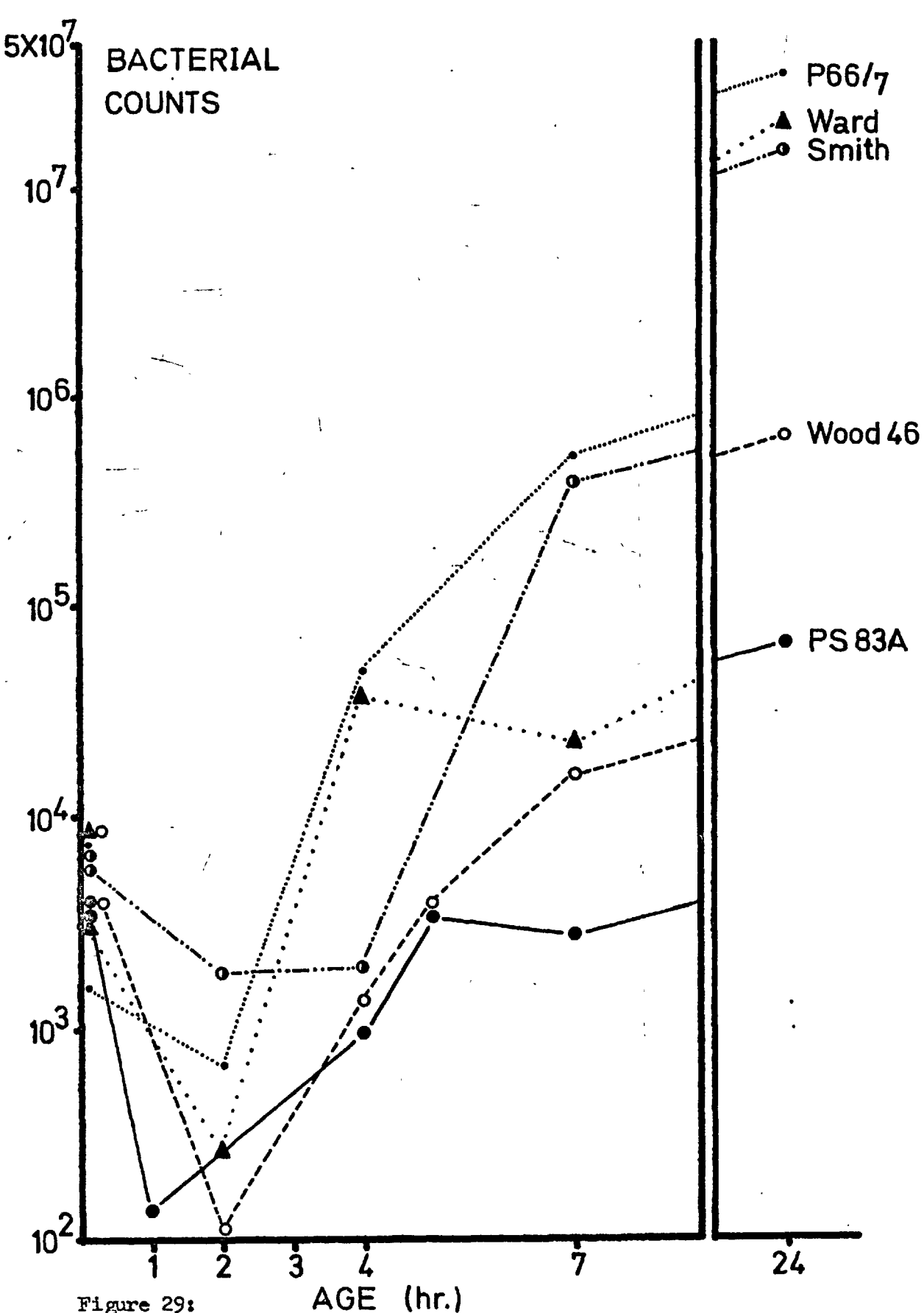


Figure 29:

Bacterial counts in the lesion after the subcutaneous injection of Staphylococcus aureus strains Smith, P66/7, Ward, Wood 46 and PS 83A mix

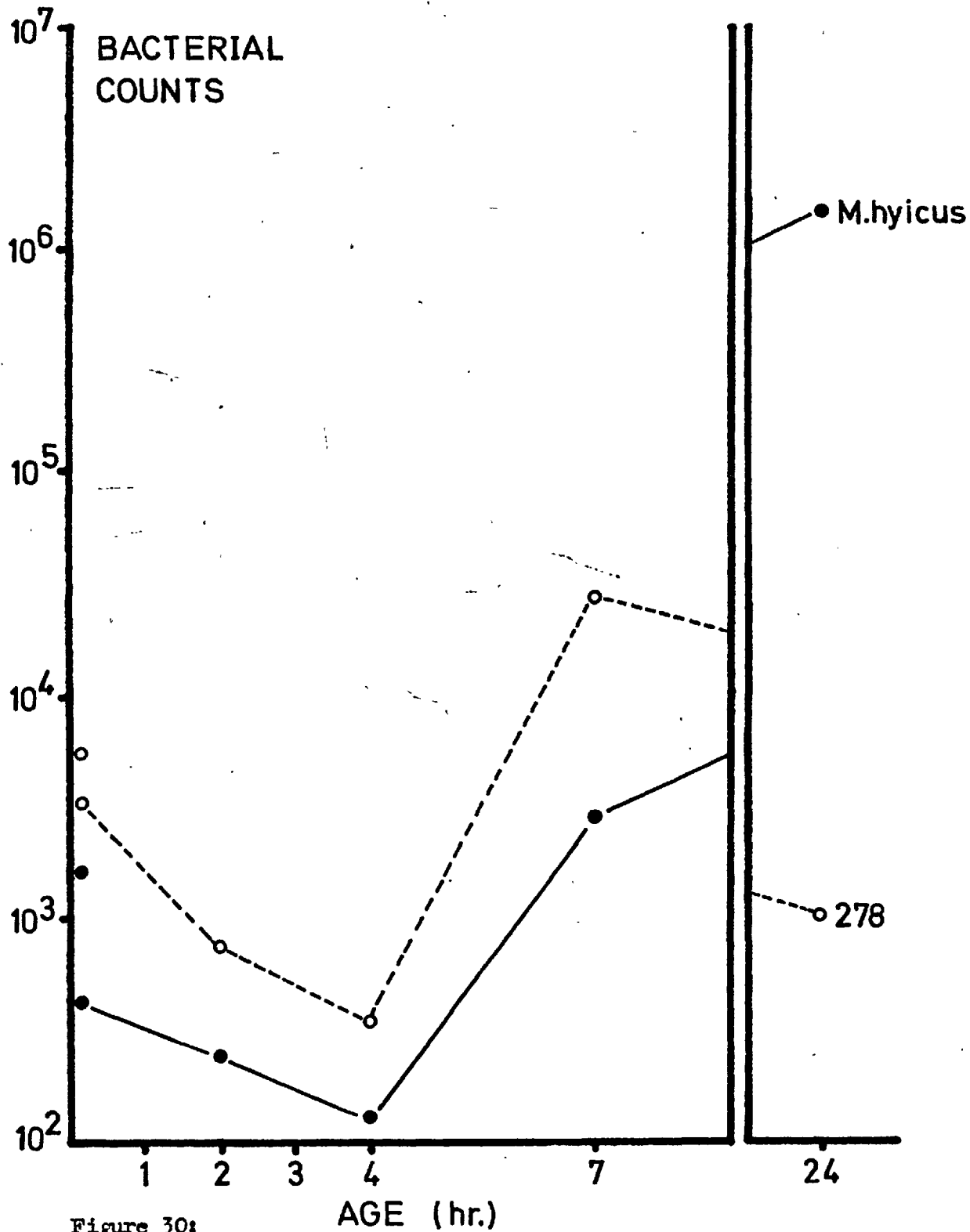


Figure 30:

Bacterial counts in the lesion after the subcutaneous injection of M. hyicus and strain 278 mixed with cotton-dust.

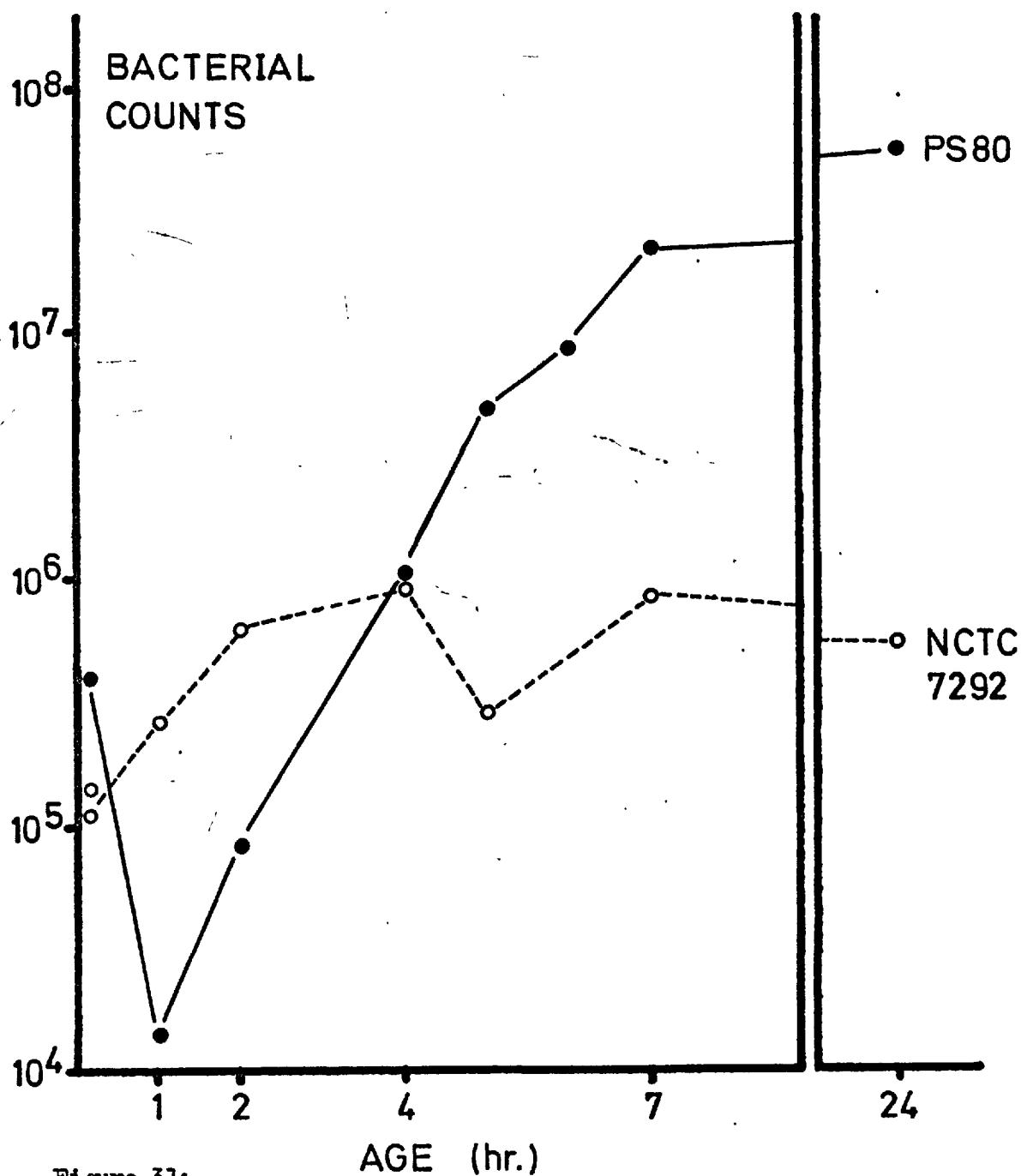


Figure 31:

Bacterial counts in the lesion after the subcutaneous injection of coagulase-negative staphylococcus NCTC 7292 and Staphylococcus aureus PS80 in the presence of cotton-dust.

Four hours after the injection the bacterial count exceeded the initial number of bacteria inoculated. By 24 hours the bacterial population had increased by 3 to 4 log units to a final count of between 2×10^6 and 3×10^7 .

The bacterial counts in the lesions of remaining five strains of Staphylococcus aureus which produced lesions of moderate to mild severity are shown in figure 29. In the lesions due to Wood 46, PS 83A and Ward, the initial drop was greater than with the 3 mouse-virulent strains and at 4 hours the bacterial count was still lower than the initial inoculum. With PS 83A even at 7 hours the population of cocci was lower than the dose inoculated. On the other hand in the lesions of Smith and P66/7 the bacterial counts at 7 hours were similar to that seen in the mouse-virulent strains.

Bacterial counts in the lesions due to M. hyicus and strain 278 are shown in figure 30. During the first 4 hours the bacterial counts were lower than the initial inoculum. It was only at 7 hours that the bacterial counts became greater than the original dose. While with M. hyicus, the 24 hour count was as high as with the mouse-virulent strains, with 278 the bacterial population at 24 hours was lower than the initial number of cocci inoculated.

Figure 31 shows the bacterial count in the lesion of NOTC 7292. In this case the bacterial count was done after injecting 10^5 cocci because even with this high dose there was hardly any

TABLE 6

Bacterial counts in the lesions of different strains of
staphylococci.

Strain	Dose inoculated	Initial drop	Time of lowest count (h)	Rate of growth k/hour	Count at	
					7 hr	24 hr.
1531	8.1×10^3	5.5×10^3	2	0.42	3.68×10^5	2.5×10^7
PS 80	3.81×10^3	2.83×10^3	1	0.52	1.83×10^5	1.68×10^7
Orbach	5×10^3	1.86×10^3	1	0.53	2.04×10^5	1.73×10^6
Smith	6.83×10^3	6.33×10^3	2	0.49	3.83×10^5	1.5×10^7
P66/7	7.83×10^3	1.66×10^3	2	0.50	5.24×10^5	3.5×10^7
Wood 46	8×10^3	3.91×10^3	2	0.36	1.59×10^4	6.66×10^5
Ward	8×10^3	3.1×10^3	2	0.30	2.3×10^4	2.17×10^7
M. hyicus	1.6×10^3	4.3×10^2	4	0.28	2.99×10^3	1.6×10^6
PS 83A	4×10^3	3.75×10^3	2	0.28	2.74×10^3	6.79×10^4
NOTC 7292	1.45×10^5	1.11×10^5	1	0.08	8.6×10^5	5.81×10^5
278	5.6×10^3	3.5×10^3	4	0.32	2.75×10^4	1.05×10^3

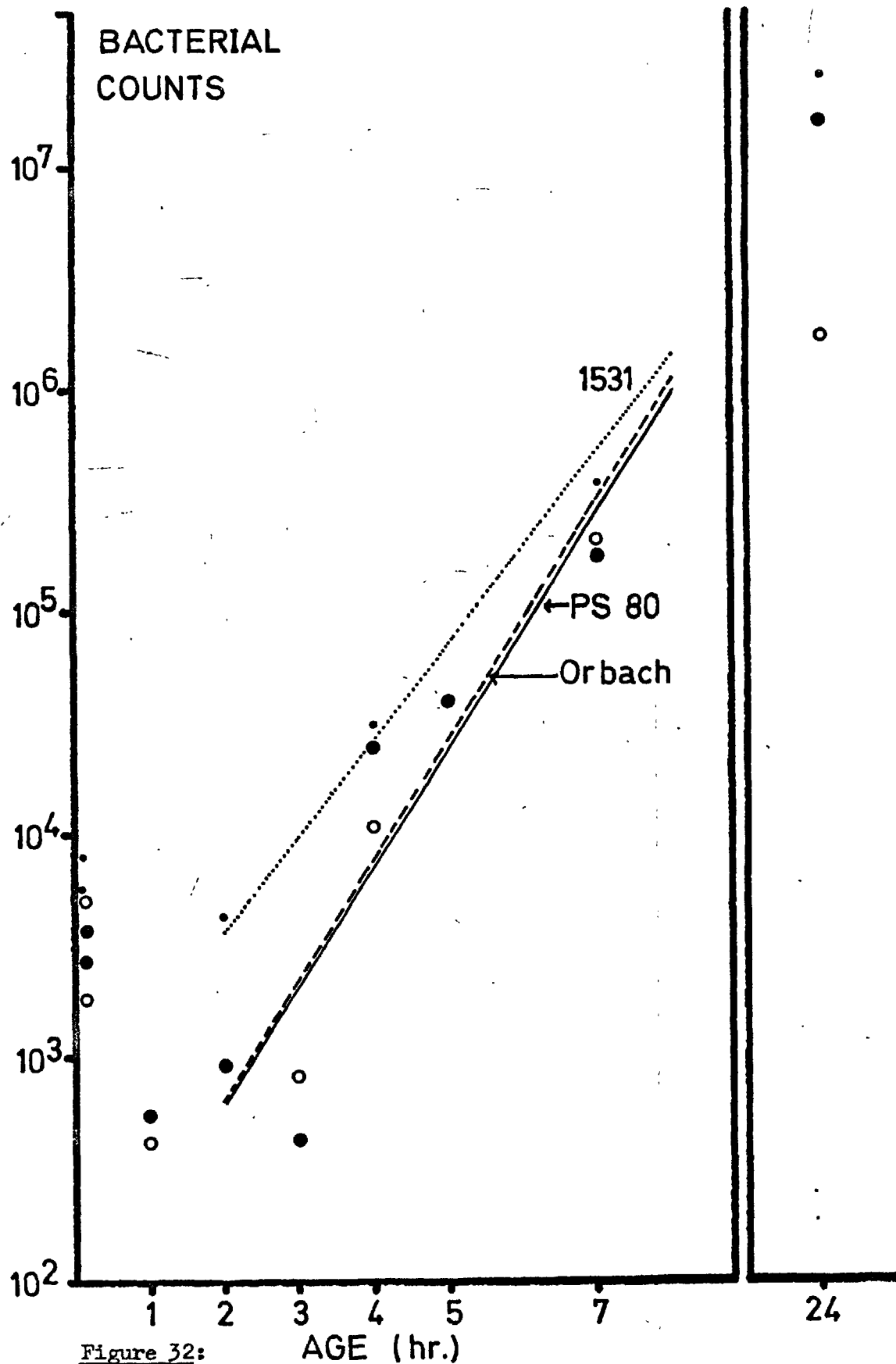


Figure 32:

Growth curves of cocci in the lesion after the subcutaneous injection of Staphylococcus aureus strains 1531, PS80 and Orbach in the presence of cotton-dust.

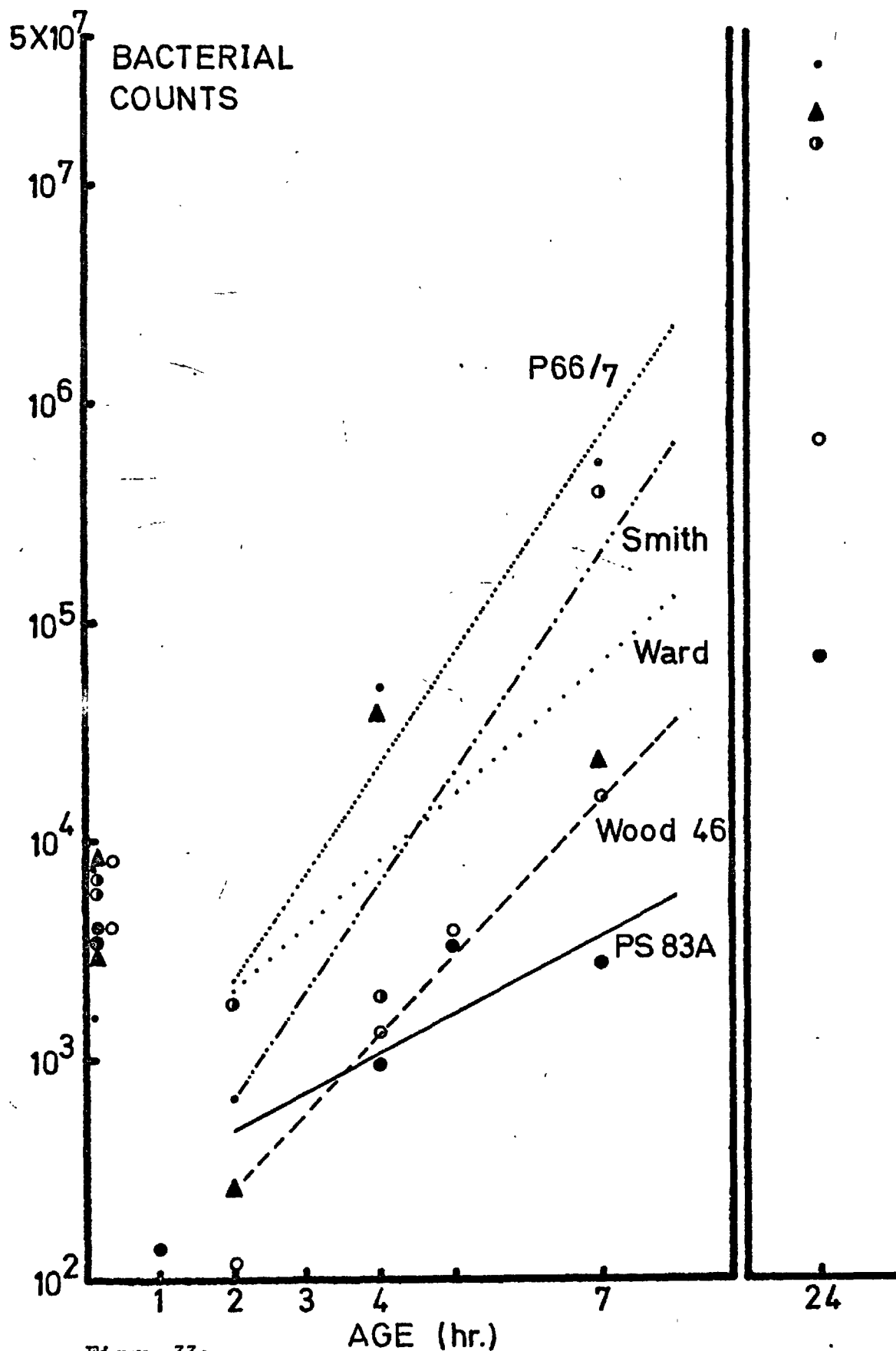


Figure 33:

Growth curves of cocci in the lesion after the subcutaneous injection of *Staphylococcus aureus* strains Smith, P66/7, Ward, Wood 46 and PS 83A mixed with cotton-dust.

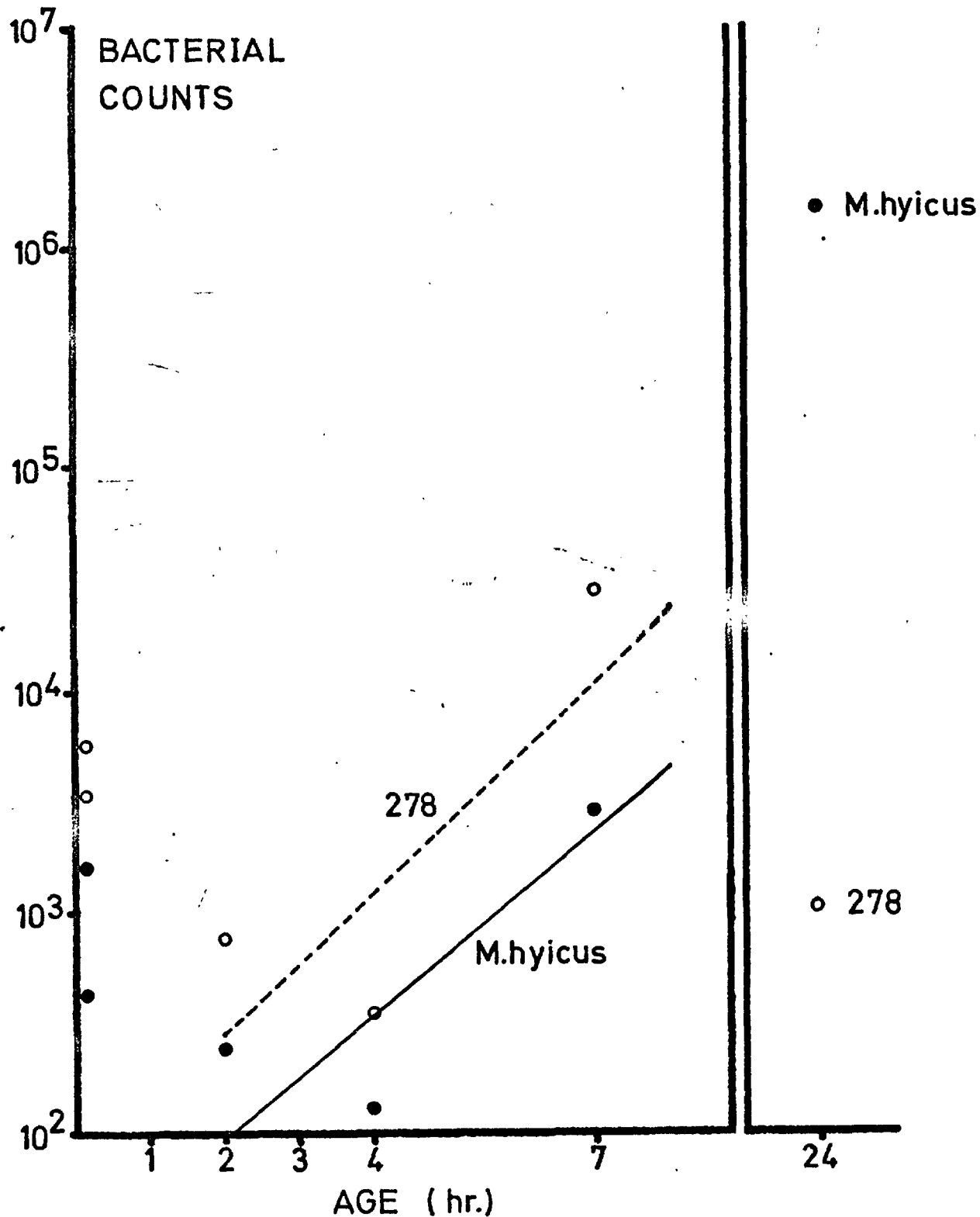


Figure 34: Growth curves of cocci in the lesion after the subcutaneous injection of M.hyicus and strain 278 mixed with cotton-dust.

