STAPHYLOCOCCAL PENICILLINASE.

A study of some aspects of the production of staphylococcal penicillinase and its association with pathogenicity.

by

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It is the object of this thesis to show evidence of different degrees of activity in penicillinase production by various fractions of penicillin resistant staphylococcal populations, the staphylococci examined being isolated from human lesions and regarded clinically as pathogenic. Having established that this differential penicillinase production occurs, it will be shown that the degree of activity is reproducible in the absence of penicillin but that various changes take place in the presence of penicillin. The induction of penicillinase activity will be demonstrated, as will variations in penicillin resistance and the effect of antipenicillinase. Evidence of differing degrees of biochemical activity associated with the differences in penicillinase activity will be sought.

SECTION 1.

INTRODUCTORY DISCUSSION.

This section consists of a general discussion of some of the aspects of the problems and work dealt with in Sections II and III.

First is a review of some of the factors of staphylococcal resistance to penicillin, including some observations on penicillinase production, and on the effect of environment on resistance.

A short discussion of the significance of B.R.L.1241 follows, and finally a section is devoted to some of the more general problems in the estimation of pathogenicity of staphylococci.

GENERAL INTRODUCTION.

The interest prompting this work arose out of consideration of the serious problem of the increasing proportion of staphylococci, particularly those isolated in hospital communities, being found resistant to penicillin.

The marked and continuing increase in their numbers must tend to limit the usefulness of penicillin as an antibiotic, but their increase may be thought to have an even more important aspect.

The serious nature of many infections with the "hospital staphylococcus" is well enough established. Jessen et al (1959) have shown that antibiotic resistant staphylococci in bacteraemia were associated with a higher mortality than were sensitive strains and that the mortality for bacteraemia acquired in hospital was higher than for that acquired outside.

The penicillin resistant strains, may be distinguishable from the sensitive strains they appear to be replacing, only in their production of penicillinase. They may, however, differ in many ways from the sensitive strains, they may be more virulent and more pathogenic in the human host.

CHARACTERS of PENICILLINASE PRODUCTION.

The characteristic of penicillin resistant organisms which enables them to survive in an environment containing concentrations of penicillin, capable of destroying penicillin sensitive organisms is, of course, the power to produce penicillinase (Abraham & Chain, 1940).

Bondi and Dietz (1948) define the degree of natural resistance of staphylococci, as depending on the ability of the organism to produce the enzyme rapidly rather than upon its ability potentially to produce large amounts of it. If sufficient amount of it is produced by an organism to destroy the antibiotic present soon after contact, growth rather than inhibition takes place.

These authors found that individual staphylococcal cells have the same order of susceptibility to penicillin as a staphylococcus which does not produce penicillinase but that large inocula of these cells are less sensitive because of the more rapid multiplication resulting in greater production of the enzyme and the subsequent rapid destruction of penicillin. By using washed cells in their experiments they showed that this was not due to preformed penicillinase being carried over in the medium.

Their findings confirm those of Luria (1946) who showed that the protection afforded by penicillinase is a mass phenomenon occurring only when bacteria are inoculated in large numbers and considered that the presence of many individuals is probably needed to yield protective amounts of penicillinase.

Kirby (1945) also found that while penicillin resistant organisms were susceptible to the bacteriostatic action of penicillin, they overcame this by destroying the penicillin when present in sufficient numbers.

Barber (1947) shows that the degree of resistance of penicillinase producing strains varies as much as 800-fold according to the size of the inoculum used.

Barber & Roywadowska-Dowyenko (1948) consider that a large inoculum may have enough "ready made" penicillinase to grow in 1000 units/ml. of penicillin, whereas a small inoculum may be inhibited by less than 1 unit/ml.

Gilson and Parker (1948) are of the opinion that while the correlation between resistance and penicillinase content is quite definite when the test inoculum is large, when it is light the correlation disappears. They consider, in fact, that the "resistance" to penicillin exhibited by penicillin resistant cells is an artefact, introduced by the method of testing and expresses the ability of the cells to dispose of the penicillin in their environment.

Proom (1945) combining penicillinase, penicillin, and organisms in various amounts, showed that whether or not any particular combination shows growth depends on at least three factors - whether the penicillin is rapidly inactivated, whether the time taken for this is greater than the time taken by the penicillin to kill the inoculum, or whether the penicillinase becomes inactivated before it has time to destroy the penicillin.