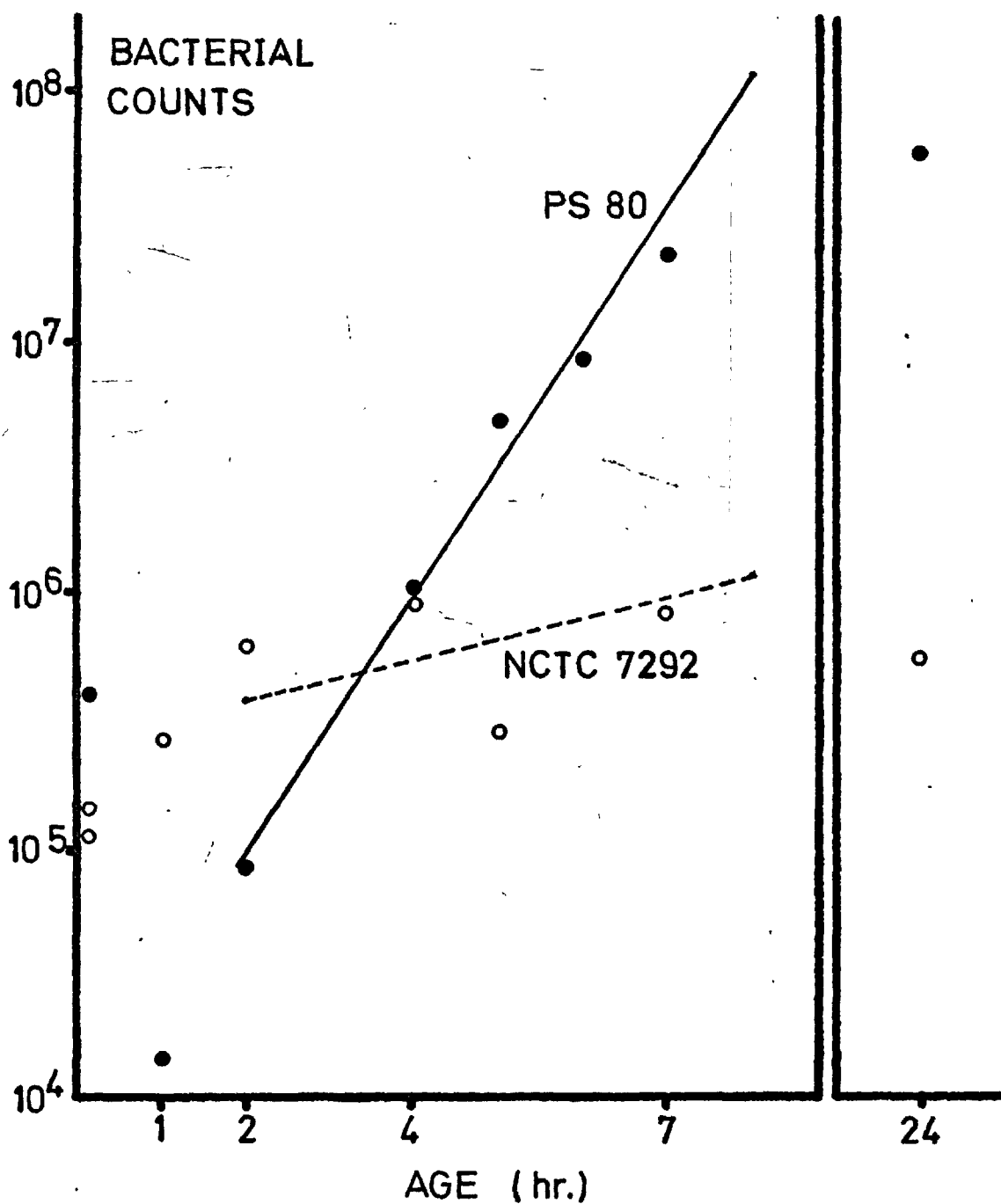


Figure 35: Growth curves of cocci in the lesion after the subcutaneous injection of coagulase-negative staphylococcus NCTC 7292 and Staphylococcus aureus PS 80 mixed with cotton-dust.

evidence of multiplication; for comparison the growth curve of PS 80 with a similar dosage is also shown. Even with such a high initial inoculum, NCTC 7292 failed to produce any significant increase in the bacterial population.

The detailed results of bacterial counts, including the growth rate (k), of various strains are shown in Table 6. The value of k was determined (figures 32 to 35) in the same way as described in the section 2. The growth rate of the three mouse-virulent strains and of Smith and P66/7, which produce moderate lesions, was higher than the strains producing milder lesions. The severity of the lesions was better correlated with the value of k and with the 7 hour count rather than with the 24 hour count, except in the case of Smith and P66/7.

(e) Relation of lesion score to the inflammatory reaction and bacterial counts.

In an attempt to demonstrate the relation of the lesion score to the 2 components of the inflammatory reaction measured, the data were plotted in the form of a "3 dimensional" graph (figure 36). The two abscissae are (1) the sum of the mean "oedema weights" during the first two hours after injection, and (2) the time at which leucocyte emigration was first detected. The height of the blocks represents the lesion score.

The three 'mouse-virulent' strains - PS 80, Orbach and 1531 elicited little or no fluid exudation during the first 2 hours after inoculation, in fact in PS 80 and Orbach the tissue weight was below

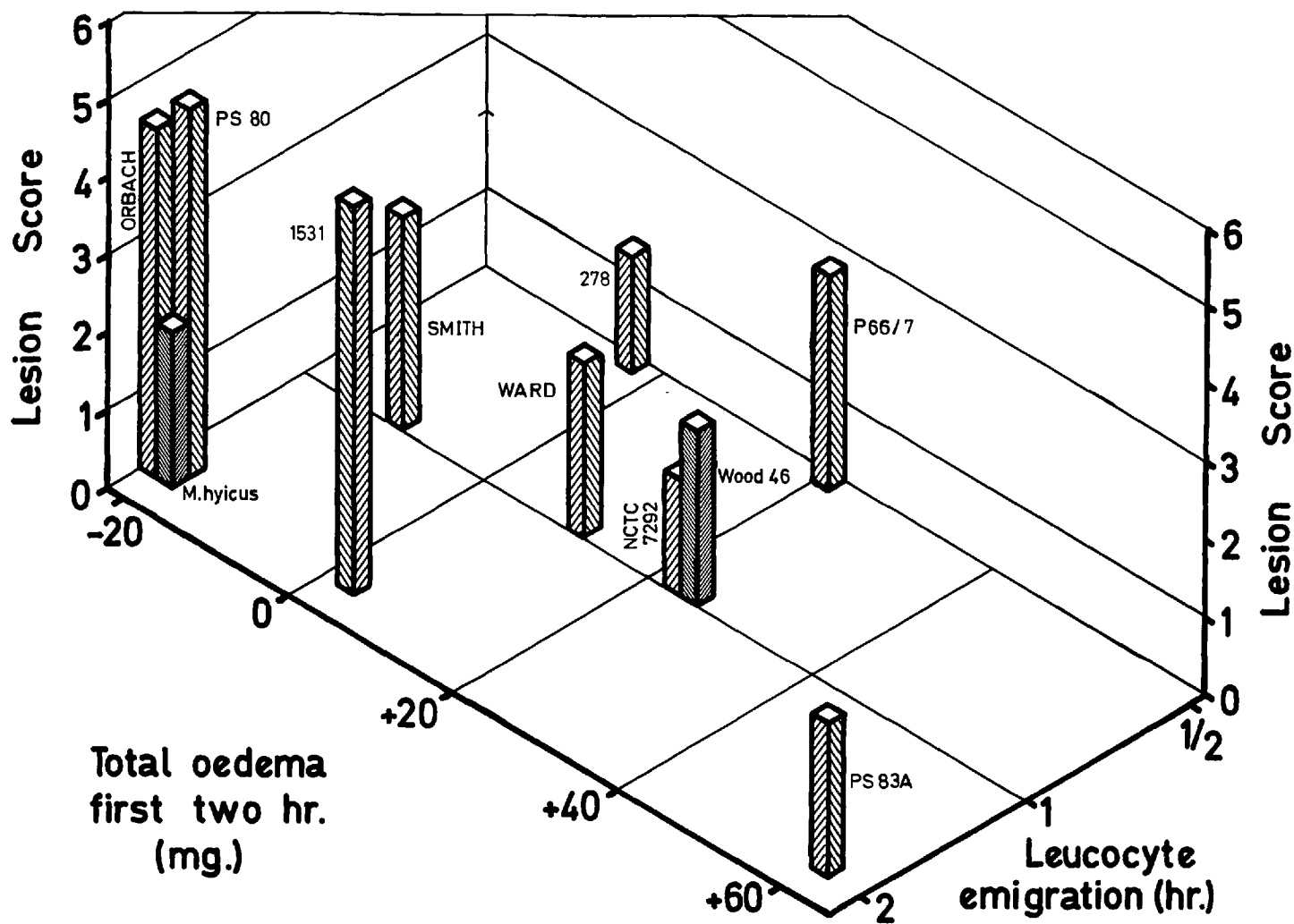


Figure 36: Relation of lesion score to the total fluid exudation for the first two hours and the time of onset of leucocyte emigration produced after the subcutaneous injection of different strains of staphylococci and micrococci.

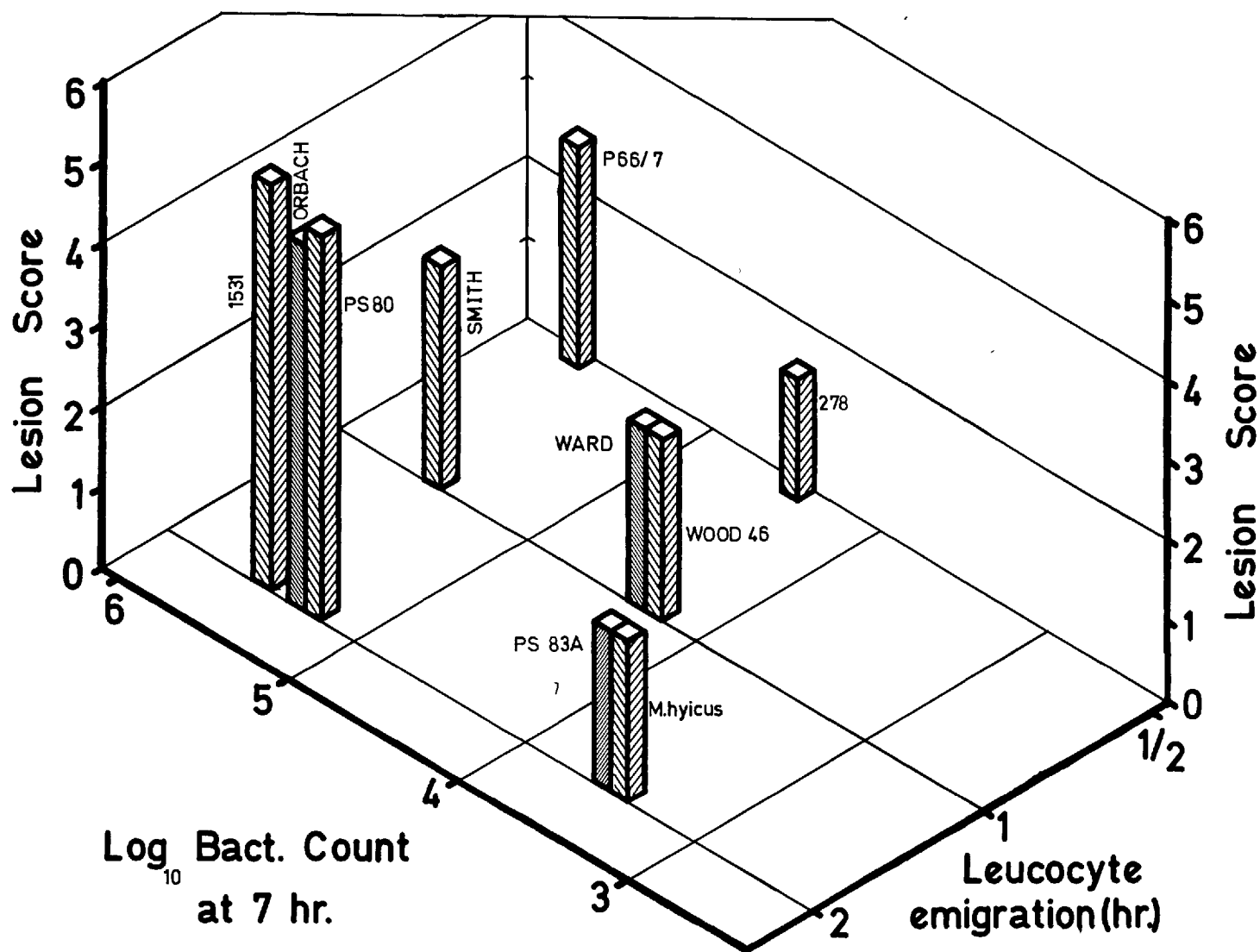


Figure 37: Relation of lesion score to the time of onset of leucocyte emigration and the bacterial count 7 hours after the subcutaneous injection of different strains of staphylococci and micrococci.

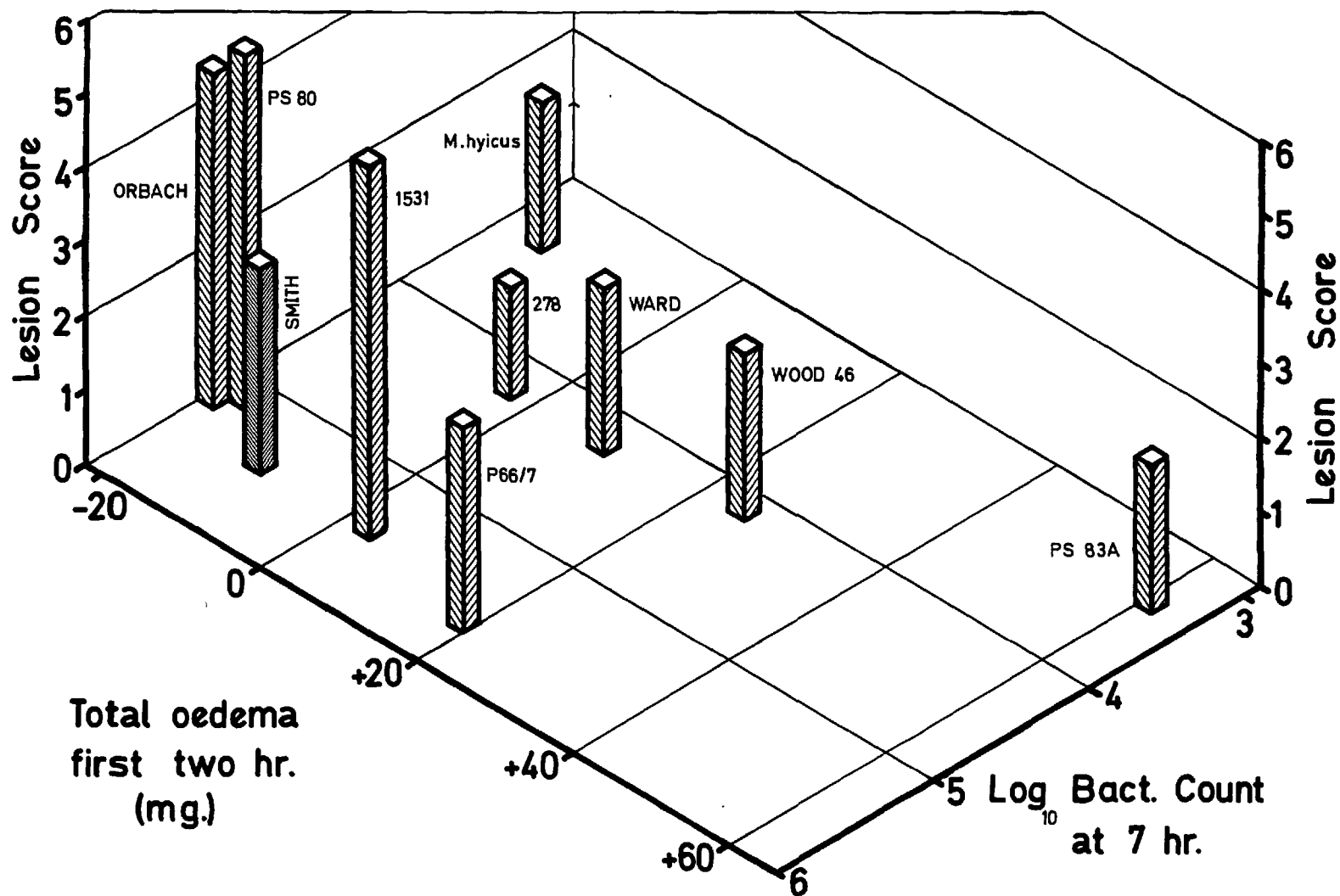


Figure 38: Relation of lesion score to the total fluid exudation for the first two hours and the bacterial count 7 hours after the subcutaneous injection of different strains of staphylococci and micrococci.

the expected weight. There was also a delayed emigration of leucocytes. The remaining strains except M. hyicus either showed exudation of fluid during the initial stages of inflammation or early emigration of leucocytes, or both. All these strains produced the milder lesions. M. hyicus, although producing a mild lesion, elicited a poor exudation of fluid as well as a delayed leucocyte emigration, that is it had these characteristics of virulent strains.

The relation of the severity of the lesion to the leucocyte emigration and the bacterial counts in the lesion at 7 hours is shown in figure 37. The two abscissae are the log of viable count at 7 hours and the time at which leucocyte emigration was noticed. The strains with high lesion scores elicited a delayed emigration of leucocytes and showed high bacterial counts in the lesion. In contrast the strains that produced poorer lesions either elicited a rapid leucocyte emigration or showed a lower bacterial count in the lesion, or both.

The relation of the severity of the lesion to early exudation of fluid and the 7 hour bacterial counts in the lesion is shown in figure 38. The two abscissae are the sum of the mean "oedema weights" during the first two hours after injection, and the log of viable counts at 7 hours in the lesion. The strains with high lesion scores showed a poor fluid exudation during the first two hours and high bacterial counts. With the exception of Smith, the remaining strains with poor lesions showed either an early exudation of fluid or a lower bacterial count or both. Smith, in

spite of producing poor exudation of fluid and a high bacterial count in the lesion, had a much poorer lesion score than PS 80, Orbach and 1531.

It is therefore concluded that, in general, with the strains producing severe lesions exudation of fluid as well as the emigration of leucocytes occurred only after an initial delay, and the bacterial counts in their lesions rose quickly to a high level. However the reverse of these findings was not true for all the strains producing poorer lesions. But no strain producing poor lesion had all the 3 characteristics seen in the lesions of "virulent strains", namely delayed exudation of fluid and emigration of leucocytes as well as high bacterial counts in the 7 hour old lesion.

4. Staphylococcal factor causing initial delay in inflammation

The factor or factors in staphylococci that can possibly be responsible for the initial delay in the inflammatory response are not known. In order to define any possible role of the structural components of staphylococci, heat killed and washed cells of Staphylococcus aureus strain PS 80 were injected with the cotton. Of the extracellular factors of staphylococci, alpha toxin which is known to cause vasoconstriction of blood vessels (Thal and Egner 1954), was also injected subcutaneously in mice mixed with cotton. The two main components of inflammatory response were estimated at intervals in both cases.

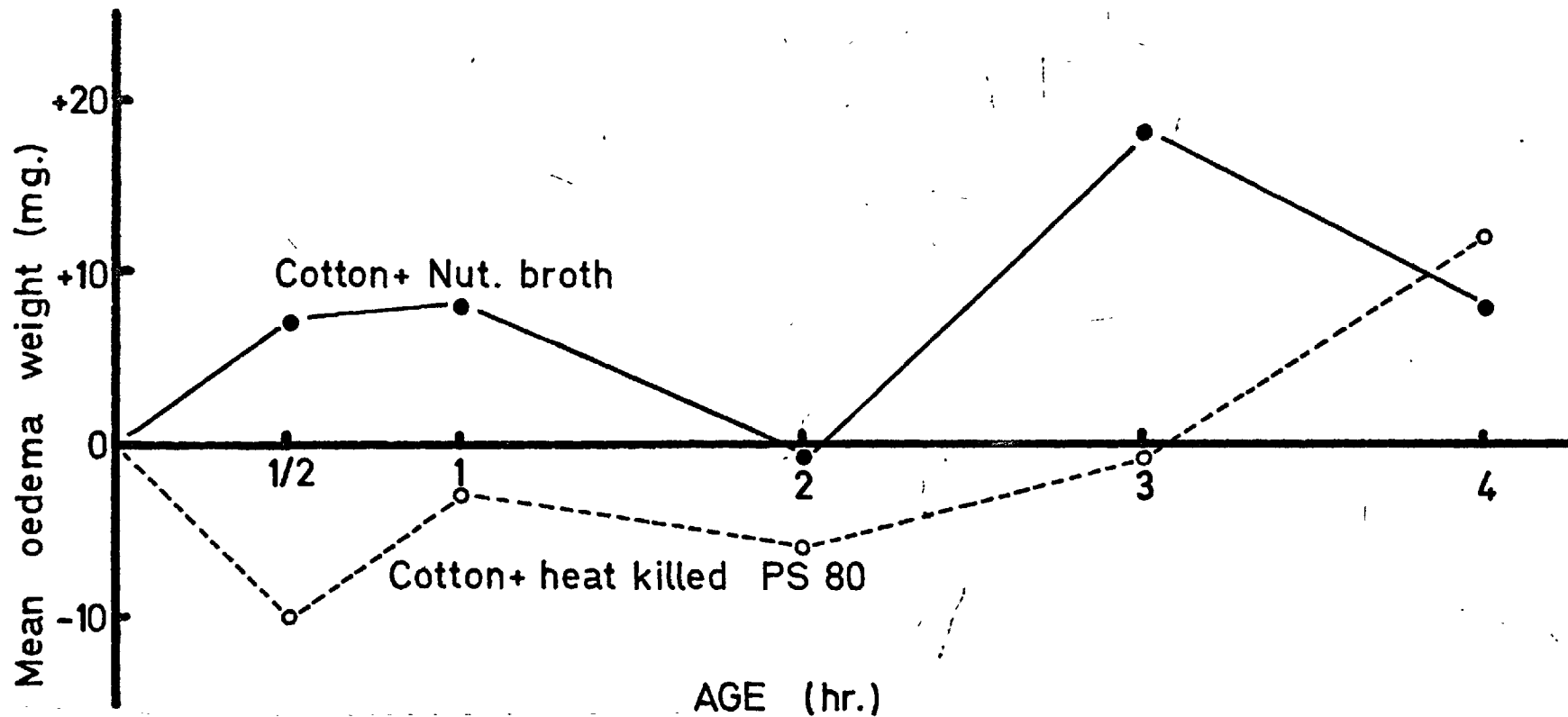


Figure 39: The mean oedema weight of tissue to the subcutaneous injection of cotton-dust with and without heat-killed Staphylococcus aureus PS 80.

(a) Inflammatory response to the injection of heat-killed *Staphylococcus aureus* PS 80.

An overnight nutrient broth culture of PS 80 was heated at $72 - 74^{\circ}\text{C}$ for one hour; it was subcultured on blood agar to verify that no viable cocci were present. The cells were washed three times and resuspended in the original volume of sterile nutrient broth. The cotton dust was inoculated with 5 μ l of this heat killed suspension and was then injected subcutaneously into mice. The changes in tissue weight after the injection is shown in figure 39. For the sake of comparison the response to the injection of cotton and nutrient broth is also shown. Each point on the graph represents the mean values from four mice. With the heat killed PS 80 there was an initial delay in the onset of exudation of fluid. When saline suspension of heat killed PS 80 was injected with cotton a similar response was obtained.

Leucocyte counts of the lesion revealed that emigration started 1 hour after the injection; the cotton dust itself was first infiltrated at 3 hours and by 7 to 8 hours the whole area of cotton dust was heavily infiltrated by leucocytes.

(b) Inflammatory response to the injection of alpha haemolysin

"Wellcome" staphylococcal alpha haemolysin was used in the experiments. Ten ml. of nutrient broth was added to the freeze dried toxin and 5 μ l of this was injected subcutaneously in mice mixed with the cotton dust. The amount of the toxin injected was

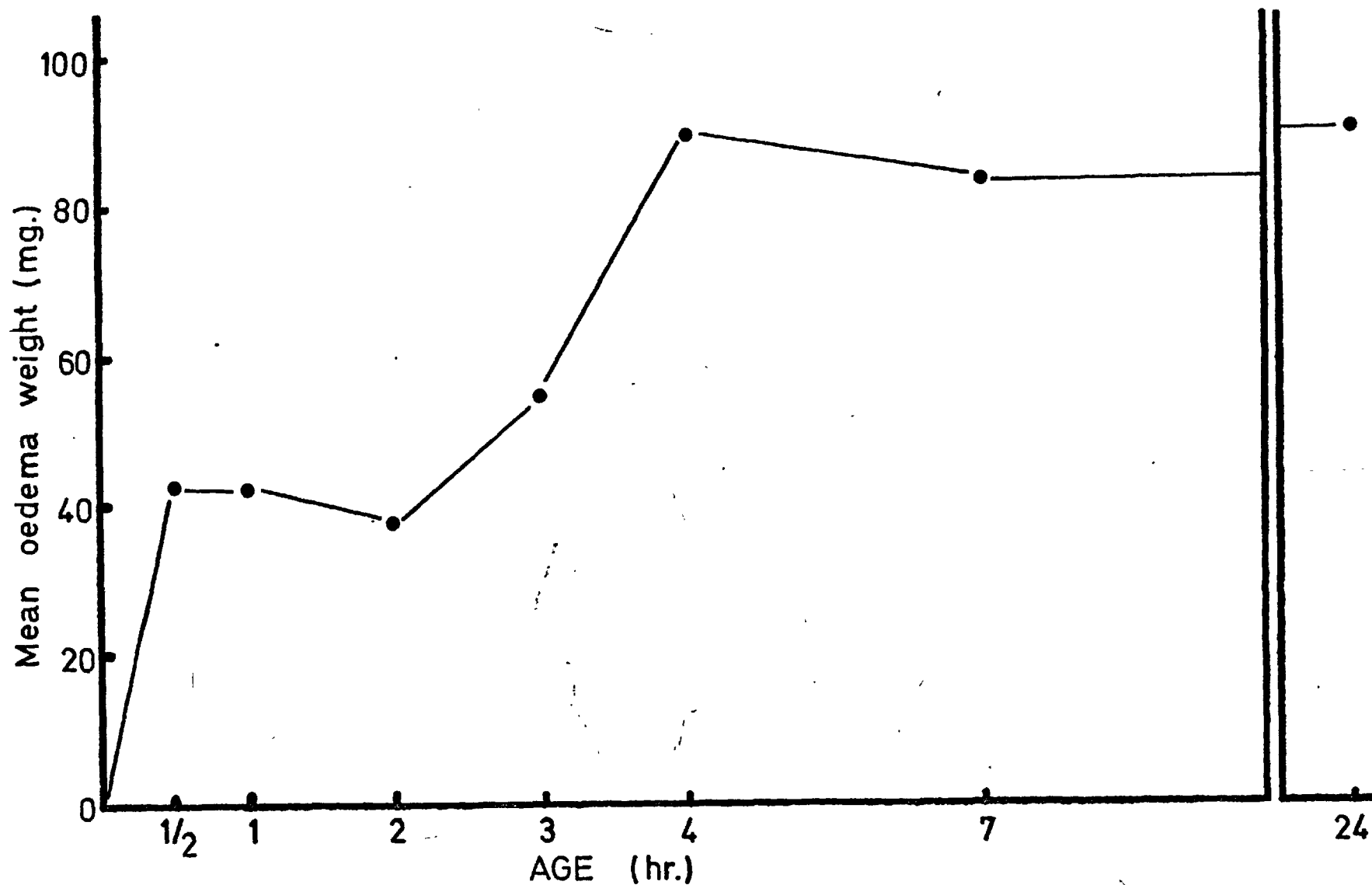


Figure 40: The mean oedema weight of tissue to the subcutaneous injection of alpha haemolysin with cotton-dust.

0.16 'welloose' units. Alpha toxin elicited rapid inflammatory exudate (figure 40). The accumulation of fluid was evident half an hour after the injection.

Leucocyte emigration in the injected site was detected at 1 hour, but the infiltration of cotton was not seen until after 7 hours and even at this time only the peripheral parts of the cotton were infiltrated. Even 24 hours after injection most of the cotton dust remained free of leucocytes only the periphery being infiltrated.

These results suggest therefore that the initial delay in the inflammatory response might well be due to some component associated with cells of staphylococci and not the alpha toxin. However, alpha toxin could be the factor responsible for inhibiting the infiltration of cotton by leucocytes in the later stages of inflammation.

5. The response of 'previously-infected' mice to staphylococcal infection.

Although, after staphylococcal infection, both immunity and hypersensitivity are known to develop in animals (for example, see Elek 1959), no detailed information is available about the effect of these changes on the inflammatory response or the bacterial population in staphylococcal lesions of these animals. This section describes the response to the injection of staphylococci in mice that had recovered from previous staphylococcal infection.

TABLE 7

Lesion score to repeated injection of PS 80

No. of injection	Dose of PS 80	No. of mice	Average lesion score/mouse
1	5.75×10^5	12	4.0
2	3.5×10^5	12	3.4
3	3.25×10^5	12	2.7
4	1.5×10^5	12	3.4
5	5.6×10^5	12	2.7
6	9.25×10^5	12	2.9
7	6.75×10^5	12	2.9

Mice that had been infected 2 to 3 times by subcutaneous injection of Staphylococcus aureus strain PS 80 mixed with cotton dust, were used. About 4 weeks after the last injection the lesions had completely healed with scar formation and the mice were ready for these investigations. They were reinjected with 10^5 staphylococci of strain PS 80 mixed with cotton dust; care was taken to avoid the injection in the scar tissue.

(a) Lesion Score

In the 'previously-infected' (P.I.) mice given a dose of 10^5 PS 80, the lesions were mild consisting mainly of swelling and pus formation. Only an occasional mouse showed small zone of necrosis, 2 - 3 mm. in diameter. The average lesion score was 2.6. This value was based on the mean from 18 mice used in three different experiments. In contrast, control mice always produced severe lesions which were characterized by necrosis and pus formation. The average lesion score varied between 4.2 and 4.8. The reduction in the lesion score in 'P.I.' mice was mainly due to partial neutralization of skin necrosis.

In view of the reports that repeated staphylococcal infection increases the susceptibility of experimental animals to staphylococci (Johanovsky 1958b, Johnson, Cluff and Goshi, 1961), twelve mice were injected with Staphylococcus aureus strain PS 80 mixed with cotton, at weekly interval for 7 weeks. The average lesion score after each inoculation is shown in table 7. In none of the repeat injections, was the severity of the lesion more than

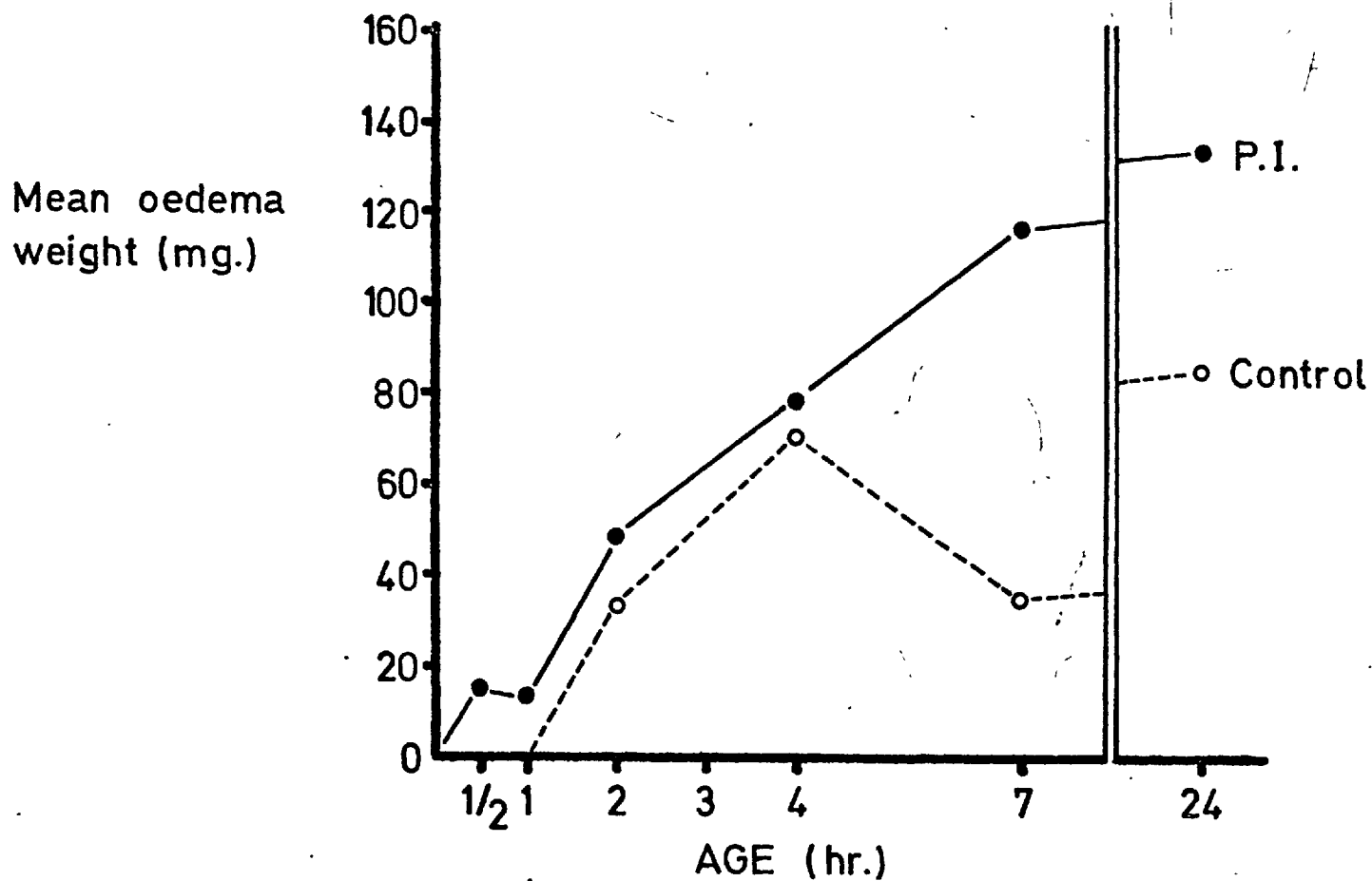


Figure 41: The mean oedema weight of tissue to the subcutaneous injection of Staphylococcus aureus PS 80 mixed with cotton-dust in previously-infected (P.I.) and control mice.

at the first injection.

When mice that had been injected previously with heat killed PS 80 were reinoculated with viable cells of PS 80, there was no reduction in the severity of the lesion, a finding similar to that reported by Noble (1965).

(b) Fluid exudation

The relation of fluid exudation with the animal's past experience with staphylococcal infection is shown in figure 41. Each point on the graph is the mean taken from 4 mice. The controls were a group of mice that had been injected with cotton and sterile nutrient broth 4 weeks previously; they were reinoculated at the same time and with the same dose of PS 80 as the 'P.I.' mice. This control was included in order to make an allowance for any possible alteration in the response of mice due to age and the previous injection of nutrient broth. In the 'P.I.' mice there was an early exudation of fluid which was evident half-an-hour after the injection. Thereafter the fluid exudation went on increasing and at 24 hours there was a considerable amount of oedema. In contrast, in the control mice, the fluid exudation started after an initial delay of 1 hour, and at 7 and 24 hours the oedema was much less than in 'P.I.' mice. With the controls no study at half-an-hour was done, however the injection of cotton and PS 80 in normal mice had always failed to elicit any exudation at half-an-hour in the previous experiments. Although the mean value for each point on the graph is based on 4 mice, fluid exudation was evident at both half and one hour in 3

MEAN OEDEMA
WEIGHT (mg.)

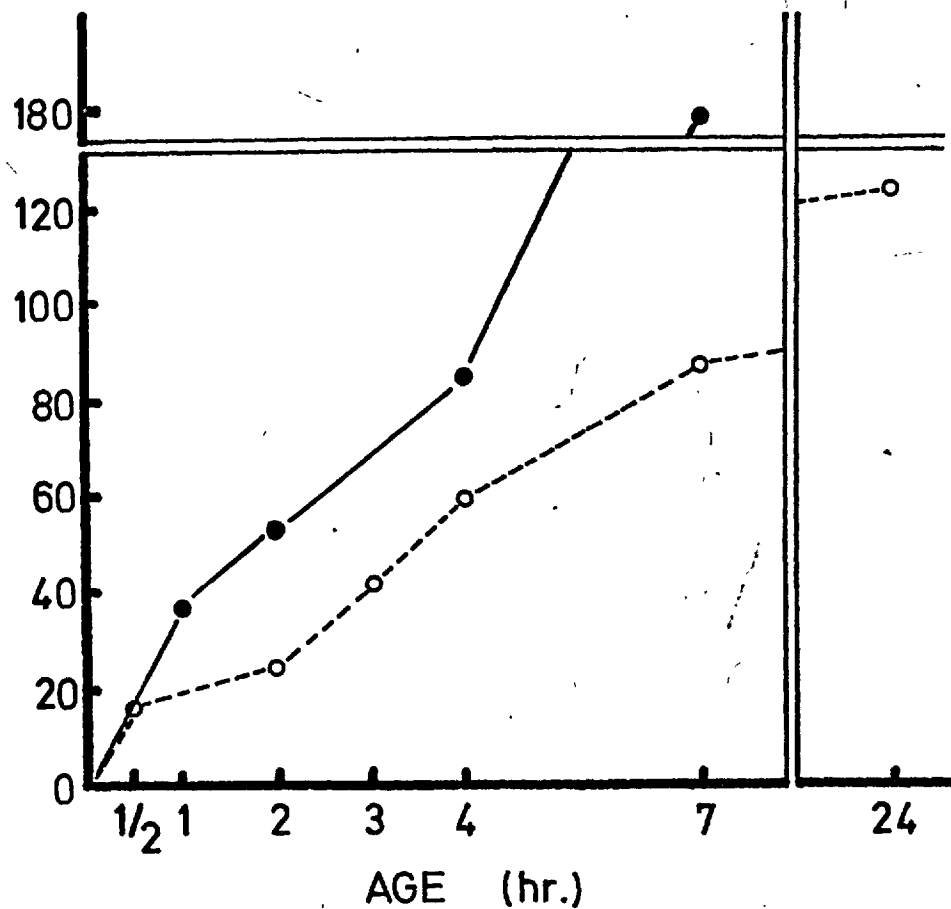


Figure 42: Two separate experiments showing the mean oedema weight of tissue in previously-infected mice to the subcutaneous injection of Staphylococcus aureus PS 80 mixed with cotton-dust.

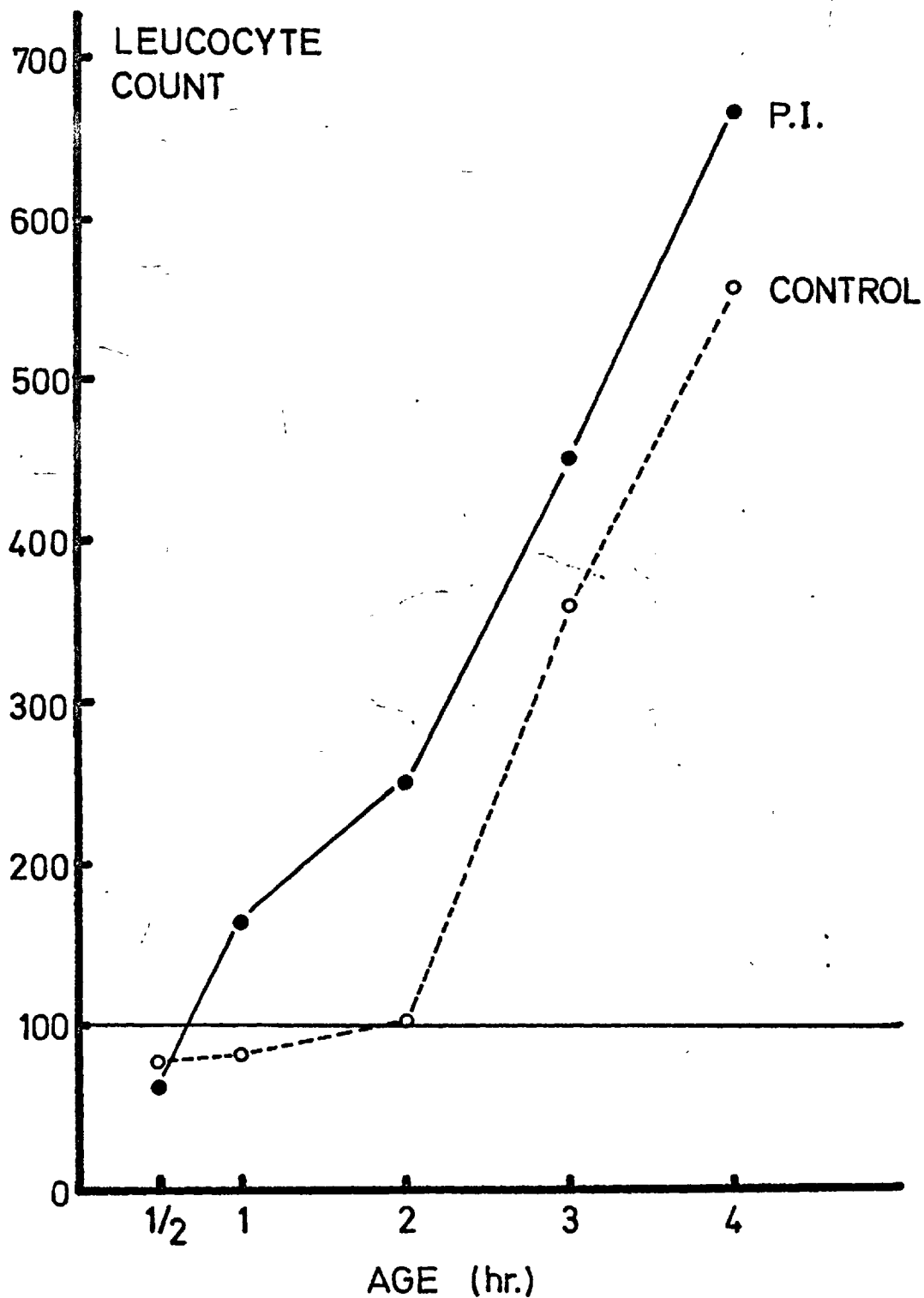


Figure 43: Leucocyte counts in the lesions of previously-infected (P.I.) and control mice to the subcutaneous injection of Staphylococcus aureus strain PS 80 mixed with cotton-dust.

Bacterial
count

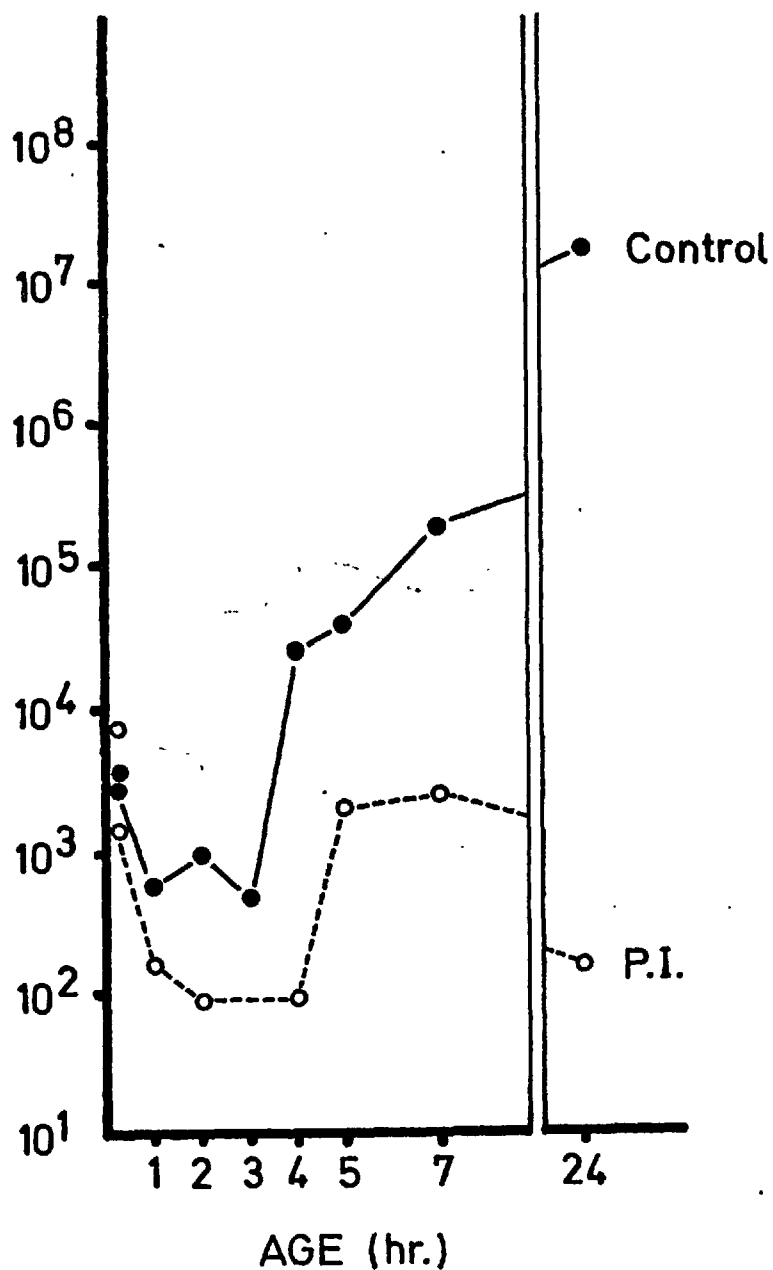


Figure 44: Bacterial counts in the lesion after the subcutaneous injection of Staphylococcus aureus strain PS 80 mixed with cotton-dust in previously-infected (P.I.) and control mice.

out of 4 'P.1.' mice. Furthermore on two other occasions mice that had experienced previous subcutaneous staphylococcal infection, showed a similar response to a second infection (figure 42). With these studies no parallel controls were included.

(c) Leucocyte emigration

The 'P.1.' mice also responded with an accelerated leucocyte response (figure 43). Leucocyte emigration at the injection site was noticeable at 1 hour and the infiltration of cotton began at 3 - 4 hours; by 7 hours the entire cotton dust was infiltrated by leucocytes. On the other hand in the control mice, the emigration of leucocyte was not seen until after 2 hours and these leucocytes remained at some distance from the edge of the cotton up to 4 hours. It was 7 hours before any significant infiltration was seen and even at 24 hours the central parts of the cotton dust remained free of leucocytes. The histological sections also showed that the tissue from 'P.1.' mice was much more oedematous than in the controls. This further supports the finding of an increased fluid exudation in mice with the past experience of staphylococcal infection.

(d) Bacterial counts

For the bacterial counts 10^3 dose of PS 80 was injected and the results are shown in figure 44. Each point on the graph represents the mean from two mice. The lower point on the ordinate represents the zero-hour count. In the 'P.1.' mice

TABLE 8

Vascular labelling after the subcutaneous injection of
Staphylococcus aureus strain PS 80 mixed with cotton-dust in mice

Time (h)	Extent of Vascular labelling *	
	In P.I. mice	In control mice
$\frac{1}{2}$	+	$\pm$
1	+	$\pm$
2	++	++
4	++	++
6	++++	++
7	+++	+++
24	+++	$\pm$

* = The extent of vascular labelling is shown
arbitrarily as $\pm$, +, ++.....++++.