A further point is raised by Housewright and Henry (1946).

By immunising rabbits they produced antipenicillinase and proved that this increased the sensitivity of organisms to penicillin where the organisms produced extracellular penicillinase. No effect was demonstrable, however, with organisms producing intracellular penicillinase only and the authors wonder how much penicillin resistance can be accounted for by intracellular penicillinase when present.

That the ability of the various cells in a staphylococcal population to produce penicillinase and to survive in an environment containing penicillin is not uniform through the population, has been indicated by several authors.

McCune, Dineen and Batten (1956) found in a population of a penicillinase producing "Giorgio" strain of staphylococci, accepted as resistant to 3.1 units/ml. of penicillin, that 90% of the population were unable to grow in 0.1 unit/ml. and occasional clones were able to grown in 10,000 units/ml. They confirmed this situation in experiments in mice where they found that penicillin caused a lowering in the bacterial census, and an increase in survival time after inoculation with the staphylococcus despite the fact that the concentrations of penicillin reached in the mice were well below the range of 3.1 units/ml. - due they considered to the fact that the infecting inoculum contained an admixture of highly susceptible cells as well as resistant ones. Rogers (1956) considers that the majority of staphylococcal strains are not homogeneous in their antimicrobial sensitivity and he suggests that the greater number of staphylococci in a microbial population may be affected by penicillin despite the insusceptibility of some micro-organisms. The suppression of most of the

staphylococcal population may be sufficient to tip the balance for the host in the management of certain infections. Some authors have published clinical findings of the apparently successful treatment with penicillin of infections with organisms apparently resistant to penicillin in vitro (Fisher, Wagner & Ross, 1955), which might seem confirmatory evidence of this.

Other variations which may occur in the level and distribution of penicillinase activity in the population are mentioned by Fairbrother, Parker & Eaton (1954), who showed that penicillinase producing strains of staphylococcus aureus can produce variants sensitive to penicillin although they accepted that penicillinase producing organisms cannot be produced in vitro from sensitive strains. Barber (1949) found that of penicillinase producing strains kept for long periods (5-12 months), approximately 50% yielded a proportion of penicillin sensitive colonies and some yielded nothing else. She points out that since a few penicillinase producing cocci may protect many penicillin sensitive cocci in the culture from the action of penicillin, testing the whole culture does not give an accurate picture of what is really happening.

It was felt in view of the evidence of variation, within a staphylococcal population, in penicillinase activity that some situation might exist, similar to the findings of Rogers (1953) on variant populations within a hyaluronidase producing culture of staphylococcus aureus. If a number of distinct fractions of population existed with

different degrees of activity as regards penicillinase production, these fractions producing most penicillinase might be considered to be the most likely, other factors being equal, to survive and multiply in an environment containing penicillin.

ENVIRONMENT and RESISTANCE.

Consideration must now be given to the ways in which environment may influence the charactersitics of an organism. Changes in character may be simplified as being due to adaptation, selection and spontaneous mutation.

Bryson (1956) discussing the genetics of antimicrobial resistance, suggests that mutations lead to differences in antibiotic sensitivity, either directly or by providing variations in the capacity for physiological adaptation through induced enzyme synthesis or otherwise, and that the establishment and maintenance of resistant strains is primarily a function of selection pressure. He suggests that the fact that from low concentrations of penicillin, the average resistant clone recovered has a correspondingly low resistance, while high concentrations of drugs commonly allow isolation of survivors of more extreme resistance, may be due to the organisms adjusting to its environment but going no further. This would certainly seem to be an argument against pure selection of ever present clones of high resistance, which while bound to form a greater proportion of a population reared in a high concentration of an antibiotic, should nevertheless be present even if in relatively diminishing proportion in lower concentrations.

Cavall-Sforza (1957) discussing the effect of environmental conditions bringing about individual adaptation, divided the adaptation into two types - post-adaptation in which a change was induced in drug resistance by the drug itself, and pre-adaptation, the selection of independently forming or pre-existing variants. He considered that a

population contained resistant individuals if single cells isolated from it, usually by plating, and allowed to grow into colonies in the absence of the selection medium, were found to be resistant.

It would seem if this is so, that if a penicillin sensitive, non penicillinase producing population of staphylococci could be found to contain a penicillinase producing clone, then the favouring and subsequent multiplication of that clone, could be an example of pre-adaptation. The situation which in fact exists in a population of a penicillinase producing staphylococcus in the presence of penicillin is surely a more complex one, in which both pre-adaptation and post-adaptation must be considered.

Dean & Hinshelwood (1957) while not denying that structural mutations leading to increased drug resistance, or improved utilization of nutrient sources, can and do occur, or the obvious consequences that mutants so arising would be rapidly selected in the appropriate environment, consider that this random mutation and selection is not the sole mechanism, or even the major mechanism for adaptation to new media, or for the development of drug resistance. They demonstrate the validity of this opinion by a number of experiments involving mass-number and time-number relationships based on adaptation to a change in nutrient source.

Barber (1957) discussing the group of naturally occurring penicillin resistant strains which she calls the "drug destructive" group, found that this type of resistance is comparatively stable, but that when sub-cultured in

vitro, penicillin destroying strains tend to yield an increasing proportion of variants, which have completely lost the capacity to produce penicillinase and are as sensitive to penicillin as the Oxford staphylococcus. She considers that staphylococcal penicillinase is not usually regarded as an adaptive enzyme but that the selection of a few penicillin resistant strains, and the dissemination of these is the main reason for the increasing incidence of penicillin resistant staphylococcal infection. She does, however, mention her experiments in which after a very long cultivation of penicillin sensitive strains of staphylococcus aureus in concentrations of penicillin of 0.005 - 0.01 units/ml. staphylococci with very weak penicillin destroying activity have been isolated - this activity, however, is still less than a hundredth of that of normal penicillinase producing strains even after one year. Barber considers, however, that with further prolonged exposure this activity might conceivably reach that of the clinically isolated strains. She points out that sub-bacteriostatic concentrations of penicillin in the tissues or abscesses of patients treated with penicillin must be very common and transfer of strains of staphylococci from patient to patient where this is the case is a method of passage and that possibly the penicillinase of staphylococci is an adaptive enzyme, for the production of which very prolonged exposure to the substrate is necessary. However, spontaneous mutation leading to the emergence of cells better able to produce the enzyme almost certainly takes

place and this, together with selection, may be the major factor in the production of penicillinase producing staphylococci.

In considering the nature of a penicillin containing environment to which a staphylococcus might be exposed, two different sets of conditions must be envisaged. First, those obtaining in the human body, and second, those obtaining in the environment in general, the air, walls, floors, bedclothes, etc. and in the human nasal passages.

The presence of sub-bacteriostatic concentrations of penicillin in all these circumstances must be admitted to be all too frequent. Gould (1958) has shown by air sampling that appreciable amounts of penicillin exist in the atmosphere of hospitals.

To consider the factors complicating the environment likely to be met with in the human host, the factors of host resistance, Bryson (1956) makes the point that evolutionary fitness in response to selection is reflected in the net reproductive and survival rate of entire genotypes, as tested against the total complex of environmental factors, and that a drug resistant cell may fail in competition with less resistant individuals in the same moderately inhibitory environment due to its being less resistant to other factors, perhaps the natural defences of the host. This might easily give a false picture of the actual rate of emergence of resistant strains in the host as these might be more readily destroyed by the host than are the less resistant parts of the population. In fact the

emergence of penicillin resistant strains during treatment is not commonly accepted.

Waisbren & Strelitzer (1959) tested the sensitivity of fourteen staphylococcal strains isolated from the blood of patients before and after the administration of antibiotics and found no change in resistance. In a further series of 152 staphylococci they found 53 gave the same sensitivity 57 were more resistant and 42 were more sensitive and concluded that the actual administration of an antibiotic to a patient does not greatly influence the antibiotic sensitivity of infecting staphylococci.

Hayes (1955) points out that the presence of a drug at the site of infection in adequate concentration is not a simple function of the dosage administered, particularly in the later stages of an infection when bacteria lying within barriers of pus, fibrin, necrotic or caseous material or granulation tissue may be exposed to a much lower concentration than that in the blood. Hayes considers that resistance of a multiple step type such as that of the staphylococcus to penicillin may arise during treatment to a level of from 2-16 fold, and advocates a sufficiently high concentration of the antibiotic to suppress this first step. It would seem reasonable that these first stage mutant bacteria, sheltered in the later stages of an infection as described, might resist the lowered concentrations of an antibiotic opposing them, but Hayes considers, in spite of this, that for practical purposes

the multiple step resistance of staphylococci to penicillin never arises during and as a result of treatment.