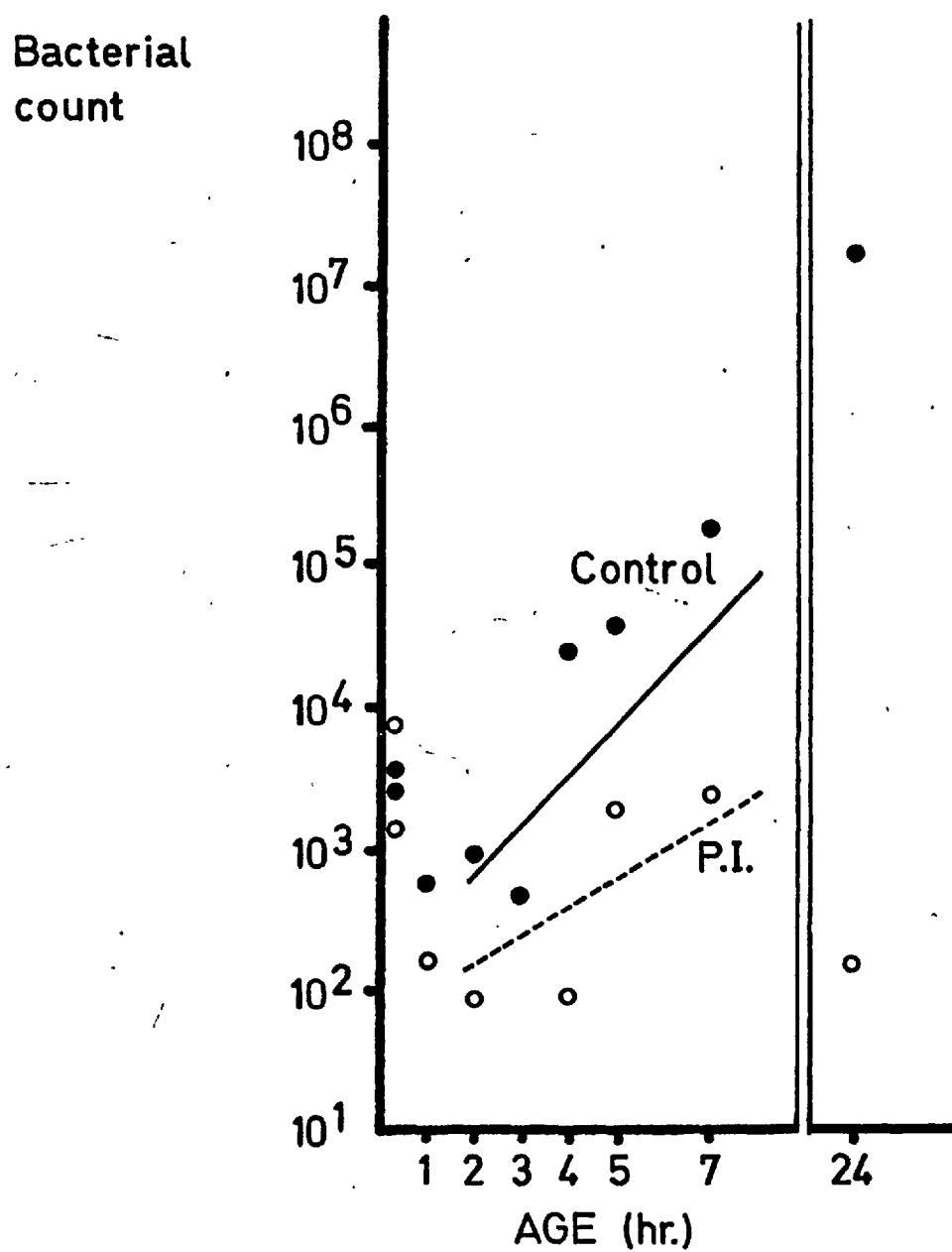
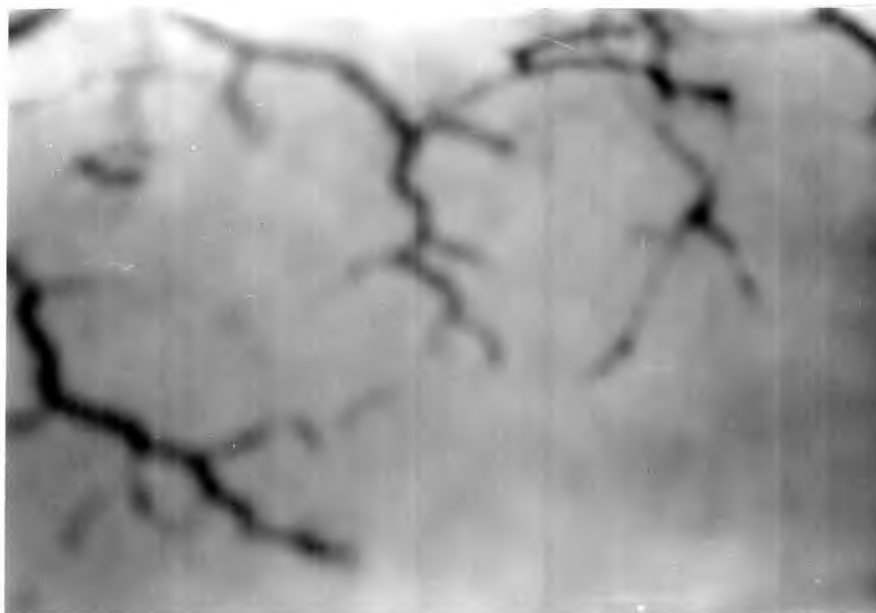


Figure 45: Growth curves of cocci in the lesion after the subcutaneous injection of Staphylococcus aureus strain PS 80 mixed with cotton-dust in previously-infected (P.I.) and control mice.

(a)



(b)



Figure 46: Vascular labelling 6 hours after the subcutaneous injection of Staphylococcus aureus strain PS 80 mixed with cotton-dust. (a) previously-infected mice. (b) normal mice. Tissue cleared in glycerine, no stain, X40.

the initial drop in the count was lower and persisted for a longer period than the control. After 4 hours, the count started rising but even at 7 hours it was lower than the 'initial inoculum'. There was presumably a further drop in the bacterial population as at 24 hours the count was lower than at 7 hours. The growth rate of oocci in (figure 45) P.l. mice was 0.20/hour as compared to 0.41/hour in the controls.

(e) Vascular labelling

The finding that the 'P.l.' mice responded with an accelerated inflammatory reaction to staphylococcal infection was further corroborated by studying the degree of vascular labelling after the intravenous injection of Pelikan-ink (see methods). The results are shown in Table 8. In the 'P.l.' mice the vascular labelling both at $\frac{1}{2}$ to 1 hour and 24 hours was more than in control mice. At 6 hours there was a considerable amount of vascular labelling in 'P.l.' mice in contrast to controls (Figure 46 a,b). Similarly while at 24 hours in the P.l. mice a large number of blackened blood vessels were seen, it had almost disappeared in the control. Carbon started leaking out of blood vessels at 1 hour after the injection in 'P.l.' mice and at 2 hours in the control mice. These times coincide with the onset of leucocyte emigration in the two cases. It was mainly the large size blood-vessels, presumably venules

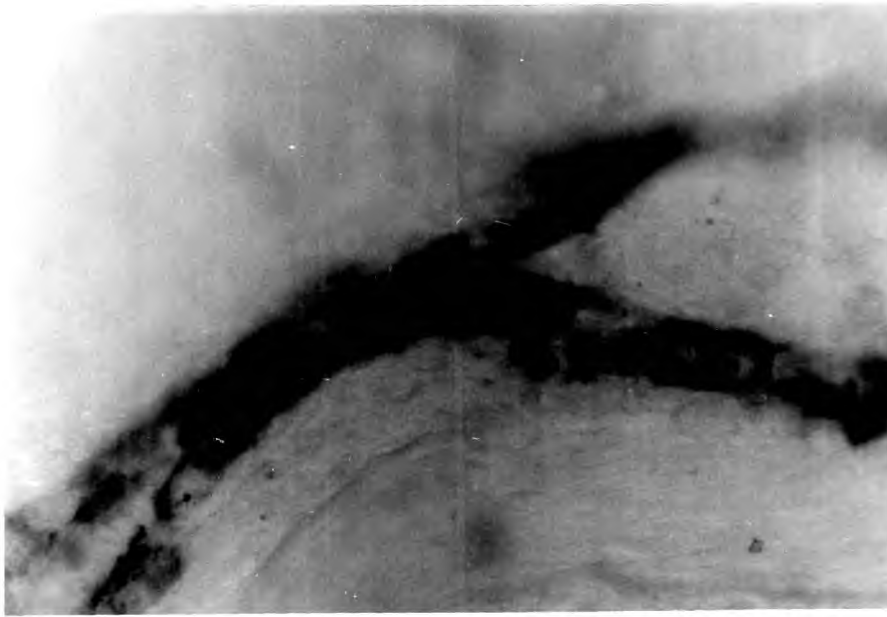


Figure 47: Carbon labelling of large blood-vessels. Cleared unstained preparation. X250.

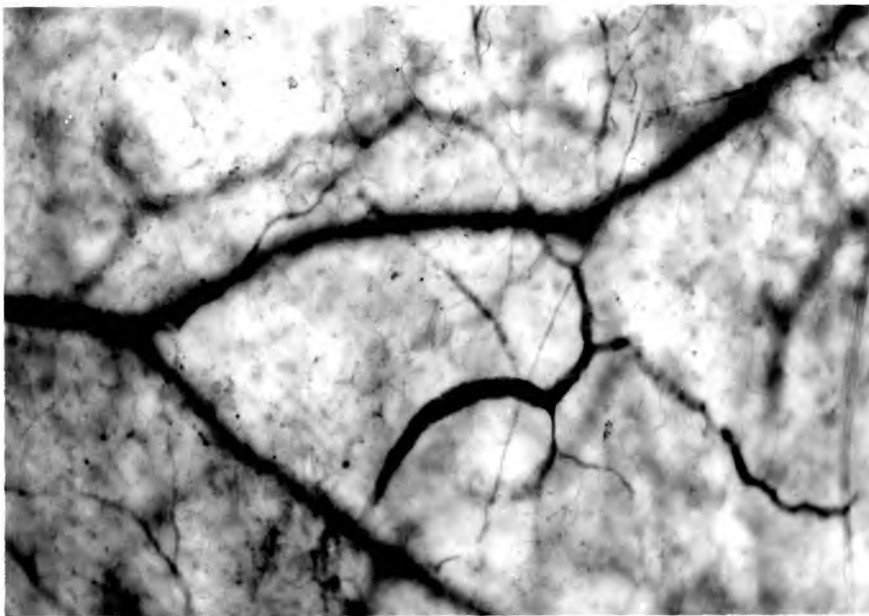


Figure 48: Normal vascular pattern of the subcutaneous tissue of mouse, after the injection of ink-gelatine mixture. Cleared unstained preparation. X40.

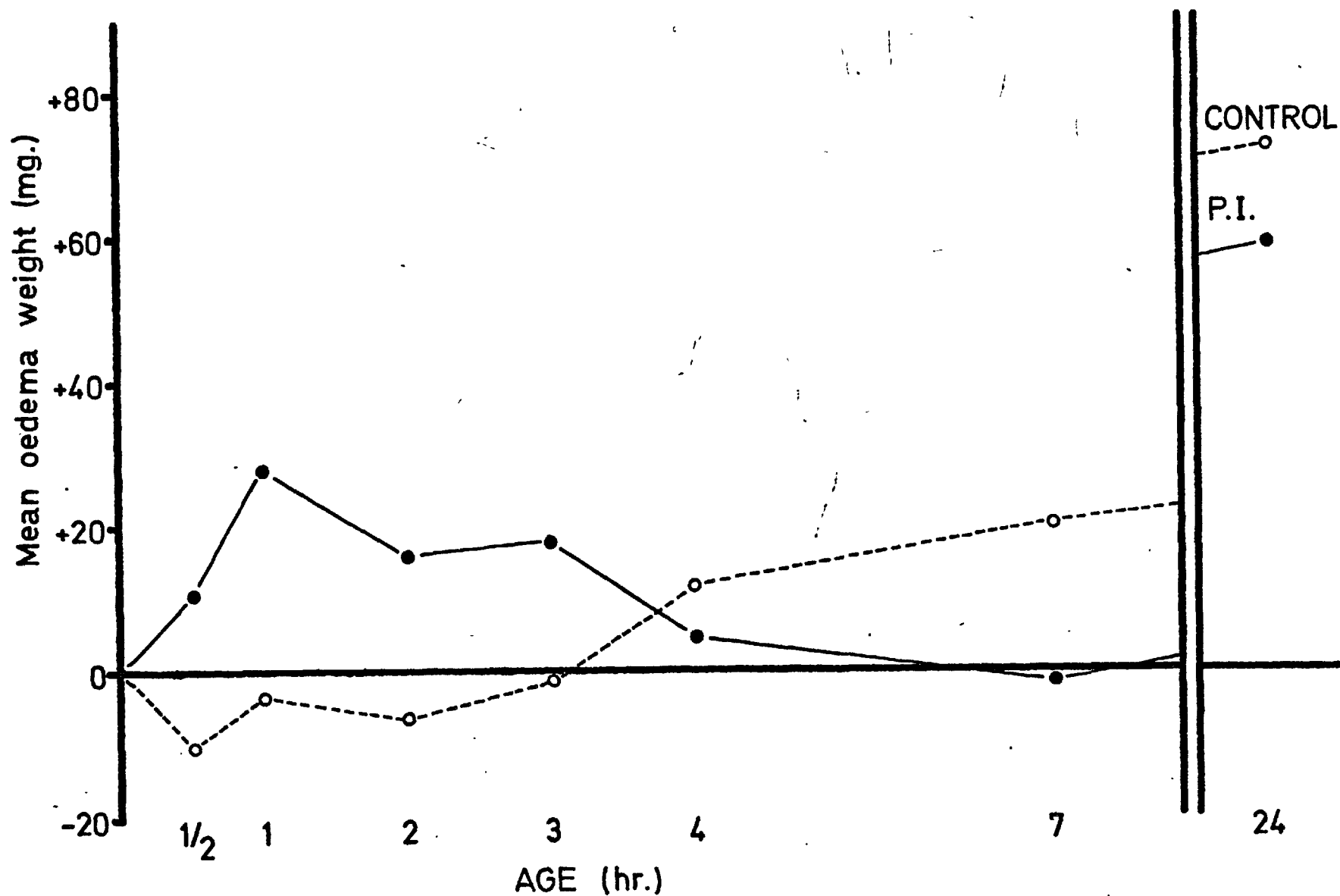


Figure 49: The mean oedema weight of tissue to the subcutaneous injection of heat-killed Staphylococcus aureus PS 80 mixed with cotton-dust in previously-infected (P.I.) and control mice.

that were affected (figure 47) in both the cases. Smaller blood vessels, presumably capillaries were also blackened to some extent, between 1 and 8 hours in the control mice and throughout the period of inflammation in the 'P.I.' mice. In order to identify the venules and capillaries the normal vascular pattern of the subcutaneous tissue is shown in figure 48.

(f) Response to the injection of heat-killed *Staphylococcus aureus* PS 80.

In order to find which antigen of the staphylococci might possibly be responsible for the accelerated inflammatory response, 'P.I.' mice were injected with heat killed PS 80 and their response was scrutinised. In the next section their response to the injection of alpha-toxin will also be described.

In the 'P.I.' mice, the exudation of fluid was evident half an hour after the injection (figure 49). Each point on the graph is the mean from 4 mice. During the first 3 hours after injection, almost all the mice showed the local exudation of fluid. For control normal mice were injected with heat killed PS 80 and cotton. In these mice the increase in tissue weight was discernible only after 3 hours. However the mean 'oedema-weight' of the tissues at 7 and 24 hours was not much different in the two cases.

In the 'P.I.' mice, the emigration of leucocytes was evident at half an hour; the cotton itself was infiltrated at 3 hours and by 24 hours it was heavily infiltrated by leucocytes. Similar responses

TABLE 9

Response of mice to the injection of 'Wellcome' brand
alpha toxin (0.16 units) mixed with cotton dust.

Time after injection (h)	Mean Oedema weight of exudate (mg)			
	P.i. mice	No. of mice	Control mice	No. of mice
$\frac{1}{2}$	56.9	6	42.8	6
1	69.4	6	42.8	6
2	57.0	6	38.1	6
24	106.8	6	90.7	6

were elicited in the control mice except that leucocyte emigration commenced at 1 hour after the injection.

(g) Response to the injection of alpha haemolysin

'Wellcome' brand alpha-haemolysin (0.16 units) was injected subcutaneously mixed with cotton dust into 'P.I.' mice. The results are shown in Table 9.

The degree of fluid exudation tended to be greater in the 'P.I.' mice both in the early stages and at 24 hours than in the mice that had no past experience of staphylococcal infection.

The histology of the tissue revealed that in the P.I. mice the emigration of leucocytes was detectable half an hour and infiltration of cotton started at 2 hours after inoculation; by 24 hours there was a massive infiltration of the entire cotton dust. On the other hand, in the control mice in spite of early emigration, the cotton was not infiltrated to any significant degree before 7 hours and even at 24 hours only the peripheral parts of the cotton were infiltrated.

'P.I.' mice showed evidence of immunity to deroanecrotic effects of alpha-toxin. While the control mice showed on average a zone of necrosis 3.1 mm. in diameter; in most cases the 'P.I.' mice had no necrosis and only rarely a pin point area of necrosis was seen.

TABLE 10

Fluid exudation in the staphylococcal lesion of serum
treated mice

Time after injection of exocel (h)	No. of mice	Difference between observed and expected weight of tissue (mg.)
$\frac{1}{2}$	4	- 5.4
1	4	- 0.3
2	4	- 0.6
24	4	98.1

6. Passive transfer experiments

(a) Serum transfer

The experimental results described in the previous sections showed that mice with past experience of staphylococcal infection exhibited some degree of protection and accelerated inflammatory response to the injection of Staphylococcus aureus PS 80 and alpha toxin. It is also known that in local anaphylactic type of reaction mediated through serum and characterized by wheal and flare there is an immediate increase in vascular permeability (McMaster 1959). It was therefore decided to transfer the serum from mice having past experience of subcutaneous staphylococcal infection with strain PS 80 to new mice by systemic injection (See Methods) and to study their response to the subsequent injection of 10^5 Staphylococcus aureus strain PS 80 mixed with cotton dust.

(1) Lesion score

Mild lesions were produced after the injection of 10^5 viable units. The average lesion score based on two experiments of 6 mice each was 3.0. The reduction in the severity of the lesion was due to neutralization of dermonecrotic effect.

(ii) Fluid exudation

The tissue weight was estimated at $\frac{1}{2}$, 1, 2 and 24 hours after the injection of oocci. The results are shown in Table 10. The value for each period was the mean from four mice.

TABLE 11

Fluid exudation after the injection of PS 80 and cotton

Time after injection of cotton (h)	Weight of oedema fluid (mg.)	
	In serum recipient group	In control group
$\frac{1}{2}$	5.7 (0.2 to 19.3)*	-6.0 (-16.7 to 3.9)
24	154.8 (120.9 to 187.2)	111.7 (74.2 to 168.9)

* = In brackets is the range of mean oedema weight

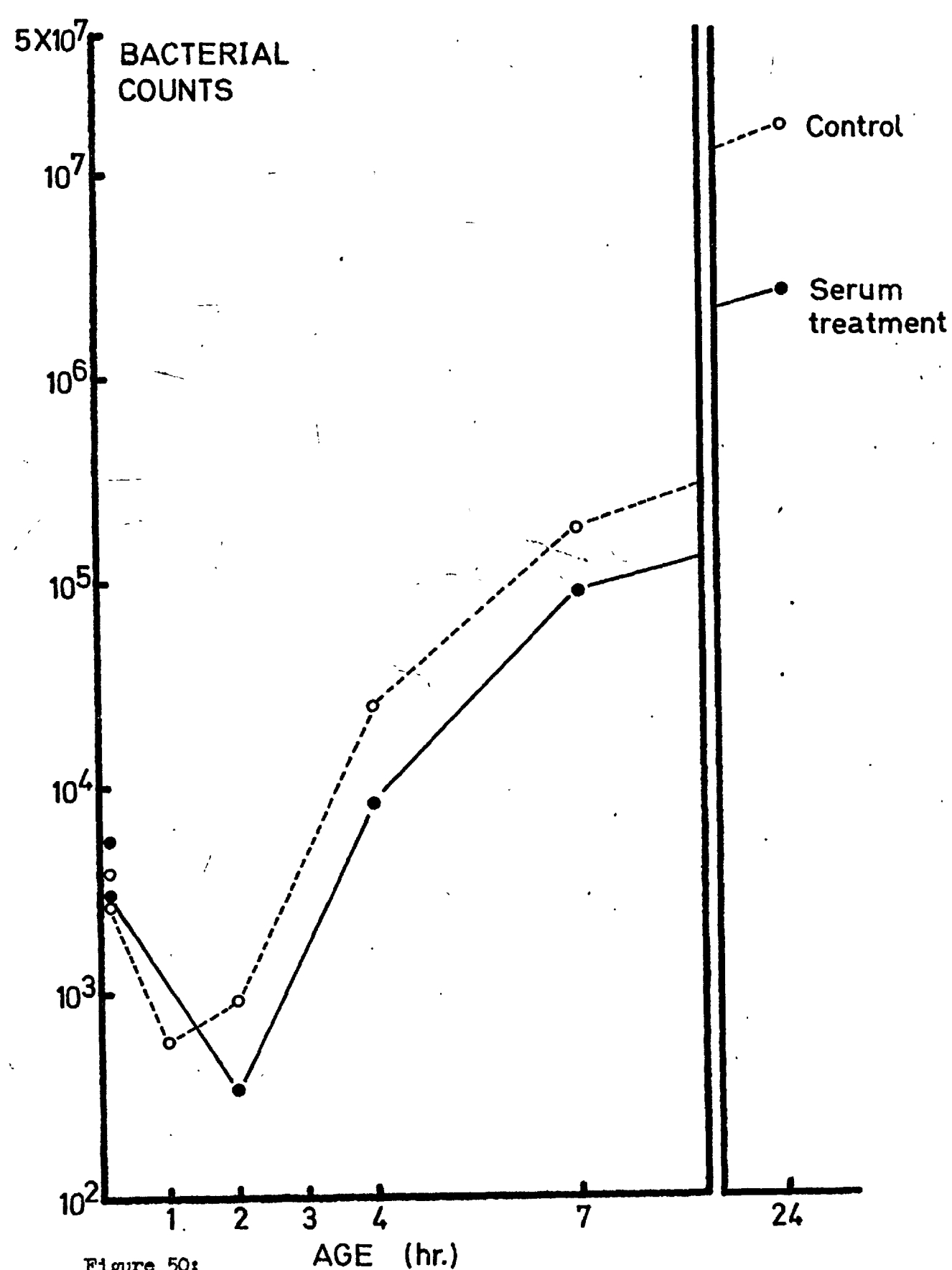


Figure 50:

Bacterial counts in the lesion after the subcutaneous injection of Staphylococcus aureus strain PS 80 mixed with cotton-dust in serum treated and control mice.

There was no evidence of fluid exudation for the first two hours after the injection. However only 2 of the 4 mice tested showed the lack of exudation in the early period and no control was included in the experiment. Next a group of untreated mice were also injected with PS 80 mixed with cotton at the time of inoculation of serum treated mice. The result is shown in Table 11. Again the values for each point are the mean from four mice. All the four mice that received serum showed some exudation of fluid at half an hour. In contrast all the control mice showed little or no fluid exudation at this stage. At 24 hours the serum treated group showed 38% more oedema fluid in the lesion than the control group. The range of mean oedema weight also indicates that the difference between serum treated and control mice may be real.

(iii) Leucocyte emigration

The emigration of leucocytes was detected at 2 hours and the cotton itself showed some evidence of infiltration even at this time. This is in marked contrast to control mice where the infiltration of cotton was not seen until 7 hours after inoculation, although the leucocyte emigration was evident at 2 hours.

(iv) Bacterial count

The counts of viable cocci in the lesion is shown in figure 50, and for the sake of comparison, the bacterial counts in the lesions of untreated mice are also included. Each point on

TABLE 12

Bacterial counts in the lesion of serum treated and control mice

Group	Initial dose	'Zero-hour' count	Time (h) of lowest count	Count at 2 hr.	Growth rate k/hour	Counts at	
						7 hr.	24 hr.
Serum treated group	5.5×10^3	3.13×10^3	2	3.4×10^2	0.45	9.1×10^4	2.59×10^6
Control group	3.81×10^3	2.83×10^3	2	9.3×10^2	0.40	1.83×10^5	1.68×10^7

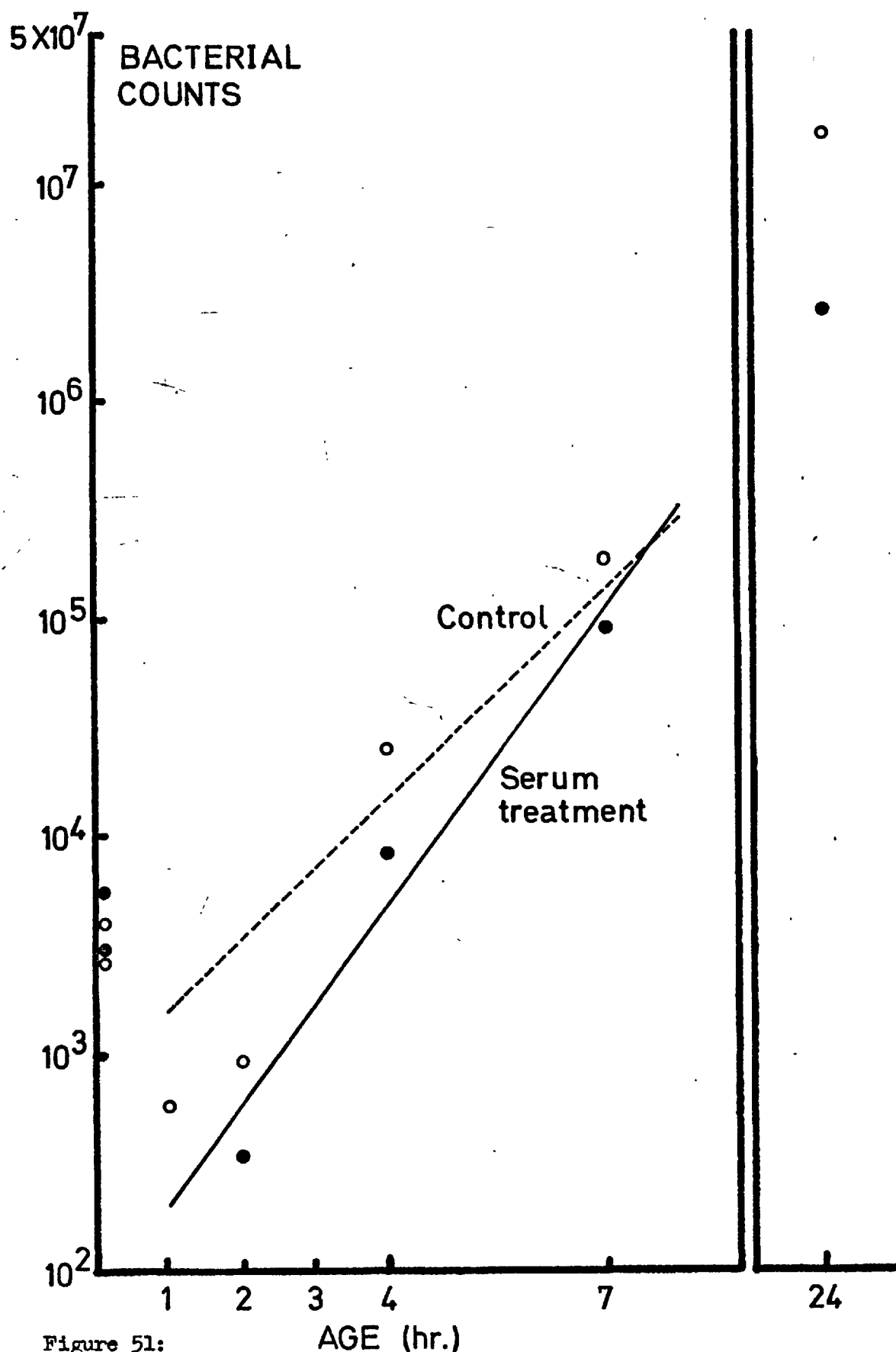


Figure 51:
 Growth curves of cocci in the lesion after the subcutaneous injection of *Staphylococcus aureus* strain PS 80 mixed with cotton-dust in serum treated and control mice.

the graph is the mean from two mice. The counts in the lesions of both the groups were similar except that the serum treated group showed a greater initial drop at 2 hours, which perhaps accounted for slightly lower counts subsequently in their lesions. The growth rate (figure 51) of cocci was same in both the groups (Table 12).

(b) Lymph-node cells transfer

Since it has been reported that in the tuberculin type of hypersensitivity reactions mediated through cells there is an increase in vascular permeability which is maximum at 24 hours after injection (Veisin and Toullet 1960), I decided to transfer lymph node cells from mice having past experience of subcutaneous staphylococcal infection with the strain PS 80, to new mice (see Methods). The response of these treated mice to the injection of Staphylococcus aureus PS 80 and cotton is now reported. In these experiments only small numbers of mice were used because of scarcity of time and material, therefore the conclusions may not have the same validity as in earlier experiments.

Mice that had received the lymph node cells showed very severe lesions after the injection of staphylococci. The lesions were characterized by large areas of necrosis. The average diameter of necrotic zone after an injection of 10^4 dose of PS 80 was 6.2 mm. and the average lesion score was 4.4. Six control mice injected at the same time with the same dose had on average a necrotic zone of

TABLE 13

Fluid exudation in lymph node treated and control mice after the injection of PS 80 and cotton

Experiment No.	Dose of PS 80	Oedema weight (mg)			
		Lymph node treated mice	No. of mice	Control mice	No. of mice
1	2.5×10^5	67.3 (35.7 to 115.3)*	4	85.3 (73.8 to 114.0)	4
2	2×10^4	55.1 (-0.4 to 99.6)	4	97.1 (59.7 to 118.3)	4

* = In brackets is the range of mean oedema weight

TABLE 14

Bacterial counts in the lesion after injection of
Staphylococcus aureus PS 80

Group	Initial count	Bacterial count at	
		7 hr.	24 hr.
Control	5.16×10^3	2.33×10^4	8.3×10^6
Lymph node cells recipient	5.16×10^3	4.25×10^4	18.9×10^6

2.2 mm. in diameter and a lesion score of 3.6. The mice given lymph node cells thus showed an evidence of increased susceptibility to staphylococcal infection.

Fluid exudation and leucocyte count were estimated only at 24 hours after the inoculation of cocci. A group of untreated mice were also included as controls. The mice receiving lymph node cells showed no evidence of increased fluid exudation at 24 hours; indeed their lesions were less oedematous than in the control mice (Table 13).

In the histological sections of the lesion at 24 hours, both with the 10^4 and the 10^5 dose of PS 80, there was only slight infiltration of the cotton dust by leucocytes. Only the peripheral parts of cotton was infiltrated and at places the leucocytes even remained away from the edge of the cotton. There is no data about the leucocyte emigration in the early period in the lymph node cell treated mice, but the 24 hour response was like that associated with virulence. For example, as already described, with very large dose of PS 80 (10^6) in normal mice, there was a similar leucocyte response at 24 hours and severe lesions. In contrast, in the untreated mice, with both the 10^4 and the 10^5 dose of PS 80, there was a heavy infiltration of the entire cotton dust by leucocytes at 24 hours.

Seven and twenty-four hour viable counts in the lesions are shown in Table 14. A group of untreated mice were included as controls. Each value is the mean taken from two mice. The bacterial counts were similar in the two groups.

TABLE 15

Response to injection of Staphylococcus aureus PS 80
and cotton in mice treated with serum and lymph node cells.

Group	Dose of PS 80	Average lesion score	Mean Oedema weight at 24 hrs.(mg)	Infiltration of cotton by leucocytes at 24 hrs.
Control	7.75×10^5	4.4	128.5	++++
Treated with lymph node cells + serum	7.75×10^5	2.5	127.1	++++

TABLE 16

Lesion score after repeat infection with a homologous
strain of staphylococci.

Strain	No. of mice	Average lesion score	
		First injection	Second injection
1531	6	5.0	3.2
Orbach	6	5.6	3.5
Smith	12	2.6	2.5
Wood 46	12	2.2	2.2
Ward	6	2.1	2.3
PS 83A	6	2.0	2.0
M. hyicus	6	2.0	2.2
278	6	1.6	1.8

(c) Transfer of lymph node cells and serum

An experiment was devised in which the mice were given not only lymph node cells but also serum from 'P.I.' mice. Each mouse was given 120 million lymph node cells and 0.25 ml. of serum by the intravenous route. Eighteen hours later, these mice, together with a group of untreated mice, were injected with a 10^5 dose of Staphylococcus aureus PS 80 in cotton dust. The result is shown in Table 15. The lesion score is an average from 6 mice each. The lesions produced in the treated mice were mild in severity. The average lesion score in the treated mice was 2.5 as compared to 4.4 in the control mice. The value of oedema weight in each group is the mean from four mice. There was no evidence of an increased exudation of fluid in the 24 hour old lesions of treated mice. However, as already described 'P.I.' mice had shown greater exudation of fluid at 24 hours than the controls. There was a heavy infiltration of the cotton dust by leucocytes at 24 hours as in the control mice.

7. Further investigations into the response of previously infected mice.

So far the response of the mice to reinfection with the strain (PS 80) used for the primary infection has been described. It was decided to investigate the response of previously infected mice to reinjection with a number of different strains of staphylococci (Table 16 and 17). In these experiments also a small number of mice were used, therefore the conclusions

TABLE 17

Lesion score after the injection of Staphylococcus aureus PS 80 in mice that had a past experience of staphylococcal infection with a heterologous strain.

Strain used for previous infection	No. of mice	Average lesion score after the injection of PS 80 in the 'P.l.' mice	% suppression of lesion score in 'P.l.' mice as compared to control	<u>Control</u> Lesion score to same dose of PS 80 in untreated mice
Orbach	6	3.0	40	5.0
Smith	6	4.2	16	5.0
Wood 46	6	4.3	14	5.0
Wood 46	6	3.1	13	3.6
PS 83A	6	5.0	N11	5.0
Ward	6	2.6	25	3.6
278	6	3.5	3	3.6

may not have the same validity as in earlier sections.

(a) Homologous reinfection

The results are shown in table 16. 'Mouse-virulent' strains like 1531 and Orbach, which produced severe lesions at the time of first injection, conferred some degree of protection to reinjection with the homologous strain. As described earlier it was mainly a protection against the skin necrosis that led to a reduction in the severity of the lesion as scored. The rest of the strains, which were 'poor-lesion' producers, did not confer any significant degree of protection against reinjection with the same strain.

(b) Heterologous reinfection

Table 17 shows the average lesion score after the injection of Staphylococcus aureus strain PS 80 into mice that had a previous subcutaneous infection with a heterologous strain of staphylococci. The value for the control is the average lesion score in the untreated mice given the same dose of PS 80 though the control figure was not always derived from mice reinjected on the same day. The maximum reduction in severity of lesion after reinjection was seen in mice that had previous infection with Orbach - a 'mouse-virulent' strain. Of the rest of the strains, only the infection with Smith, Wood 46 and Ward induced a slight reduction in the severity of the lesion to subsequent injection of PS 80. However, mice

TABLE 18

Lesion score after the injection of Staphylococcus aureus strain
PS 80 in mice that had two subcutaneous staphylococcal infection
with a heterologous strain.

Two past injections with	No. of mice	Average lesion score after PS 80 injection in 'P.I.' mice	% Suppression of lesion score in 'P.I.' mice as compared to controls	<u>Control</u> Comparable lesion score to same dose of PS 80 in untreated mice.
278	12	2.3	52	4.8
M.hydous	6	3.4	29	4.8
Smith	6	2.4	50	4.8
Ward	6	2.3	36	3.6
Wood 46	6	3.2	11	3.6

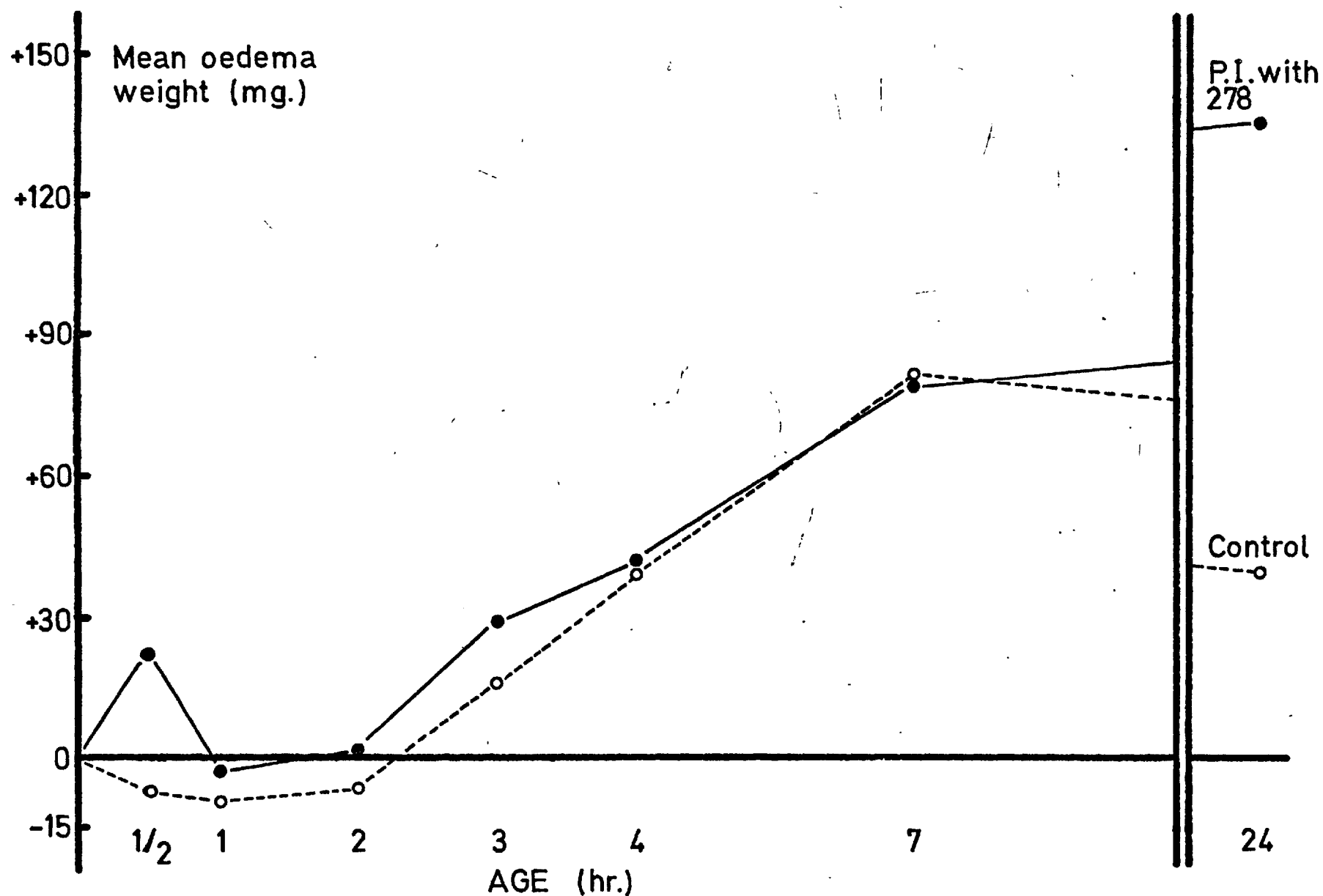


Figure 52: The mean oedema weight of tissue after the subcutaneous injection of Staphylococcus aureus PS 80 mixed with cotton-dust in mice previously-infected (P.I.) twice with strain 278 and control mice.

that had two previous infections with a 'poor-lesion' producing strain, exhibited some reduction in the severity of lesion to challenge with PS 80 (Table 18).

Strain 278, which is a micrococcus, also conferred some degree of protection against Staphylococcus aureus PS 80, although the strain 278 fails to produce either delta or alpha haemolysin in vitro. Therefore the inflammatory response after injection of PS 80 was studied in mice that had had two previous subcutaneous injection of 278. Figure 52 shows the tissue weights. Each point on the graph is the mean from 4 mice. There was an early exudation of fluid which became evident half an hour after injection. All the four mice tested at this time showed fluid exudation. The oedema weight varied from 3 to 38 mg. in these mice. This was followed by a phase in which there was no fluid exudation; thereafter the exudation of fluid went on increasing and at 24 hours the lesions were very oedematous. For comparison the response of untreated mice to the injection of PS 80 is also shown in the graph. Thus the mice that had previous infection with 278 showed an early as well as accelerated exudation of fluid. Histology of the lesion revealed that emigration of leucocytes started two hours after the injection; cotton began to be infiltrated at four hours. However by 7 hours only the peripheral parts of the cotton was infiltrated; the entire cotton dust was seen to be infiltrated only at 24 hours.

In view of the finding that strains of staphylococci that

TABLE 19

Lesion score with and without colchicine treatment

Strain	Colchicine treated mice			Control mice	
	No. of mice	Average lesion score	% enhancement of lesion score as compared to controls	No. of mice	Average lesion score
Ward	6	3.0	43	6	2.1
Wood 46	6	4.0	90	6	2.1
PS 83A	6	1.7	32	6	2.5
278	6	2.0	33	6	1.5

produced big necrotic lesions induced the maximum degree of protection in mice against reinfection, it was decided to try to enhance the severity of the lesions produced by strains like PS 83A and Wood 46 which are 'poor-lesion' producers by treating them with colchicine (see Methods). In the next section a detailed description of the anti-inflammatory action of colchicine will be given. The average lesion score in the colchicine treated and control mice after the injection of coeci is shown in table 19.

The lesions were greatly enhanced in the case of Wood 46. Wood 46 produced large quantities of alpha toxin and delta toxin in vitro. Although strains PS 83A and Ward also produced alpha as well as delta haemolysin in vitro, their lesions were not enhanced to that extent. Strain 278 produced neither of these haemolysins and its lesions were also not much enhanced. When colchicine treated mice had recovered from the subcutaneous infection with Ward or Wood 46; they were reinjected subcutaneously with PS 80 and cotton. The lesion score to 10^5 dose of PS 80 was 4.5 in the case of Ward and 4.0 in the case of Wood 46. Thus there was no evidence of an increased resistance to reinfection with PS 80 in these mice even after some enhancement of the first infection.

8. The effect of anti-inflammatory drugs on the course of staphylococcal infection in mice.

This section describes the effect of a number of anti-inflammatory drugs (see Methods) on the two components of inflammation

TABLE 20

Mean oedema weight of tissue in the drug treated mice 4 hours after the injection of 10⁵ dose of Staphylococcus aureus strain PS 80 mixed with cotton dust.

Exp. No.	Treatment	No. of mice	Mean Oedema weight (mg)	Mean blue Diameter (m.m)
1	Control	5	14.08(-11.4 to 24.6)*	9.3(7.3 to 10.8)*
	Cortisone acetate	6	11.3 (-25.9 to 44.4)	9.4(6.9 to 11.4)
	Betamethasone disodium phosphate	5	-0.4 (-30.7 to 15.4)	7.1(2.8 to 9.7)
	Dexamethasone-21-phosphate disodium salt	4	-12.4 (- 1.6 to -37.6)	8.7(6 to 11.8)
2	Control	5	64.8 (9.3 to 140.1)	11.8(9.9 to 14.0)
	Butazolidin	5	7.4 (-13.1 to 52.2)	7.9(6.0 to 11.9)
	Prednisolone disodium phosphate	5	8.3 (1.5 to 43.2)	8.5(5.2 to 11.4)
3	Control	4	11.7 (- 7.6 to 32.8)	8.8(5.6 to 10.9)
	Sodium salicylate	4	7.9 (- 9.0 to 25.4)	3.8(Cotton only blue to 8.0)
4	Control	4	65.3 (42.3 to 79.0)	13.7(10.9 to 15.8)
	Colchicine	4	18.4 (-17.3 to 50.7)	7.7(5.0 to 11.0)
	Sodium salicylate	4	36.6 (10.4 to 46.5)	12.2(9.4 to 14.9)
5	Control	6	41.8 (13.0 to 66.4)	13.3(11.4 to 15.0)
	Chlorpromazine hydrochloride	5	39.3 (6.3 to 79.3)	12.6(8.9 to 17.3)
	Quinine hydrochloride	5	46.7 (31.8 to 65.7)	15.5(10.9 to 18.9)
	Colchicine	5	10.3 (-1.4 to 32.3)	8.7(7.7 to 9.7)
6	Control	4	18.5 (16.0 to 20.2)	-
	Indomethacin	4	25.6 (2.9 to 60.6)	-

* = In brackets the range of mean oedema weight and mean blue diameter in the mice tested is given.

- = not tested

and the lesion produced by Staphylococcus aureus strain FS 80. However this is only a preliminary study and some of the results are difficult to interpret probably due to small number of animals used and due to the fact that very little is known about the mode of action of these drugs. Nevertheless, the results are reported as they were considered to be of some importance. Fluid exudation and the emigration of leucocytes into the lesion were estimated at only one time, namely 4 hours after inoculation except in the mice treated with indomethacin, where the inflammatory response 24 hours after the injection was also studied. The response at 4 hours was chosen for study, because of the evidence that in experimental bacterial infections most of the reactions determining the ultimate size of the local lesion are complete in 4 - 5 hours (Miles, Miles and Burke 1957). In all experiments a group of untreated mice were included as controls. The various anti-inflammatory drugs were tested in separate experiments.

(a) Fluid exudation

The oedema weight in the lesions of drug treated mice was compared with the oedema weight in controls. The detailed results are given in Table 20. As in each group only small number of animals were used and mice within any group often showed a great variation in the oedema weight of the tissue, very few differences between the mean oedema weight of drug-treated and

TABLE 21

Oedema weight of the tissue in drug treated mice 4 hours after the injection of 10^5 dose of Staphylococcus aureus strain PS 80 mixed with cotton dust, expressed as ratio of oedema weight in controls.

Exp. No.	Treatment	Oedema weight in exp. as ratio of weight in controls
1	Cortisone acetate	0.81
	Betamethasone disodium phosphate	< 0.1
	Dexamethasone-21-phosphate disodium salt	< 0.1
2	Butazolidin	0.12
	Prednisolone disodium phosphate	0.13
3	Sodium salicylate	0.68
4	Colchicine	0.29
	Sodium salicylate	0.57
5	Chlorpromazine hydrochloride	0.94
	Quinine hydrochloride	1.1
	Colchicine	0.25
6	Indomethacin	1.3

TABLE 22

Total leucocyte count/H.F. in the tissue sections of drug treated mice 4 h. after the injection of *Staphylococcus aureus* strain FS 80.

Exp. No.	Treatment	Total leucocytes/ H.P.F.	Leucocyte count in drug treated mice as ratio of counts in controls
1	Control	319	
	Colchicine	104	0.33
	Sodium salicylate	285	0.90
	Chlorpromazine hydrochloride	458	1.43
	Quinine hydrochloride	518	1.62
	Butazolidin	461	1.44
2	Control	456	
	Prednisolone disodium phosphate	208	0.46
	Sodium salicylate	647	1.41
3	Control	227	
	Indomethacin	297	1.30
4	Control	517	
	Dexamethasone-21-phosphate disodium salt	122	0.24
	Betamethasone disodium phosphate	196	0.38
	Cortisone acetate	237	0.46

control mice were significant. In table 21 the results of oedema weight in the drug treated mice are expressed as ratio of the oedema weight in the controls. A ratio of 0.5 or less was arbitrarily regarded as indicative of a reduction in the fluid exudation 4 hours after the injection of staphylococci.

Of the drugs tested colchicine, butasolidin, betamethasone, dexamethasone and prednisolone gave oedema weights which had a ratio of 0.5 or less to the controls. Although there was also a suggestion of some suppression of fluid exudation in mice treated with cortisone or sodium salicylate the oedema weight always had a ratio of more than 0.5 to the controls. Treatment of mice with chlorpromazine or quinine failed to suppress the fluid exudation. Similarly indomethacin treatment had no effect on fluid exudation either at 4 or 24 hours after the injection of cocci. The oedema weight of the tissue in indomethacin treated mice 24 hours after the inoculation of cocci was 82.3 mg; the weight in the untreated mice at the same time was 102.1 mg.

(b) Leucocyte count

The results of leucocyte counts are shown in Table 22. Each value represents the mean from the histological preparations of two mice and in each tissue section the counts were done in 3 to 4 fields. Again, if the drugs that reduce the leucocyte count to half or less of the control are regarded as suppressing the leucocyte emigration, only colchicine, cortisone, prednisolone, betamethasone and dexamethasone could be considered as effective.

TABLE 23Staphylococcal lesions in mice after the administration
of anti-inflammatory drugs

Exp. No.	Treatment	No. of mice	Average lesion score	Mean diameter of necrosis (mm)	Mean diameter of necrosis in treated mice as ratio of diameter in controls
1	Control	6	4.8	4.2	
	Quinine hydrochloride	6	4.0	2.8	0.66
	Chlorpromazine hydrochloride	6	4.3	3.1	0.7
	Betamethasone disodium phosphate	6	4.6	5.7	1.4
	Dexamethasone-21- phosphate disodium salt	6	5.3	6.2	1.5
	Butazolidin	6	5.3	8.7	2.1
	Cortisone acetate	6	5.1	8.9	2.1
	Prednisolone disodium phosphate	6	5.6	9.5	2.3
2	Control	6	3.5	2.2	
	Sodium salicylate	6	4.8	6.1	2.8
	Colchicine	6	5.8	9.8	4.4
3	Control	6	3.0	1.3	
	Sodium salicylate	6	4.5	3.5	2.7
4	Control	6	3.8	2.8	
	Indomethacin	6	4.5	4.5	1.6

(c) Lesion score

The severity of the staphylococcal lesion after the administration of various drugs was evaluated 24 and 48 hr. after the injection of cocci and the results are shown in table 23. In all cases the higher lesion score was due to a bigger zone of necrosis than in control mice. A better estimate of the lesion enhancement was therefore obtained by measuring the mean diameter of necrotic lesion. The mean diameter of necrosis in the drug treated mice is also expressed as ratio of mean diameter of necrotic lesion in controls (table 23).

Apart from chlorpromazine and quinine hydrochloride, all the drugs tested enhanced the severity of lesions to some extent. However if only the drugs producing an area of necrosis two times or more of the controls are regarded as enhancing the lesion severity then only cortisone, prednisolone, butasolidin, sodium salicylate and colchicine were able to enhance the severity of staphylococcal lesions. The lesions in mice treated with dexamethasone and betamethasone were enhanced 1.5 and 1.4 times of controls respectively. It is noteworthy that of the four corticosteroids tested betamethasone and dexamethasone had reduced the fluid exudation and leucocyte emigration to a greater extent than cortisone and prednisolone and yet the staphylococcal lesions in both dexamethasone or betamethasone-treated mice were smaller than in the mice treated with cortisone or prednisolone. It may be that the enhancement of staphylococcal lesions by corticosteroids is not solely due to suppression of inflammation.

TABLE 24

The effect of anti-inflammatory drugs on staphylococcal infection

Drug	Enhancement of lesion	Reduction in fluid exudation	Reduction in leucocyte emigration
Quinine hydrochloride	-	-	-
Chlorpromazine hydrochloride	-	-	-
Cortisone acetate	+	=	+
Prednisolone disodium phosphate	+	+	+
Betamethasone disodium phosphate	-	+	+
Dexamethasone-21-phosphate disodium salt	-	+	+
Butazolidin	+	+	-
Colchicine	+	+	+
Sodium salicylate	+	-	-
Indomethacin	-	-	-

(d) Relation of the two components of inflammation to lesion severity.

The effects of the anti-inflammatory drugs are summarized in table 24 on the basis of the criteria of reduction in the two components of inflammation and enhancement in the lesion severity already described. Of the drugs tested chlorpromazine, quinine and indomethacin affected neither components of inflammation at 4 hour nor the severity of the lesion. Sodium salicylate failed to reduce either of the two components of inflammation in the dose used for the experiment, yet it enhanced the severity of lesion. However sodium salicylate had shown some reduction (62% of the control) in the fluid exudation and if larger number of mice were used in the experiment the results might have been more valid. Of the four corticosteroids tested, cortisone reduced leucocyte emigration without reducing the fluid exudation, while betamethasone, dexamethasone and prednisolone reduced both the components of inflammation yet only cortisone and prednisolone were able to produce the lesions twice or more as big as the controls. However even with dexamethasone and betamethasone the lesions were slightly bigger than in controls. Colchicine reduced both the components of inflammation and also enhanced the lesion. Butaxolidin reduced fluid exudation without affecting leucocyte emigration and increased the lesion severity.