It would seem then that the present apparent dominance of penicillin resistant, penicillinase producing staphylococci, may be the outcome of the influence of all the factors mentioned - selection, spontaneous mutation and adaptation, but that the selection and mutation involved must be considered in the widest possible field of total environment. Minute concentrations of penicillin must be present not only in tissues and lesions of patients, but in all the numerous potential culture media in the host's own environment. Selection must be proceding in these conditions and the fraction of the total population most fitted to survive will be that most actively able to produce penicillinase or most adaptable to produce increased quantities of penicillinase by induction. Furthermore, any spontaneous mutants capable of increased penicillinase production or of increased inducibility must be even more readily selected.

B.R.L. 1241.

Since the completion of the experimental work of this thesis a considerable advance has been made in penicillin therapy. The development of 6 - amino penicillànic acid by Batchelor et al (1959) was followed by the announcement of the synthesis of sodium 6 - 2,6, dimethoxybenzamido penicillanate monohydrate, B.R.L. 1241 (Douthwaite & Trafford, 1960, Knox, 1960, Stewart, 1960, Rolinson et al 1960, and other authors), a penicillin to which all staphylococci, penicillinase producers or not are uniformly sensitive at a concentration of about 1.25 - 2.5 ug./ml.

A study of the reports of B.R.L. 1241 however makes it obvious that it will be a considerable time at least until the use of penicillin of the older types will cease. The high cost, the rapid excretion and lowering of the blood level requiring frequent dosage, the required intramuscular route in considerable volume, the high effective concentrations - 100 times that of Penicillin G. to inhibit the Oxford staphylococcus, are all limiting factors. Douthwaite and Trafford (1960) mention also that cases of hypersensitivity to penicillin are still sensitive to B.R.L. 1241; that injections are painful and that prolonged treatment has caused local reactions. The possibilities for further improvement on the basis of the original acid are, of course, immense but the possibility of nature's producing an answer perhaps in the form of an enzyme cannot be overlooked.

Despite this, one would in no way venture to minimise an achievment of the magnitude of this development. Some authors (Elek & Fleming, 1960) have gone so far as to saturate an entire environment, air, walls and bedding with the antibiotic for six months in a campaign to stamp out nasal carriage of resistant staphylococci. They considered after attempts to produce mutants resistant to B.R.L. 1241 had failed, that the emergence of such staphylococci was highly unlikely although they could not say it was impossible. They based this opinion on the molecule's being arranged in a manner not occurring in nature.

Barber (1960) and Novick (1960), however, sound a warning against over optimism in this respect and an editorial in the Lancet (1960) advocates reasonable caution and control of the use of the new antibiotic.

There is, it seems, no doubt that at present the older penicillins remain the drugs of choice in the treatment of many infections, so that their presence in the environment generally and in the human host, will be likely to continue in the foreseeable future.

ESTIMATION of PATHOGENICITY.

Once it is established that a staphylococcal strain is divisible into fractions of its total population which can be distinguished by some character one from another - in this case penicillinase activity, the next problem must be to see if these fractions are distinguishable also in other respects. For the purpose of this work these other characters have been confined to a few which have some recognition as having a bearing on or association with the pathogenicity of the organism, and a few which may be taken as indices of general enzyme activity.

The validity of the criteria of pathogenicity chosen will be discussed individually in the appropriate sections of the work, but some general observations appear here.

The problem is considerable and does not appear in the light of present knowledge, to be capable of any really satisfactory solution. There are, however, a considerable number of pointers and indications of virulence, many of which have received almost universal acceptance and it is some of these tests which were first considered. Any potential criterion of pathogenicity must be considered in the light of its apparent relation to some aspect of pathogenic action of the staphylococcus. The frequency of its association with staphylococci isolated from and associated with human lesions and the frequency with which such staphylococci exist not associated with it are also important. Further, to be of real value any such substance

or toxin must be capable of quantitative estimation by a reliable and not too specialised technique.

The following quantitative estimations of the in vitro production of toxins and enzymes were decided upon. Coagulase, alpha haemolysin, delta haemolysin, hyaluronidase, fibrinolysin, deoxyribonuclease and antigen-antibody line production. Qualitative tests for the presence of lipase and phosphatase were also carried out. Two techniques of mouse inoculation were used, intravenous through a tail vein and intramuscular into the posterior aspect of the thigh.