In an attempt to demonstrate the relation of the lesion severity in the drug-treated mice to the two components of the inflammatory

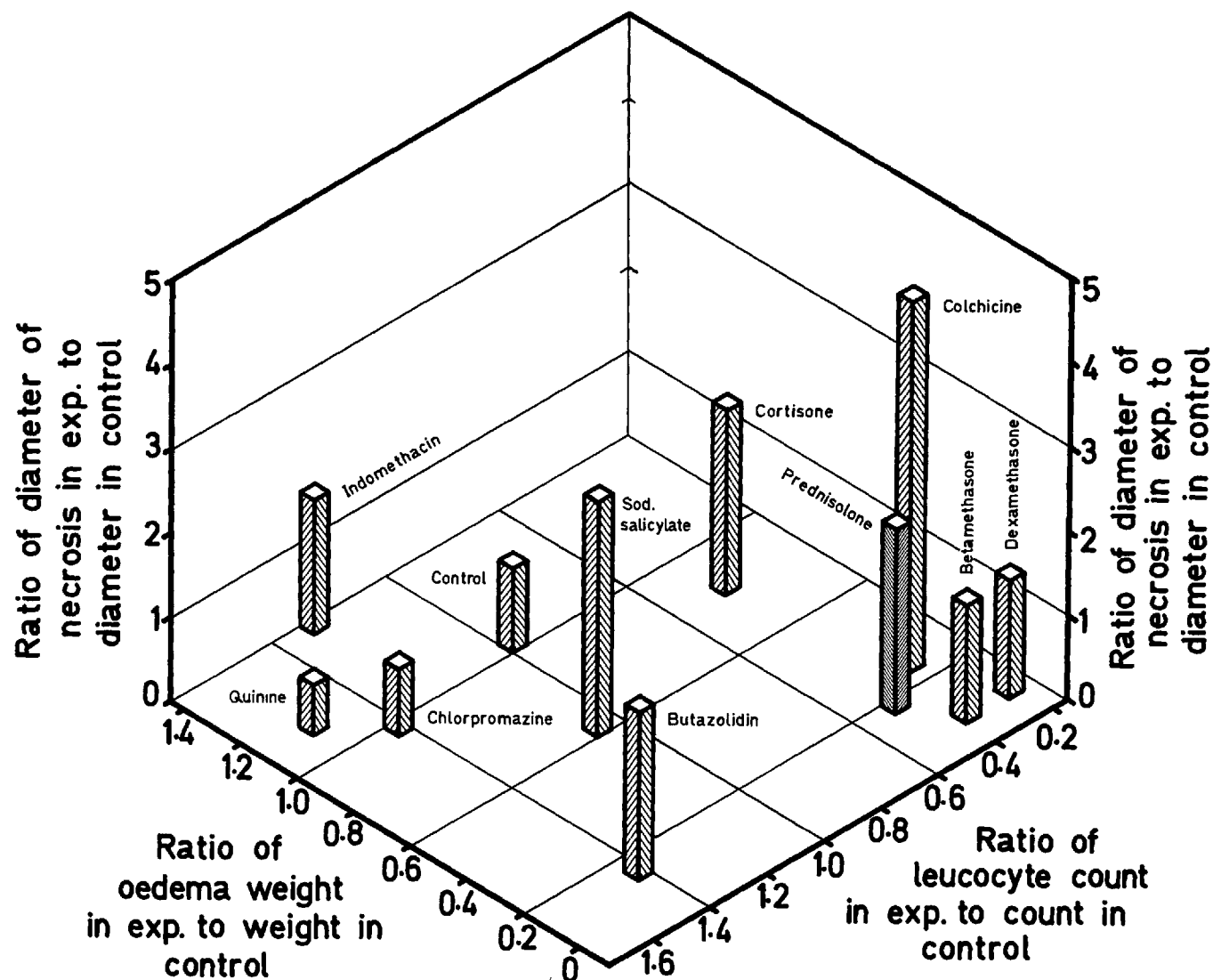


Figure 53: The effect of anti-inflammatory drugs on the two components of inflammation and the severity of lesion produced by the subcutaneous injection of *Staphylococcus aureus* strain PS 80 in mice.

TABLE 25

The relation of lesion severity in cortisone treated mice
with the dose of Staphylococcus aureus strain PS 80.

Dose of PS 80	Cortisone treated mice			Control mice		
	No. of mice	Average lesion score	Mean diameter of necrosis(mm)	No. of mice	Average lesion score	Mean diameter of necrosis(mm)
6×10^5	6	5.1	8.9	6	4.8	4.2
5.6×10^4	6	5.3	9.1	6	3.6	4.2
5.66×10^3	6	2.8	*	6	2.5	*
9×10^2	6	0.5	nil	6	2.5	<1.0

* = Of the 6 mice tested only two showed necrosis,
of 4.6 mm and 12.8 mm in diameter respectively

* = Of the 6 mice tested only one showed a necrotic
zone of 2 mm in diameter

reaction measured, the data were plotted in the form of a '3 dimensional' graph (figure 53). The two abscissae are (1) the mean oedema weight in experiments as ratio of the mean oedema weight in controls and (2) the leucocyte count in experiment as ratio of the count in controls. The height of the blocks represents diameter of necrosis in drug treated mice as ratio of diameter of necrosis in controls.

The drugs enhancing the staphylococcal lesions reduced one or both of the components of inflammation except sodium salicylate which did not reduce any of the components of staphylococcal inflammation at 4 hours yet led to a bigger lesion than the controls.

These observations suggested that besides the anti-inflammatory activity, these drugs may have metabolic or some other effects which might enhance the staphylococcal lesion. That this may well be true was suggested by the fact that the action of cortisone acetate in enhancing the severity of lesion seemed to be dependent on the number of staphylococci injected. With low dose of PS 80, cortisone treatment either failed to enhance the lesion severity or even gave rise to smaller lesions than in the control mice (Table 25). With high dose of PS 80 ($10^4 - 10^5$) the cortisone treated mice showed large areas of necrosis and a higher lesion score than the control. With 10^3 dose, the average lesion score in the cortisone treated animals was due mainly to two mice out of 6 tested. With the lowest dose (10^2) the lesion score in cortisone treated mice was much lower than in the control.

TABLE 26

Bacterial counts in the lesions of drug treated and control mice after the injection of *Staphylococcus aureus* PS 80 mixed with cotton dust.

Treatment	Initial dose	zero-hour count	2 hr. count	Rate of growth k/hour	Counts at	
					7 hr.	24 hr.
	4.0×10^5	not tested	8.13×10^4	0.51	2.41×10^7	5.91×10^7
No drug (Control)	3.0×10^4	1.68×10^4	4.67×10^3	0.40	5.6×10^5	1.95×10^7
	3.83×10^3	2.81×10^3	9.3×10^2	0.40	1.83×10^5	1.68×10^7
	2.5×10^5	3.94×10^4	3.91×10^4	0.53	1.98×10^7	1.41×10^8
Cortisone	5.33×10^4	8.5×10^3	2.35×10^4	0.41	2.86×10^6	2.6×10^7
	5.66×10^3	1.3×10^3	6.05×10^2	0.29	1.75×10^4	7.49×10^7
Colchicine	6.0×10^3	2.7×10^3	2.28×10^2	0.40	2.5×10^4	3.09×10^7
Butasolidin	7.4×10^3	1.9×10^3	3.5×10^2	0.40	3.4×10^4	3.08×10^7

BACTERIAL COUNTS

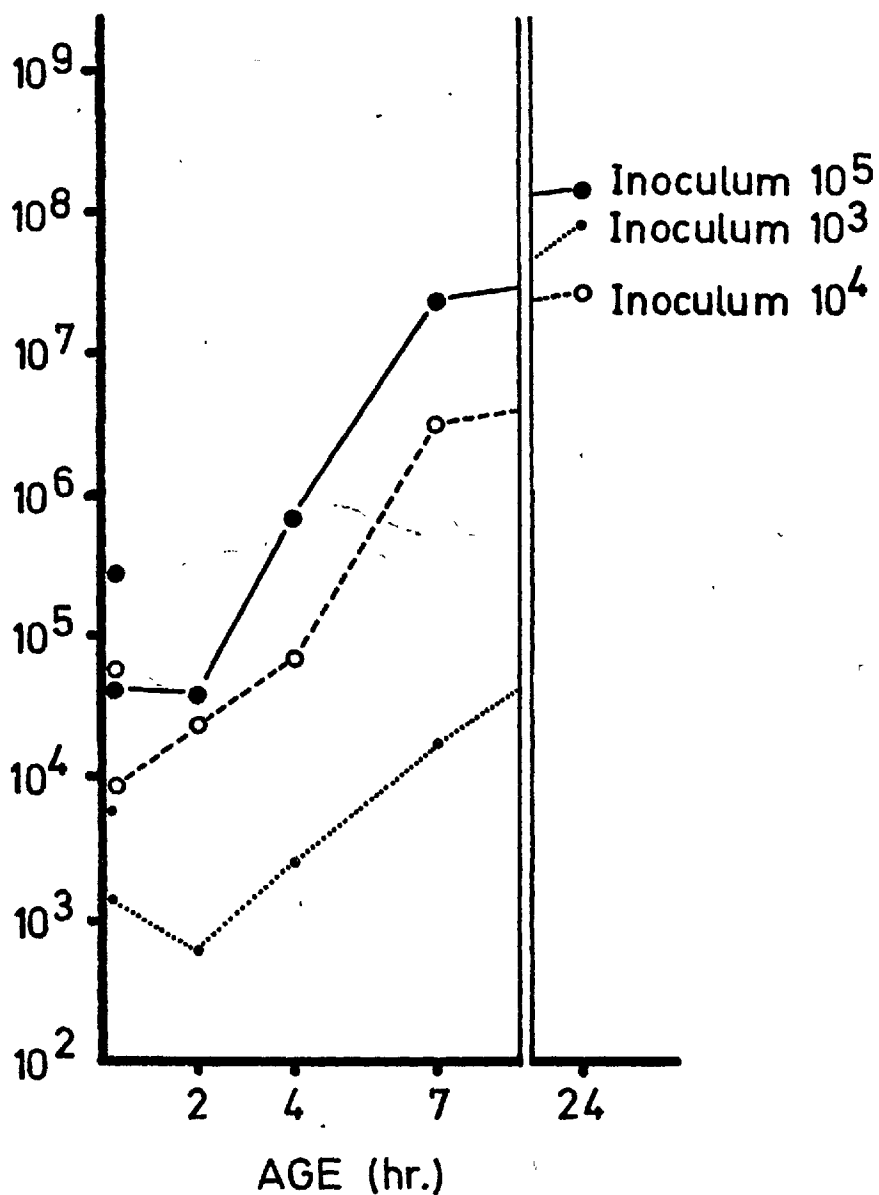


Figure 54: Bacterial counts in the lesion of cortisone treated mice given different inocula of Staphylococcus aureus strain PS 80 mixed with cotton-dust.

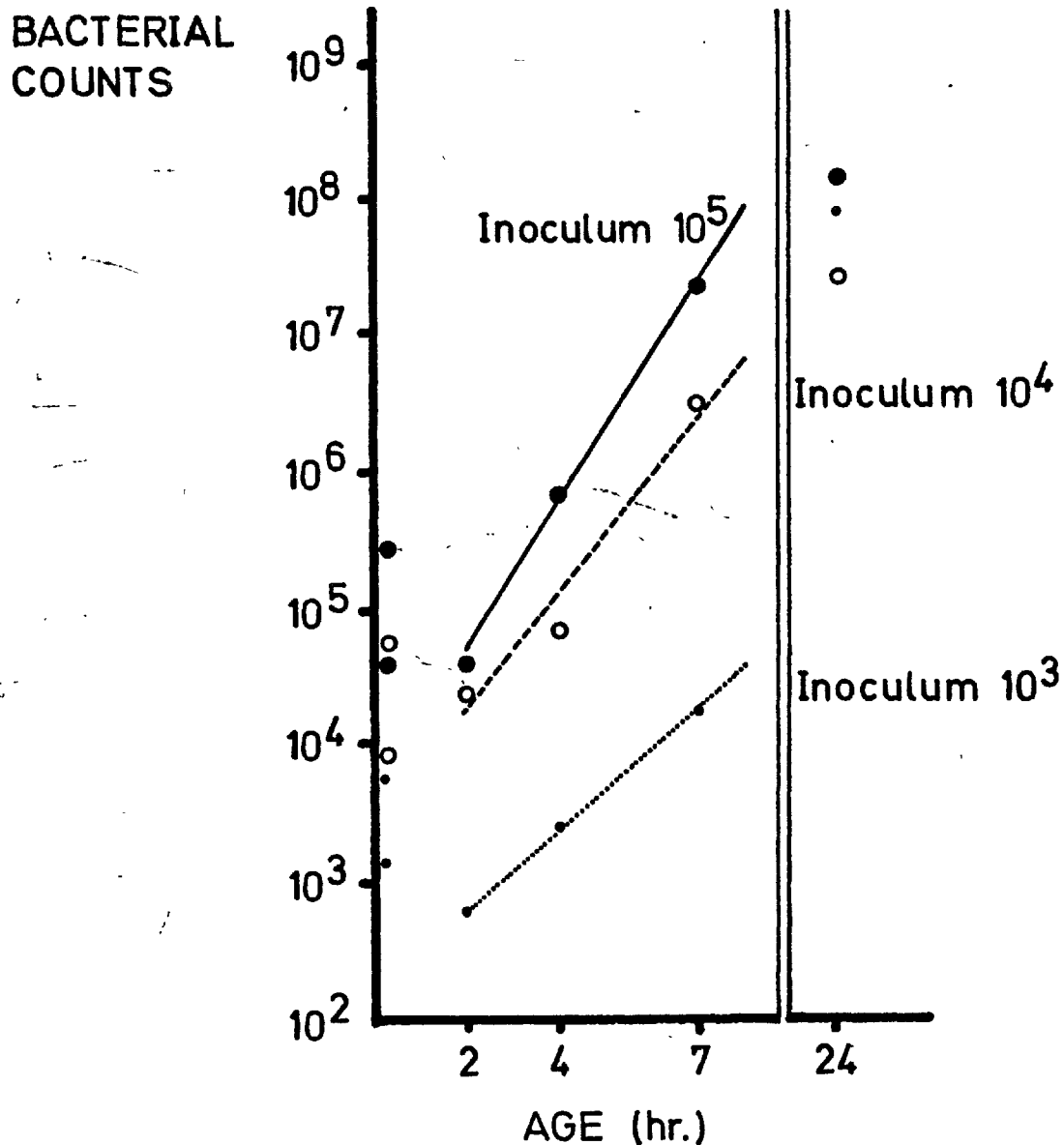


Figure 55: Growth curves of cocci in the lesion after subcutaneous injection of varying doses of Staphylococcus aureus strain PS 80 mixed with cotton-dust in cortisone treated mice.

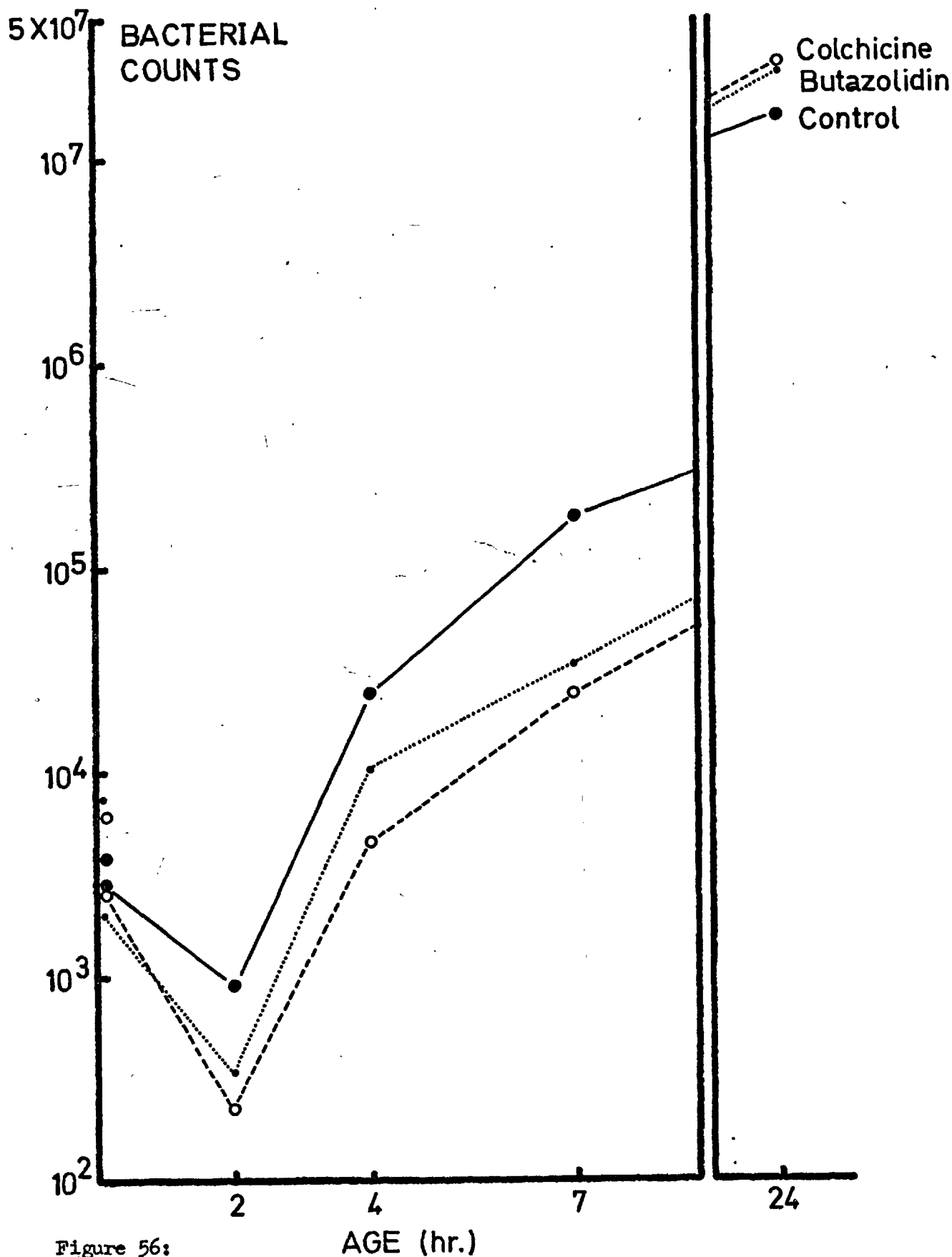


Figure 56:

Bacterial counts in the lesion after subcutaneous injection of *Staphylococcus aureus* strain PS 80 mixed with cotton-dust in colchicine and butazolidin treated and control mice.

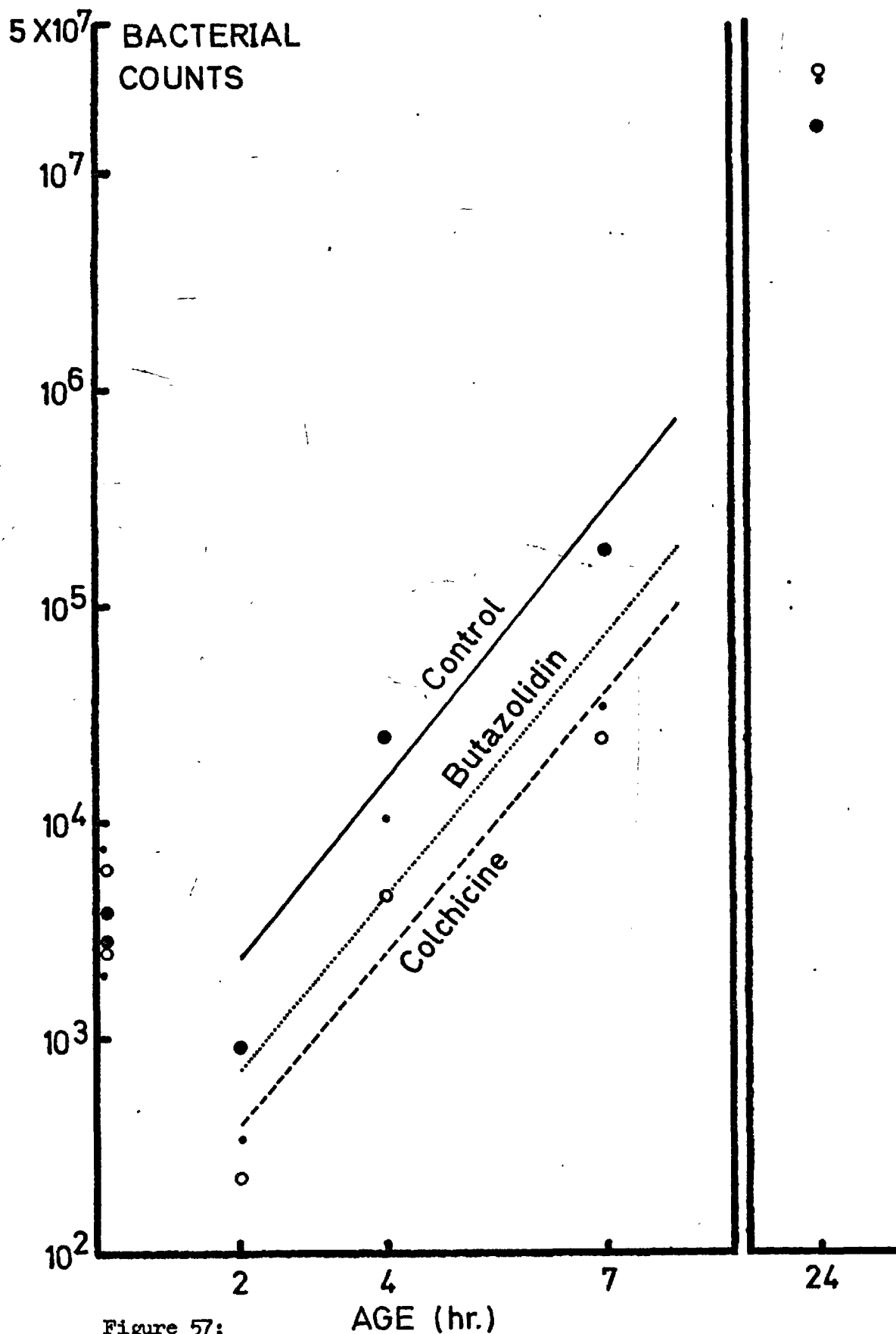


Figure 57:

Growth curves of cocci in the lesion after the subcutaneous injection of *Staphylococcus aureus* strain PS 80 mixed with cotton-dust in colchicine and butazolidin treated and control mice.

(c) Bacterial counts

Bacterial counts in the lesions were done in cortisone, butasolidin and colchicine treated mice. The results are shown in figures 54 to 57 and table 26. The table also shows the bacterial counts in the lesion of untreated mice for the sake of comparison. Each point on the graph represents the mean from two mice. As the action of cortisone in enhancing the lesion severity was found to be dependent on the number of staphylococci injected, bacterial counts in cortisone-treated mice were done after injecting varying numbers of PS 80. With colchicine and butasolidin the bacterial counts of the lesion were done only with an original dose of 10^3 Staphylococcus aureus strain PS 80, because as already described in earlier experiments the differences in growth patterns of cocci were best revealed at this dose.

It is noteworthy that in all cases the bacterial counts in the drug treated mice were similar to those seen in the lesions of control mice except with the 10^3 dose in cortisone treated mice, where the rate of growth of cocci per hr (k/hr) was 0.29 as compared to 0.40 in controls. At 24 hours the bacterial counts were very similar in all cases irrespective of treatment or dose of PS 80.

9. The effect of anti-inflammatory drugs on subsequent reinfection with staphylococci.

As already described many of the drugs tested increased the severity of the staphylococcal lesion and it seemed possible that

TABLE 27

The effect of anti-inflammatory drugs on the subsequent staphylococcal infection.

Exp. No.	Treatment at first infection only	No. of mice	Average lesion score	
			1st infection (PS 80 10 ⁵)	2nd infection (PS 80 10 ⁵)
1	None (control)	6	4.8	3.0
	Quinine hydrochloride	6	4.0	3.1
	Chlorpromazine hydrochloride	6	4.3	2.6
	Betamethasone disodium phosphate	6	4.6	3.2
	Cortisone acetate	6	5.1	2.6
	Dexamethasone-21-phosphate disodium salt	6	5.3	2.3
	Butazolidin	6	5.3	2.8
	Prednisolone disodium phosphate	6	5.6	2.6
2	None (control)	6	3.5	3.3
	Sodium salicylate	6	4.8	3.1
	Colchicine	6	5.8	2.1

Lesion score
2nd. infection

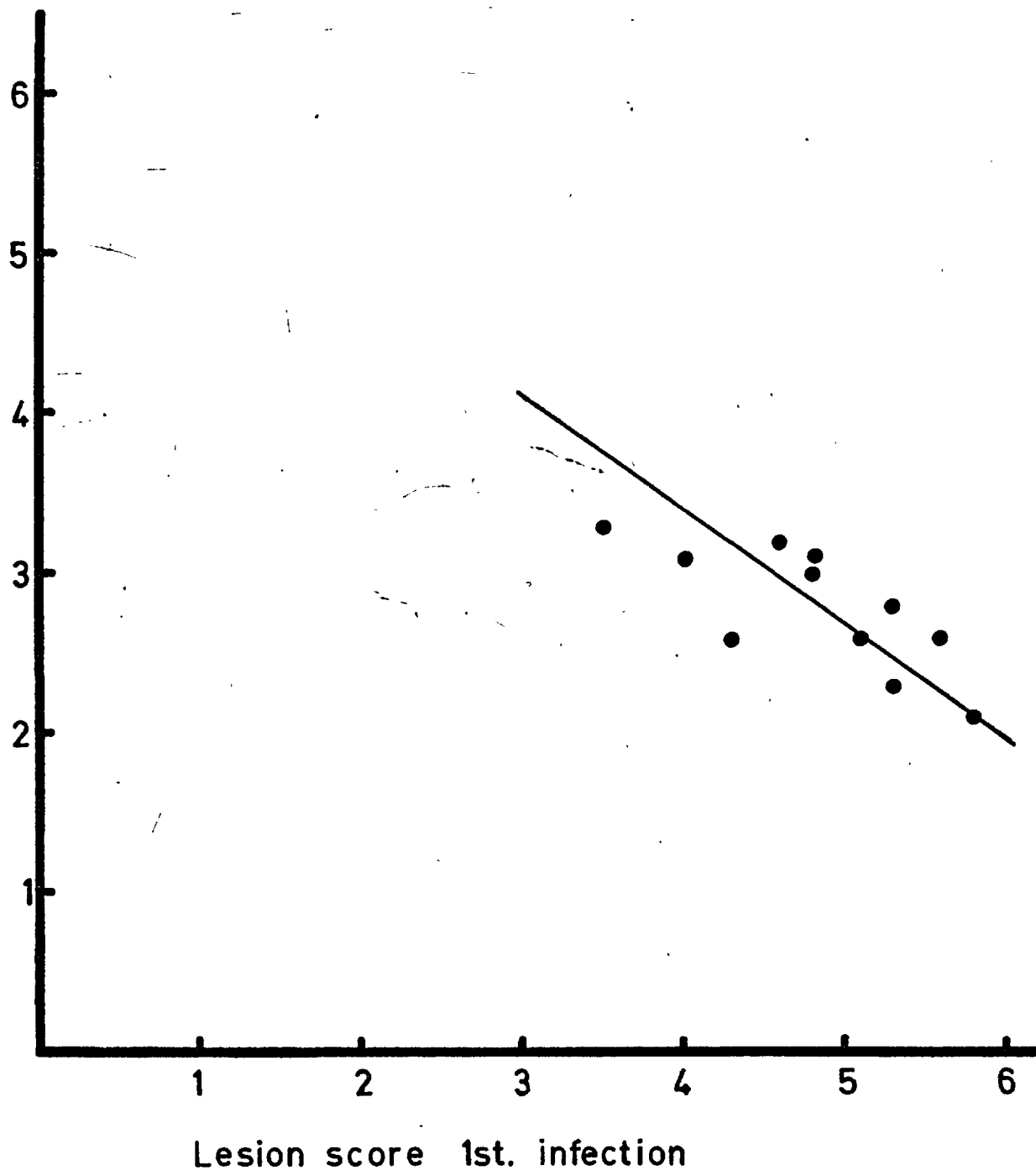


Figure 58: Correlation of lesion score after the first infection with the lesion score after the second infection with Staphylococcus aureus strain PS 80 mixed with cotton-dust.

the greater lesions might have induced better immunization of the mice against staphylococci. However, some of the drugs tested are also claimed to alter the immunological response of animals. Therefore the severity of lesion after reinfection of PS 80 in mice that had received the drugs at the time of first infection was studied. The result is shown in table 27. The mean diameter of necrosis is not shown as in many cases after the second infection there was only a pin-point area of necrosis and it was difficult to measure the necrotic zone with any accuracy.

The mice that had been treated at the time of first infection with the drugs like cortisone, prednisolone, colchicine, butasolidin and sodium salicylate, which enhance the lesions showed less severe lesions than controls after the second injection of staphylococci. However the treatment with drugs like chlorpromazine and dexamethasone at the time of first infection also reduced the lesion severity to the second injection of staphylococci.

Figure 58 shows the correlation of lesion score at the time of first infection with the lesion score at the time of second infection. The points, though scattered, tend to fall around a straight line; in general a greater lesion score at the time of first infection was associated with a lower lesion score after the second injection of staphylococci.

10. The effect of anti-inflammatory drugs on 'previously infected' mice.

As 'previously-infected' mice exhibited accelerated

TABLE 28

Staphylococcal lesions in 'previously-infected' mice after
the treatment with anti-inflammatory drugs.

Exp. No.	Treatment	No. of mice	Average lesion score	Average diameter of necrosis mm.	Diameter of necrosis as ratio of diameter of necrosis in controls
1	None (control)	6	2.6	0.9	
	Colchicine	6	3.0	1.4	1.5
	Butazolidin	6	3.0	1.4	1.5
	Sodium salicylate	6	2.0	0.5	0.5
	Quinine hydrochloride	6	2.0	0.70	0.7
	Chlorpromazine hydrochloride	6	3.1	1.6	1.7
	Prednisolone disodium phosphate	6	2.4	0.6	0.7
2	None (control)	6	2.5	0.6	
	Cortisone acetate	6	5.1	6.3	10.5
	Betamethasone disodium phosphate	6	4.0	2.3	3.8
	Dexamethasone-21-phosphate disodium salt	6	4.3	4.6	7.6
3	None (control)	6	2.7	0.5	
	Indomethacin	6	3.1	1.2	2.4

TABLE 29

The effect of anti-inflammatory drugs on fluid exudation in 'previously-infected' mice to the reinjection of Staphylococcus aureus PS 80.

Exp. No.	Treatment	No. of mice	Mean Oedema weight (mg)	Mean blue diameter (mm)	Mean Oedema weight as ratio of oedema weight in controls
1	None (control)	6	50.3(21.5 to 76.8)*	17.1(15.4 to 19.8)*	
	Colchicine	6	27.4(7.3 to 51.1)	11.5(9.4 to 14.6)	0.54
	Betasolidin	6	10.4(2.8 to 18.6)	9.5(6.4 to 13.0)	0.2
	Sodium salicylate	6	27.3(13.4 to 36.9)	10.5(7.4 to 12.6)	0.64
	Quinine hydrochloride	6	22.3(8.0 to 50.2)	9.5(7.4 to 13.4)	0.5
	Chlorpromazine hydrochloride	6	49.4(25.8 to 105.1)	15.5(12.9 to 18.0)	0.98
	Prednisolone disodium phosphate	6	34.8(-23.2 to 81.4)	10.9(8.3 to 14.4)	0.70
2	None (control)	6	35.3(1.6 to 87.8)	9.48(7.4 to 11.6)	
	Cortisone acetate	6	23.0(-0.2 to 53.4)	12.38(8.9 to 14.9)	0.6
	Betamethasone disodium phosphate	6	39.6(-5.5 to 123.1)	11.32(9.3 to 14.9)	1.1
	Dexamethasone-21-phosphate disodium salt	6	20.3(4.4 to 39.6)	10.68(7.4 to 12.4)	0.6

* = The range of mean oedema weight and mean blue diameter is given in bracket

inflammatory response and protection against skin necrosis after the reinjection of staphylococci, it was decided to study the effect of treating such mice with anti-inflammatory drugs at the time of second infection. Mice that had had a previous subcutaneous staphylococcal infection with the strain PS 80 were treated with anti-inflammatory drugs.

(a) Lesion score

The average lesion score and mean diameter of necrosis in 'previously-infected' mice after the reinjection of Staphylococcus aureus PS 80 and drug treatment is shown in table 28. Of the drugs tested cortisone, betamethasone, dexamethasone and indomethacin enhanced the severity of lesions on the criteria that the diameter of necrosis in these drug treated mice was more than double of the controls. The maximum enhancement was seen after treatment with cortisone, betamethasone and dexamethasone when lesion 4 to 10 times bigger than controls were produced.

(b) Fluid exudation

The result of fluid exudation 4 hours after the injection of PS 80 in these mice is shown in table 29 and 30. Although there was some reduction in the fluid exudation after treatment with colchicine, butasolidin, sodium salicylate and quinine hydrochloride only butasolidin and quinine reduced the fluid exudation to half or less of controls. With indomethacin the fluid exudation was also estimated at 24 hours in addition to 4

TABLE 30

The effect of indomethacin on fluid exudation in 'previously-infected' mice to the reinjection of Staphylococcus aureus Strain PS 80.

Treatment	No. of mice	Mean Oedema weight			
		At 4 hr.		At 24 hr.	
		weight (mg)	as ratio of oedema weight in controls	weight (mg)	as ratio of oedema weight in controls
None (control)	12	131.8 (62.0 to 176.0)*		194.8 (79.2 to 321.3)	
Indomethacin	12	81.7 (5.9 to 144.2)	0.6	116.9 (45.3 to 173.3)	0.60

* = The range of mean oedema weight is given in brackets

TABLE 31

The effect of anti-inflammatory drugs on total leucocytes/
HFF in the 'previously-infected' mice.

Treatment	Total no. of leucocytes/ HFF	Leucocyte count as ratio of count in controls
None (control)	495	
Cortisone acetate	313	0.6
Betamethasone disodium phosphate	169	0.4
Dexamethasone-21-phosphate disodium salt	256	0.52
Prednisolone disodium phosphate	267	0.54
Chlorpromazine hydrochloride	587	1.2
None (control)	237	
Colchicine	242	1.02
butasolidin	216	0.9
Sodium salicylate	352	1.5
Quinine hydrochloride	453	1.9
Indomethacin	269	1.1

TABLE 32

The effect of anti-inflammatory drugs on staphylococcal infection in 'previously-infected' mice.

Drug	Reduction in fluid exudation	Reduction in leucocyte emigration	Enhancement of lesion
Colchicine	-	-	-
Butazolidin	+	-	-
Cortisone acetate	-	-	+
Prednisolone disodium phosphate	-	-	-
Betamethasone disodium phosphate	-	+	+
Dexamethasone-21-phosphate disodium salt	-	-	+
Sodium salicylate	-	-	-
Quinine hydrochloride	+	-	-
Chlorpromazine hydrochloride	-	-	-
Indomethacin	-	-	+

hours after injection (table 30), because of reports that indomethacin specifically suppresses the fluid exudation of the delayed hypersensitivity reaction (Walters and Willoughby 1965). Although there was some reduction in fluid exudation both at 4 and 24 hours, it was not reduced to half of the control.

(c) Leucocyte count

The total leucocyte counts/HPF in the tissue sections of 4 h. old lesion are shown in table 31. Of the drugs tested only betamethasone was able to reduce the leucocyte emigration.

(d) Relation of the two components of inflammation with the severity of lesion.

The effects of anti-inflammatory drugs on the response of 'previously-infected' mice are summarized in table 32. On the basis of criteria of reduction in the two components of inflammation and enhancement in the lesion severity already described, only betamethasone reduced leucocyte emigration and only butasolidin and quinine hydrochloride reduced fluid exudation. However the enhancement of lesion was seen only after the treatment with betamethasone, cortisone, dexamethasone and indomethacin. Thus of the drugs enhancing the lesion only in the case of betamethasone the increased severity of lesion was related to reduced leucocyte emigration. These findings suggest that the enhancement of lesion by these drugs is not solely due to their anti-inflammatory activity.

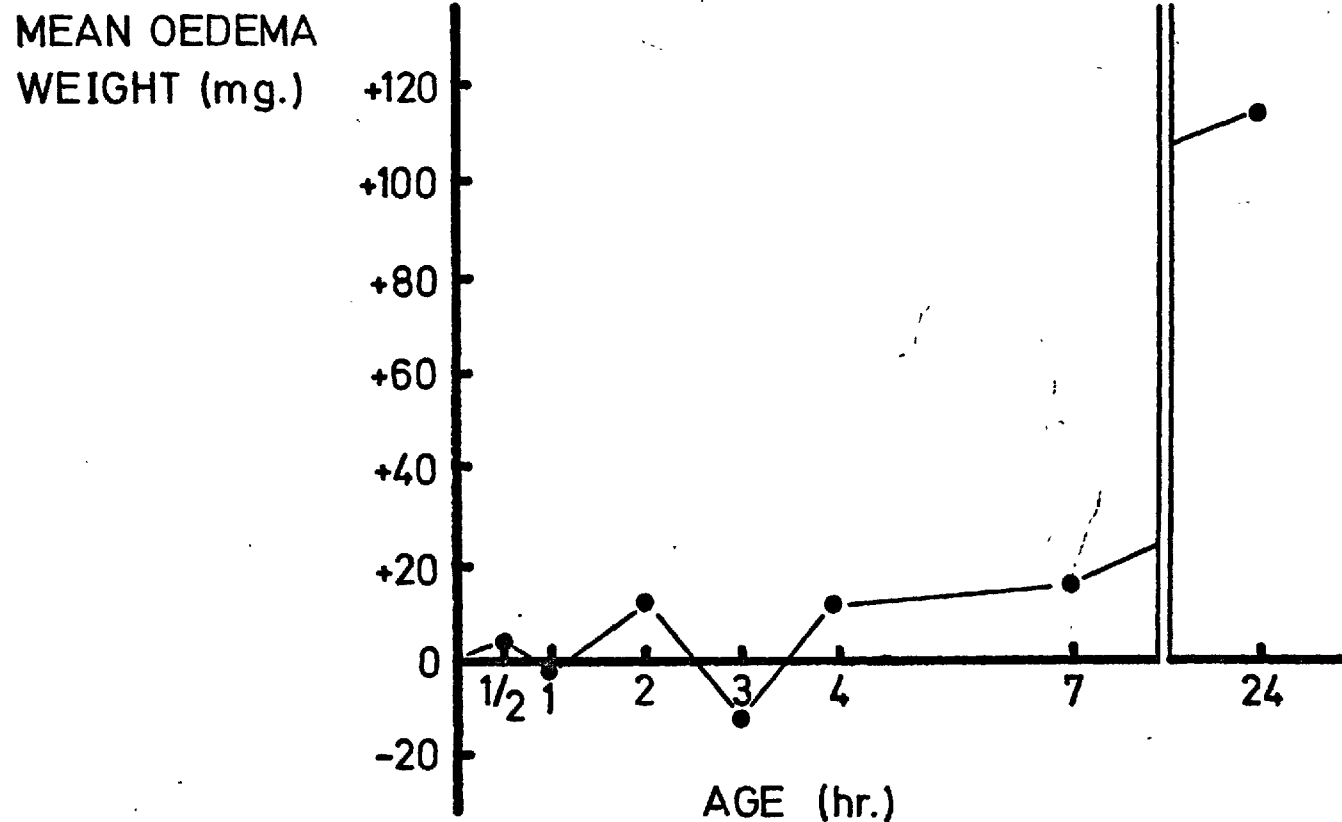


Figure 59: The mean oedema weight of tissue after the subcutaneous injection of 10^5 Staphylococcus aureus PS 80 mixed with cotton-dust in endotoxin treated mice.

11. The effect of endotoxin on staphylococcal infection

Endotoxin-treated mice (see methods) were injected with 10^5 dose of Staphylococcus aureus strain PS 80 mixed with cotton dust.

(a) Lesion score

In endotoxin treated mice, very severe lesions were produced after the injection of staphylococci. The average lesion score from 16 mice in three different experiments was 5.66. The lesions were mainly necrotic. The mean diameter of the necrotic zone was 11.6. i.e., three times as big as in the controls. The average lesion score and the average diameter of the necrotic zone in untreated mice with similar dose of PS 80 was 4.8 and 4.19 mm. respectively.

(b) Fluid exudation

The oedema weight of staphylococcal lesion in endotoxin treated mice is shown in figure 59. There was very little exudation up to the first 5 hours of challenge. Some exudation of fluid started after 5 hr, but was slight even up to 7 hr. The lesions 24 hr. after the injection of cocci were oedematous. Thus endotoxin delayed the onset of exudation of fluid.

(c) Leucocyte emigration

The leucocyte response did not start until about 4 hours after the injection of cocci. Although the cotton itself

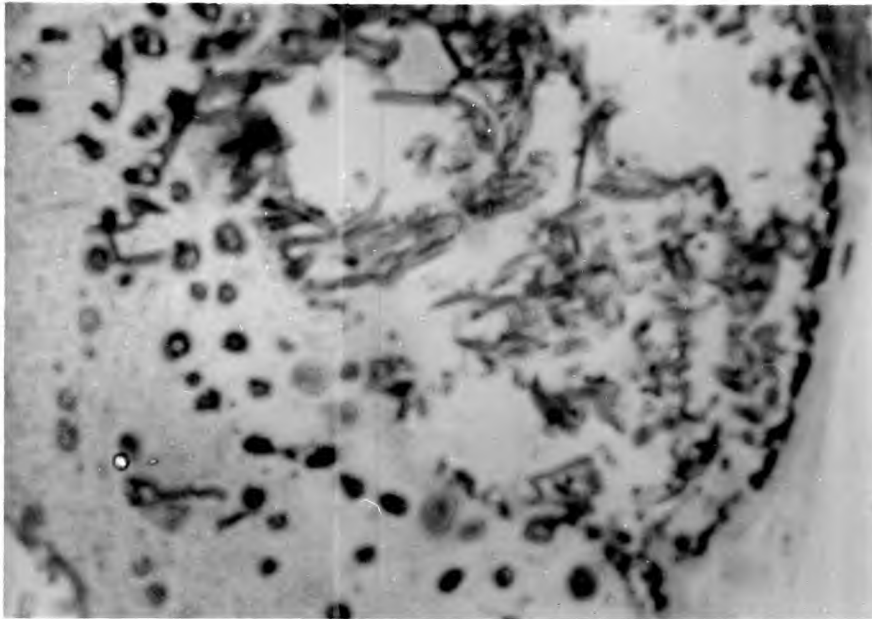


Figure 60: Twenty-four hour old lesion in endotoxin treated mice after the subcutaneous injection of 10^5 Staphylococcus aureus strain PS 80 mixed with cotton dust. The lack of infiltration of cotton-dust by leucocytes. At places leucocytes are at some distance away from the edge of the cotton-dust. X40.

TABLE 33

The effect of endotoxin and anti-inflammatory drugs on
the dermonecrosis produced by alpha haemolysin.

Treatment	No. of mice	Average diameter of necrosis (mm.)	Diameter of necrosis as ratio of diameter of necrosis in control
None (control)	6	3.8 (2.4 to 4.6)*	
Cortisone acetate	6	4.5 (2.0 to 7.3)	1.2
Colchicine	6	3.3 (2.5 to 7.2)	0.87
Endotoxin	6	5.2 (4.4 to 6.6)	1.3

* The range of diameter of necrosis is
given in brackets

showed evidence of slight infiltration at 4 hours, it was not complete even at 7 or 24 hours, and only a small part of the periphery of the cotton dust was infiltrated by leucocytes. At places in sections of 24 hour old lesion the emigrated leucocytes remained at some distance from the edge of the cotton dust (figure 60).

(d) Possible mechanism of action of endotoxin in enhancing the severity of staphylococcal lesion.

Besides affecting the inflammatory response, there may also be other mechanisms by which endotoxin enhances the staphylococcal lesion. In view of the large areas of necrosis produced by staphylococcal injection in the endotoxin treated mice the possibility that endotoxin might be enhancing the action of alpha haemolysin was considered.

'Wellcome' brand alpha haemolysin (0.16 units) along with cotton dust was injected subcutaneously in mice that had been treated with endotoxin. As a control alpha haemolysin was also injected in the untreated mice. For comparison mice treated with cortisone acetate and colchicine were also included in the experiment. The mean diameter of necrosis is shown in Table 33. In none of the treated mice the zone of necrosis was twice as big as in the controls. Therefore the enormously big areas of necrosis produced after injection of staphylococci in endotoxin treated mice seemed not to be due to enhancement of the dermonecrotic action of alpha haemolysin.

TABLE 34

The effect of dibenylamine on the staphylococcal lesion of
endotoxin treated mice.

Mean diameter of necrotic zone (mm)		
	Endotoxin treatment	Colchicine treatment
Control group	14.3 (6.3 to 21.1)*	9.3 (5 to 17.4)
Dibenylamine group	8.5 (5 to 12.5)	7.16 (4.6 to 13.5)

* = The range of diameter of necrosis is given in
brackets.

In view of the similarity between the effects of the interaction of staphylococci and endotoxin to the effects of adrenaline upon endotoxin-prepared skin of rabbits (Thomas 1959), an experiment was done using the adrenergic blocking agent 'dibenyline'. Twelve mice were given 'dibenyline' (Smith, Kline & French) intraperitoneally in 2 doses of 50 μ g at 24 and 2 hr. before the injection of endotoxin or colchicine. As controls another 12 mice were treated only with endotoxin or colchicine. All the 24 mice were injected subcutaneously with 10^5 Staphylococcus aureus strain PS 80 mixed with cotton dust. The severity of the lesion is expressed in terms of mean diameter of necrotic zone in table 34. There was some reduction in the severity of staphylococcal lesion in the endotoxin treated mice after dibenyline administration. Dibenyline reduced the diameter of necrosis in endotoxin treated mice to 60% of the controls as compared to only 78% of the controls in colchicine treated mice. Thus dibenyline did not completely abolish the infection enhancing activity of endotoxin; even after its administration, the lesions in endotoxin prepared mice were twice as big as in the untreated mice injected with a comparable dose of 10^5 PS 80.

VII DISCUSSION

Staphylococcal disease seems an uncommon event when one considers the almost constant contact that this microbe has with the human host. Therefore the factors that predispose the host to staphylococcal infection are important areas for investigation. It is almost impossible to study the interplay of these factors with those of the host and bacteria in vitro, and although attempts have been made to analyze some of these interactions by mixing blood serum and phagocytic cells with staphylococci, it is not an exact reproduction of events in vivo, where there are continual changes not only in the bacterial population but also in the cellular and humoral defence factors of the host at the lesion site. It is difficult to carry out experiments in man therefore mice were used for the present study. Although the results of animal experiments can not be extrapolated for humans, it was thought worthwhile to study the pathology of staphylococcal infection in mice as little is known about the dynamics of staphylococcal infections and there are examples of some bacteria where virulence in animals and man run parallel. By studying the inflammatory response of the mice and bacterial population changes of the lesion at intervals after the injection of cocci, some aspects of host-parasite interaction can be elucidated.

The role of cotton-dust in promoting staphylococcal infection

There is ample evidence that the presence of a foreign body in the tissue enhances staphylococcal infection (Klek and Conen 1957, James and MacLeod 1961, Noble 1964). However the mechanism by which a foreign body promotes the infection is not understood. This work illustrated that when cotton dust was mixed with the cocci injected into the tissues there was a delay in the exudation of fluid and the emigration of leucocytes. The difference in the inflammatory response to staphylococci injected with or without cotton-dust was evident in the first hour after injection. With cotton dust, in addition to a delayed emigration of leucocytes there was also a marked delay in the infiltration by phagocytic cells of the region of bacterial growth. Twenty four hours after injection of 10^6 cocci, there was very little cellular infiltration of the cotton dust, which contained masses of bacteria. A similar finding of the absence of granulocyte infiltration in the lesion has been reported by Cluff (1965) when staphylococci were injected in large quantities intradermally in rabbit or when a third-degree burn of a rabbit's skin was infected with staphylococci.

The beneficial effect of the presence of cotton dust to the invading cocci was reflected in the bacterial counts of the lesion. There was a rapid growth of cocci after an initial lag period and

by 7 hours the count had increased two log units over the original dose inoculated; at 24 hours the count had increased more than 100 fold. In the absence of cotton dust there was no such multiplication and the 24 hour count remained lower than the initial inoculum. A similar multiplication of staphylococci injected subcutaneously in mice was reported by Taubler and Kapral (1964) in the presence of sutures.

The rapid growth of cocci in the presence of cotton dust can be correlated with the deficient cellular emigration and fluid exudation during the early stages of infection; the fluid as well as the cellular components of inflammatory exudate seem to be important determinants of the destruction of staphylococci (Gladstone and Glenzrees 1960, Cohn 1962a,b,c). Due to the unrestricted growth during the early stages after inoculation, there is presumably an elaboration of various extracellular factors, including leucotoxic factors which are then able to keep the leucocytes away from the site of bacterial multiplication. This further promotes the growth of staphylococci.

The deficiency in leucocyte infiltration of the cotton-dust was presumably due to alpha toxin because when commercial alpha toxin was injected with the cotton dust, there was a similar lack of infiltration of the cotton dust by leucocytes. In the mice with past experience of staphylococcal infection, that is

having some immunity against staphylococci, the leucocytes infiltrated the cotton containing alpha toxin. However 'Wellcome' brand of alpha toxin used in the experiment is not a pure product, so it is possible that this effect is due to some other factors. It would be useful to carry out these experiments with purified alpha haemolysin. P-V leucocidin is known to be ineffective against mouse leucocytes (Gladstone and van Heyningen 1957), therefore if other factors are involved it may either be delta haemolysin or some other unknown leucotoxic component of staphylococci.

James and MacLeod (1961) suggested that the potentiating effect of the suture material was due to the inflammatory reaction provoked by the sterile suture material. In order to test the effect of cotton-dust alone in eliciting an inflammatory reaction, sterile cotton-dust with sterile nutrient broth was inoculated subcutaneously in mice, there was little exudation of fluid in the first 2 hours of injection. The main phase of vascular permeability was after that period and it reached its maximum 7 hours after inoculation. The leucocyte emigration started 1 hour after injection; however cotton was infiltrated by leucocytes at 3 hours and by 24 hours a large number of leucocytes had infiltrated the entire cotton. As already discussed the infiltration of cotton dust during the later stages of inflammation due to staphylococci is prevented presumably by alpha toxin and the cotton itself does not appear to

play a direct role in this. Penn and Ashford (1963) who studied the inflammatory response to implantation of cotton pellets in the rat also reported that the main phase of increased vascular permeability occurred after 2.5 to 3 hours. Thus there is no doubt that cotton-dust by itself elicits inflammatory reaction, but that is after a lapse of several hours and in this work the importance is attached to the early inflammatory reaction, and it seems that the infection enhancing effect of the cotton-dust is due to the initial delay in the inflammatory reaction caused by cotton dust.

The mechanism by which the cotton dust delays the onset of inflammation can only be postulated. It is possible that it may be due to the increased tissue pressure due to the mechanical presence of cotton dust. The observation of Andriole and Lytton (1965) that under conditions of increased tissue pressure there is a decreased capillary blood flow and delayed emigration of leucocytes is pertinent.

Besides the delay in the onset of inflammation in the presence of cotton dust other factors that may be responsible for the enhancement of staphylococcal infection should also be considered. An important effect of the cotton dust is to concentrate the cocci in a small volume of space occupied by the cotton pellet. In contrast, when cocci are injected without cotton, they are distributed in a much larger volume of space.

There was no evidence in this work to support the possibility

that in the absence of cotton dust, cocci disseminated to other parts of the body. However Fisher and Robson (1963) report that about 0.2% of staphylococci injected subcutaneously in a mouse without any foreign body find their way into the blood stream. Nevertheless the above findings indicate that most of the staphylococci injected subcutaneously whether in the presence or absence of cotton dust remain confined to the local site of injection.

The possibility that some humoral defence factors from the blood could not penetrate the cotton plug could not be substantiated; it was found that the cotton plug was penetrated within minutes after the intravenous injection of Evans blue. Whether the concentration of humoral factors in the deeper parts of the cotton was the same as on the surface could not be ascertained.

It is also not known whether phagocytosis was in any way affected in the presence of the cotton dust. In vitro experiment on phagocytosis of staphylococci in the presence and absence of cotton dust may serve to clarify this point.

The response to different strains of staphylococci.

The vascular permeability response of the mouse to the subcutaneous injection of Staphylococcus aureus strain PS 80 was found to be monophasic. Some investigators have reported a biphasic response after the injection of Staphylococcus aureus

in experimental animals (Rigdon 1941, Burke and Miles 1958). However these workers injected staphylococci by the intradermal route and without any foreign body. The experiments were also carried out on species of animal other than mouse. All these differences in the experimental conditions may account for the absence of primary phase of permeability; Miles (1961) has suggested that the primary phase depends on the species of animal.

One of the striking features of this study is the notable difference found in the inflammatory response elicited by different strains of staphylococci. Some strains produced an early inflammatory response even in the presence of cotton dust, that means these strains by themselves produced so much inflammation that they could overcome the delaying effect of cotton.

The initial delay in fluid exudation in the presence of cotton dust was found to be a characteristic of 'mouse-virulent' strains such as PS 80, Oxbach and 1531. With all the three strains a delay in the inflammatory reaction was also correlated with rapid bacterial growth and the production of severe lesions. The finding that with these strains the weight of the tissue during the first hour was less than the weight of the control tissue is in agreement with the work of Burke and Friberg (1961) who have reported a decrease in the dry mass of the 'inflammatory exudate' during the early stages of staphylococcal inflammation. The reason for this initial drop in the weight of the tissue after

injection is not known; it could be due to a decreased blood flow at the site of injection.

In all cases, there was an initial drop in the bacterial counts before the growth of cocci in vivo was apparent. This initial drop in the bacterial counts did not seem to be either due to dissemination or clumping of the cocci. No viable cocci could be cultured from any of the viscera tested. However as 'zero-hour' counts of lesion have emphasized, there was a difficulty in recovering all the cocci from the site of injection even if the animal was examined immediately. Therefore a trivial dissemination could well have been missed. Kapral (1965) has reported that in the staphylococcal peritonitis of mouse there was clumping of cocci and the counts of colony forming units remained low till the death of the animal. In the present work the low counts were only seen during the first 1-3 hours after which there was a rapid rise in the bacterial count with the three 'mouse-virulent' strains. It appears likely that the initial drop in the count was due to the destruction of cocci.

After the initial drop in the bacterial count, there was a rapid growth of cocci even with a small dose of 10^2 PS 80. With this dose the 24-hour count was similar to that seen with an initial inoculum of 10^5 . However with the 10^2 dose of PS 80 lesions were much milder than with the 10^5 dose. This indicates that the ultimate size of the lesion is determined during the early stages of infection. There was some evidence to support this view. With a high dose of PS 80, 4 hours after injection the leucocytes

remained away from the cotton and the bacterial counts in the lesion had increased well above the dose inoculated. On the other hand with a 10^2 dose of PS 80, leucocytes started infiltrating the cotton dust 3 - 4 hr. after injection and the bacterial count in the lesion at 4 hours was still lower than the dose inoculated. However the bacterial count in vivo is actually the number of cocci resulting from bacterial multiplication minus the numbers lost from the lesion due to destruction or dissemination and there is no satisfactory way to find it.

It has already been shown that alpha haemolysin was ultimately responsible for dermonecrosis. It was however not a factor involved in the initiation of infection, because alpha haemolysin by itself elicited early exudation of fluid and rapid emigration of leucocytes. For the initiation of infection the factor which delays the onset of inflammation should be considered important. Although cotton-dust itself delays the onset of inflammation there also appears to be a factor in some staphylococci which also delays the inflammatory reaction. It was shown in the experimental work that heat-killed PS 80 injected along with cotton-dust elicited the exudation of fluid after an initial delay of 3 hours that is after a much longer delay than the injection of sterile cotton-dust alone. The importance of this factor was illustrated by the injection of Staphylococcus aureus strains such as Wood 46 and Ward. Both were capable of producing alpha and delta haemolysin in vitro; moreover

Wood 46 produced largest quantities of alpha haemolysin amongst all the strains used for this study. Yet both these strains failed to produce severe lesions. This seemed to be because both these strains stimulated an early inflammatory response which in turn accounted for the low bacterial counts in the lesion, presumably because of a rapid destruction of cocci. On the other hand a strain that delayed the onset of the inflammatory reaction but lacked the capability to produce alpha haemolysin also failed to produce severe lesions, as exemplified by Micrococcus hyicus. It seems therefore that no single factor of staphylococci can be held responsible for "virulence". A fully virulent strain must possess both the factor causing an initial delay in the inflammatory response and the factors needed for ultimate destruction of tissue.

Similarly it appears that for protection against staphylococcal infection, antibodies to both factors are important. Staphylococcus aureus strain Wood 46 produces only mild lesions in mice, with minimal necrosis. However when colchicine treated mice are injected with Wood 46 a fair degree of necrosis is evident which is presumably due to alpha haemolysin. But these mice were not protected against subsequent infection with PS 80 which also has the ability to delay the onset of inflammation. Wood 46 presumably lacks the factor responsible for the initial delay in inflammation.

It was also found that either of the two components of inflammatory response was probably capable of limiting the multiplication of cocci in the early stages of infection. For example, Staphylococcus aureus strain PS 83A elicited early exudation of fluid but delayed emigration of leucocytes and strain 278 which is a micrococcus evoked the opposite reaction; in both infections the bacterial counts were low and mild lesions were produced. PS 83A was capable of producing both alpha and delta haemolysin in vitro. Strains like the coagulase-negative strain NOTC 7292 which both evoked an early inflammatory response and also lacked the capacity to produce alpha haemolysin, produced the mildest lesion.

Besides the inflammatory response, other host factors such as phagocytosis and the ultimate destruction of cocci within the phagocyte seem to be important. For example strains like Smith, which elicited an early emigration of leucocytes, and P66/7, which elicited an early exudation of fluid as well as a leucocyte response, nevertheless showed a high bacterial count in the lesion during early stages of infection. Smith strain is known to possess an antiphagocytic surface antigen (Morse 1962a, Koenig 1962, Rogers and Kelly 1962).

There are no reports in the literature correlating the early exudation of fluid during the course of staphylococcal inflammation with the severity of the lesion. There are a few

brief reports about the relative delay in the influx of polymorphonuclear leucocytes in the lesion site with Staphylococcus aureus infection as compared to Staphylococcus albus (Delaunay 1942, Cohn 1962a). However no attempt was made in the past to distinguish strains of Staphylococcus aureus differing in their virulence on the basis of either the delayed leucocytic emigration or delayed fluid exudation. The findings in this work illustrate that amongst the strains of Staphylococcus aureus tested virulence could be partly related to the ability of a strain to delay the onset of inflammation.

The exact factor or factors in the 'mouse-virulent' strains responsible for causing the delay in the inflammatory reaction is not known. It seems to be due to some component associated with the cells of staphylococci as the heat-killed and washed cells of Staphylococcus aureus strain PS 80 elicited an inflammatory response only after an initial delay of 3 hours. A search is therefore needed for cell wall components of staphylococci that can delay the onset of inflammation. There are a few reports of the presence of endotoxin-like substance in staphylococci (Arndt, Young and Ritts 1963, Neter et al. 1960). This may be pertinent in that endotoxin from Gram-negative bacteria is known to delay the inflammatory process (Miles and Niven 1950, Conti et al 1961). It would also be interesting to investigate whether the cell-wall 'aggressin' of Staphylococcus aureus described by Fisher (1965) delays the onset of inflammation; this 'aggressin'

was claimed to promote the multiplication of cocci.

Although alpha toxin was shown to be involved in preventing the leucocyte infiltration of cotton in the later stages of inflammation and presumably it then rendered the leucocytes ineffective, it is not responsible for the initial delay in the inflammatory reaction.

The protective role of the inflammatory reaction against bacterial infection has been investigated by other workers (Miles, Miles and Burke 1957, Burke and Miles 1958). From a study of nine different bacterial infections, including Staphylococcus aureus, in the skin of guinea-pigs these workers concluded that the maximum death-rate of injected bacteria occurred during the first 2 hours of infection and that after 4 - 5 hours most of the reactions determining the ultimate size of the local lesion had occurred. However the conclusion that the maximum death of injected bacteria occurred during the first two hours was based on indirect evidence and no actual bacterial counts of the lesion were done. Burke and Miles (1958) observed further that during the first 2 hours of this "decisive period" macroscopic signs of inflammation were scanty and a fully developed acute inflammatory response was seen only after this "decisive period". However Miles and co-workers used intradermal injection and had used small numbers of bacteria which might have been the cause of slower evolution of inflammatory reaction. When Burke and Miles (1958) injected large numbers of

bacteria, there was a fully developed inflammatory response at 2 hours and in this case the period of maximal bacterial destruction correlated well with the maximum vascular response.

Response of 'previously-infected' mice to staphylococci.

Mice having past experience of staphylococcal infection showed an altered reactivity to the reinjection of staphylococci. These mice were protected against the dermonecrotic effects of staphylococci and alpha haemolysin. However the 'previously-infected' mice were not protected against staphylococcal abscess formation. These mice also exhibited an accelerated inflammatory response to staphylococci which was characterized by an early exudation of fluid evident half an hour after the injection, in contrast to normal where it started after an initial delay of two hours. At 24 hours there was much more oedema fluid in the lesion of 'previously-infected' mice than in control mice. The emigration of leucocytes started 1 hour after injection in contrast to 2 hours after injection in normal mice and the cotton dust which contained the cocci was infiltrated 3 - 4 hours after injection i.e., much earlier than in the normal mice. With a 10^3 dose of Staphylococcus aureus PS 80 there was a slower rate of growth of cocci in the lesion of 'previously-infected' mice than in normals and 24 hour bacterial counts were lower than the original inoculating dose.

The protection against dermonecrosis in 'previously-infected' mice

was mediated by serum presumably due to the presence of antibodies to alpha haemolysin; when serum from 'previously-infected' mice was transferred to normal mice it conferred protection against the dermonecrotic effects of staphylococci in the treated mice. Similarly the accelerated exudation of fluid and the rapid leucocyte emigration as well as the infiltration of the cotton dust containing staphylococci was due to an immunologic response mediated by circulating antibodies. Serum from 'previously-infected' mice was able to confer these altered reactions on normal mice.

The accelerated increase in vascular permeability exhibited by 'previously-infected' mice seems to be due to a hypersensitivity reaction to staphylococci. Other workers have also reported that animals with previous staphylococcal infection show both the immediate and delayed type of hypersensitivity reaction to the injection of staphylococci and their products (Julianelle and Wiegand 1935b, Julianelle and Hartmann 1936, Johnson, Cluff and Goshi 1961, Goshi, Smith, Cluff and Norman 1963, Bodel and Atkins 1964). The finding of Voisin and Toullet (1960) that increase in vascular permeability is an essential feature of both the immediate as well as tuberculin type of hypersensitivity is relevant to the present discussion.

The nature of the antigen or antigens responsible for sensitization can only be surmised. This work has illustrated that both the alpha haemolysin and some component associated with the cells

of staphylococci were involved, because heat-killed staphylococci produced an early accelerated emigration of fluid in the 'previously-infected' mice. Similarly alpha haemolysin evoked a greater emigration of fluid in the first hour as well as at 24 hours in the 'previously-infected' mice than in normals. The sensitizing antigen associated with the cells of staphylococci seemed also to be present in a micrococcus strain 278. Mice 'previously-infected' with 278 showed an early accelerated inflammatory response and reduced lesion severity after the injection of Staphylococcus aureus PS 80. Pacheco (1932) found that repeated intradermal injection of guinea-pigs with heat-killed staphylococci led to an accelerated inflammatory response to subsequent infection with Staphylococcus aureus. Goshi, Smith, Cluff and Norman (1963) have also demonstrated that rabbits immunised with alpha haemolysin developed large inflammatory reactions to the injection of this toxin, in contrast to non-immune animals. Other staphylococcal antigens incriminated in hypersensitivity reactions include polysaccharide A for the immediate type of reaction (Julianelle and Hartmann 1936) and a protein antigen common to both the pathogenic and non-pathogenic strains of staphylococci for the delayed reaction (Julianelle and Wiegand 1935b).

The rapid emigration of leucocytes in the staphylococcal lesion of 'previously-infected' mice could be due to a chemotactic effect exerted by staphylococcal antigen-antibody

complex. Boyden (1962) has recently shown that mixtures of antibody and antigen have a strong chemotactic effect on polymorphonuclear leucocytes. On the other hand the rapid leucocyte infiltration of the cotton dust containing staphylococci seems to be due to neutralisation of staphylococcal alpha toxin, because in the 'previously-infected' mice the leucocytes rapidly infiltrated the cotton dust containing either alpha haemolysin or staphylococci. However as already discussed the 'Wellcome' brand of alpha toxin is only a partially purified product and the findings should be confirmed by the use of pure alpha haemolysin. Goshi, Cluff and Norman (1963) using purified alpha haemolysin have demonstrated prompt infiltration of the area of staphylococcal inoculum by polymorphonuclear leucocytes in the animals immunised with alpha haemolysin toxoid, in contrast to normals.

The accelerated inflammatory response of the 'previously-infected' mice led to low bacterial counts in the lesion, previously because of the destruction of cocci. In these mice there was a greater initial fall in the bacterial count which remained low for a longer period than in controls. The increased ability to destroy staphylococci could not be passively transferred by serum.

Cluff and his associates (Johnson, Cluff and Goshi 1961) found that repeated staphylococcal infection of rabbit skin is associated with increased susceptibility to reinfection due to the

development of delayed hypersensitivity. In this study the mouse showed no increased susceptibility to repeated staphylococcal infection. However when living lymph node cells from 'previously-infected' mice were transferred to normal mice, the treated mice exhibited a markedly increased susceptibility. Even a dose of 10^4 PS 80 produced severe necrotic lesions in these mice, while the same dose in normal mice produces only a moderate lesion. However mice treated with lymph node cells did not show an accelerated exudation of fluid at 24 hours as might be expected in a delayed hypersensitivity reaction. Jchanovsky (1958b) has reported transfer of increased susceptibility, as well as skin reactivity, to staphylococci by spleen cells or peritoneal exudate cells from the sensitized animals.

In the present studies when lymph node cells from sensitized mice were transferred along with the serum from 'previously-infected' mice there was no increased susceptibility to staphylococci in the treated mice, in fact they exhibited protection against dermonecrosis. This may mean that in the presence of antibodies to products of staphylococci, the increased susceptibility mediated by lymph node cells is not manifested.

So far the effect of reinfection with a homologous strain of Staphylococcus aureus has been discussed. Some degree of protection was also conferred against the infection with a heterologous strain of staphylococcus. The degree of protection

seemed to be related to the severity of the first lesion and repeated infections with the strains of staphylococci producing mild lesions gave better protection against reinfection with a 'mouse-virulent' strain than one infection only.

Type of blood-vessels affected during staphylococcal inflammation

Several investigators have reported on the type of blood vessels affected after thermal and chemical injury (Wells and Miles 1963, Hurley and Spector 1965, Spector, Walters and Willoughby 1965). However there is a lack of information about the type of blood-vessels involved during the staphylococcal inflammation. In this study, utilising colloidal carbon as a vascular marker it was shown that the leaking blood-vessels were mainly venules, although some capillary involvement was also seen. Majno et al (1961) showed that the vascular labelling was due to the accumulation of carbon particles between endothelial cells and their basement membrane. The carbon labelling of blood-vessels was first seen at half an hour after injection but the maximum labelling was 6 - 7 hours after inoculation which is the time of maximum permeability, judged by the measurement of oedema fluid. Similarly the first leakage of carbon from the blood vessels, which was seen at 1 hour in 'previously-infected' and 2 hours in 'non-immune' mice, coincides with the onset of leucocyte emigration. This technique was also found to be useful in demonstrating the extent of vascular labelling. For example

in 'previously-infected' mice a large number of blackened blood vessels were seen at 24 hours which was quite in contrast with the normals where the carbon labelling had almost disappeared by this time.

The effect of anti-inflammatory drugs and endotoxin on staphylococcal infection.

The inflammatory reaction of the host can be delayed not only by the presence of a foreign body, but also by treating the animals with anti-inflammatory drugs. Those anti-inflammatory drugs that reduced one or both components of staphylococcal inflammation e.g., colchicine, butasolidin, cortisone acetate and prednisolone, were found to enhance the severity of the lesions. However, betamethasone and dexamethasone, in spite of their anti-inflammatory activity, did not increase the lesion severity in the dosage used. Both these drugs were able to enhance the lesion severity in the 'previously-infected' mice. Even in normal mice the betamethasone and dexamethasone produced slightly bigger lesions than in controls but the lesions were less than double the size of controls. Drugs like quinine hydrochloride, chlorpromazine hydrochloride and indomethacin which did not alter the inflammatory response during the first 4 hours, failed to increase the susceptibility of the mice to staphylococci. However indomethacin was able to enhance the severity of lesions in 'previously-infected' mice.

The role of inflammatory cells as the major agent in the destruction of staphylococci is well known (Cohn 1962 a,b); the

role of the fluid part of the exudate in the response to staphylococcal infection is not well known. The results of treating mice with anti-inflammatory drugs suggest that the fluid part of the exudate also plays an important role. For example treatment of mice with butasolidin, which was found to reduce the fluid exudation without affecting leucocyte emigration, enhanced the severity of staphylococcal lesion. However it is not known whether butasolidin affects phagocytosis or eventual destruction of cocci inside the cell. That the 'infection-enhancing' effect of most of the anti-inflammatory drugs may well be due to its action on host defence mechanism at several levels could therefore be not discounted.

The action of corticosteroids in enhancing the staphylococcal infection was associated with decreased leucocyte emigration which presumably resulted in greater multiplication of cocci; however this could not be substantiated by the method of bacterial counting used in this study. Another complex aspect of cortisone action was that, with low dose of cocci, the lesions were milder in cortisone treated than in normal mice. This may be due to the stabilizing effect of cortisone on lysosomes (Weissmann and Thomas 1964) and perhaps in spite of the reduced inflammatory response the tissue defences were adequate to deal with the small numbers of cocci injected. As already suggested there may be some other mechanism reducing the host resistance besides the anti-inflammatory activity of these

drugs. Sodium salicylate treatment in the dose employed in this study neither reduced fluid exudation nor leucocyte emigration yet the severity of staphylococcal lesion was enhanced. It is not known what is the exact mechanism by which this drug enhances the staphylococcal infection.

In the mice having past experience with staphylococcal infection, the treatment with anti-inflammatory drugs was also able to enhance the severity of the lesion but the severity was much less than in the normal mice treated with these drugs. This reduction in the lesion severity was due to partial neutralisation of skin necrosis. The anti-inflammatory drugs were also found to be less effective in reducing the inflammatory response in 'previously-infected' mice.

The infection-enhancing activity of endotoxin has been attributed to a delay in the emigration of leucocytes (Miles and Hiven 1950, Conti et al 1961, Cohn 1962b). The findings of this work illustrated that endotoxin treatment reduced both the fluid exudation and the leucocyte emigration and allowed the staphylococci to produce big necrotic lesions. The large areas of necrosis seemed to be related to increased alpha haemolysin production presumably as a result of decreased destruction of cocci due to altered inflammatory response. That the large necrotic areas in the endotoxin-treated mice were not due to increased susceptibility of these mice to alpha haemolysin was shown by the fact that alpha haemolysin itself produced similar areas of necrosis in normal and endotoxin treated mice.

There are strong reasons to believe that besides the anti-inflammatory activity other actions of endotoxin may also be involved in enhancing the infection. Cohn and Morse (1960) have reported an altered phagocytic activity of polymorphonuclear leucocytes under the influence of endotoxin. There is also considerable evidence that the effects produced by endotoxin are mediated at least partly through epinephrine (Thomas 1959, Brunson 1964, Siltzer and Freedman 1965). This seemed to be true because in the present study it was found that administration of adrenergic blocking agent dibenylamine in endotoxin treated mice reduced the severity of staphylococcal lesion. However the enhancing activity of endotoxin was not completely abolished and the lesions were still twice as big as in the untreated mice. These observations will support the suggestion that other factors like the delay in the inflammatory response also play a part in the mode of action of endotoxin.

VIII CONCLUSIONS

The inflammatory reaction of the host immediately following the introduction of staphylococci seems to be one of the important determinants of the final outcome of infection. Staphylococcal infection is enhanced in the presence in the tissue of a foreign body like cotton dust which delayed the onset of both the fluid exudation and leucocyte emigration in response to staphylococci. The initial advantage enjoyed by invading cocci on account of the delay in inflammation was reflected in rapid growth of bacteria in the lesion and increased severity of the final lesion.

No single factor was recognized as responsible for virulence in staphylococci. The virulence of strains of Staphylococcus aureus for mice was found to be due partly to some component associated with the cells of staphylococci which delayed the onset of inflammation. For the initiation of staphylococcal infection, factor or factors causing a slight delay in the onset of inflammatory response were necessary but for the final lesion production, factors like alpha haemolysin were needed.

Repeated staphylococcal infection of mice led to immunity as well as hypersensitivity to reinfection. Immunity presumably due to serum antibodies to alpha haemolysin protected the mice against the dermonecrotic and leucotoxic effects of alpha haemolysin. The rapid emigration of leucocytes in the staphylococcal lesion of 'previously-infected' mice seemed also an immunological response

mediated by serum antibodies.

Hypersensitivity led to an accelerated inflammatory reaction in the sensitized mice to reinfection with staphylococci and this reactivity could be transferred to normal mice by the serum from 'previously-infected' animals. Sensitising antigens seemed to be alpha haemolysin as well as some component associated with the staphylococcal cells. An increased susceptibility to staphylococcal infection was found in the mice that had received living lymphoid cells from 'previously-infected' mice. The increased susceptibility to staphylococci in the treated mice was manifested only in the absence of serum antibodies.

The anti-inflammatory drugs that reduced either the exudation of fluid or the emigration of leucocytes during the first four hours of infection also enhanced the severity of staphylococcal lesion. The enhancement of staphylococcal infection by endotoxin was mediated partly by a delay in the inflammatory reaction and partly through epinephrine.

IX APPENDIXMedia

Broth: Oxoid nutrient broth

Serum agar: Oxoid blood agar base containing 5% horse serum.

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

Diluent: Oxoid $\frac{1}{4}$ strength Ringer's solution.

Formula

Sodium chloride	2.25 gms.
Potassium chloride	0.105 gms.
Calcium chloride	0.12 gms.
Sodium bicarbonate	0.05 gms.
Distilled water	1000 ml.

(pH 7.0)

Sterilised by autoclaving for 15 minutes at 15 lb. per square inch.

Depilating powder

Barium sulphide	7 parts
Fleur	7 parts
Talcum powder	7 parts
Soap powder	1 part

This powder was made into a paste with water.

Glycerine jelly:

Equal parts of glycerine and 10% gelatin solution were mixed. A few crystals of phenol were added to the mixture.

Ink-gelatine mixture:

Gelatine (5 gm.) was dissolved in 100 ml. of warm distilled water. To it was added 5 gm. of potassium iodide. This is fluid at room temperature. Pelikan ink (142 mg/ml.) was diluted tenfold with this gelatine mixture and was used for injection.

Staining procedures:Haematoxylin and Eosin

Deparaffinize with xylene, absolute alcohol.

Stain with Ehrlich's haematoxylin 2 - 3 minutes.

Blue in tap water.

Counter stain with 1% aqueous eosin 1 minute.

Wash with running water.

Blot, dehydrate in absolute alcohol.

Clear in xylene.

Mount in Canada balsam.

Toluidine blue

Deparaffinize with xylene, absolute alcohol and water.

Stain with 1% aqueous toluidine blue 1 - 2 minutes.

Wash in water

Dehydrate with absolute alcohol.

Clear in xylene.

Mount in Canada balsam.

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