It is appreciated that certain other investigations with a bearing on the problem might have been done. The absence of such a test from those above does not imply that it is thought to be of no value. Certain tests such as leucocidin production (Gladstone & Van Heyningen, 1957) are very difficult technically and were not considered suitable for that reason.

It is difficult to know the true significance of the presence or absence in vitro of an enzyme or toxin. It is also difficult to devise conditions and techniques which will accurately and repeatedly give a quantitative estimation.

Elek (1959) points a warning against assuming the presence of a toxin to be significant when he says "Although there is every reason for regarding the virulence of an organism as being an expression of its biochemical activity, not all the products necessarily contribute to virulence.

The possibility of an accidental association of a character with virulence must always be borne in mind; such an association may be very constant". He mentions some further sources of error in technique, such as non-specific effects, removal by filtration, differences in the constitution of the medium and conditions of incubation, disappearance or destruction of toxins by lytic enzymes produced by the organisms, different times of harvesting and what he calls bacterial variation.

Blair (1952) points out a further basic difficulty in that staphylococci which elaborate no demonstrable exotoxin, may produce local lesions indistinguishable from those produced by strains which elaborate a potent toxin.

Smith & Dubos (1956a) consider that the power of staphylococci to cause disease in man and in experimental animals, is well known to exhibit a high degree of correlation with several in vitro characteristics of these bacteria - as examples they quote the production of coagulase, alpha toxin and pigment and the ability to ferment mannitol and to hydrolyse phenolphthalein phosphate. They feel, however, that there is doubt whether these characteristics have any causal relation to or are merely correlated with the property of virulence.

Lack & Wailling (1954) after a study of 435 strains found that although hyaluronidase, coagulase and alpha toxin production in a broth culture were remarkably constant under standard conditions, individual colonies showed marked variation in yield of enzyme - this they considered

was one of the greatest obstacles to the study of pathogenesis. They suggest that pathogenicity is more frequently associated with a broad spectrum of toxins rather than with a high production of any one toxin. The same authors point out further difficulties in the estimation of pathogenicity. They observe that strains which do not produce staphylokinase and others which do not produce alpha haemolysin during in vitro passage, have done so after mouse passage, and that conversely, strains stored for long periods in vitro lose their capacity to produce staphylokinase and alpha haemolysin before they lose their power to produce coagulase.

Christie, North & Parkin (1946) consider that the tendency shown by many strains to throw variants with fewer of the properties characteristic of pathogens than the parent strains, suggests that the fully pathogenic staphylococcus showing all these properties is the original organism, and that as one property after another is lost, the organism degenerates to the typical non pathogen with none of the special properties possessed by the typical pathogen. Obviously if this is so, there must be a danger of the loss of a character of virulence or of some degree of it at any time during its examination.

A further factor is considered by Dubos (1956) when he states "Even the recovery of a culture from a lesion may at times yield erroneous or at least incomplete information concerning the characteristics of the staphylococci which were initially responsible for the disease. It seems possible

for example, that the organisms that initiate the disease process may become profoundly altered in some of their characteristics during sequestration in an abscess. A culture directly recovered from an abscess may have lost temporarily some of the very properties that had first endowed it with invasive power.

The tests chosen for the estimation of pathogenicity in this work are tests which have a close apparent association with pathogenicity. If the association is merely an accidental relationship, then at least the increased or decreased production of toxins and enzymes so closely associated with pathogenicity must be of interest and if, as is possible, some or all of the products estimated have an actual causal relationship with pathogenicity, the findings will be the more valuable.

A number of workers have concerned themselves with possible changes in the biochemical and toxic activities of staphylococci in the course of the development of <u>in vitro</u> resistance to penicillin and other antibiotics. Spink, Ferris & Vivino (1944) found that an increased resistance to penicillin is accompanied by the development of strains more susceptible to the bactericidal action of whole blood and possibly to other defence mechanisms of the host. Krzywy, Stanecki & Fast (1958) are reviewed as having found little change in biochemical characters during the development of penicillin resistance, while Richou, Kourilsky & Chirol (1958) failed to produce change in haemolysin production in staphylococci held in prolonged

contact with penicillin.

North & Christie (1945) found no correlation in a large series of organisms tested, between resistance to penicillin and other biochemical properties.

In the case of naturally resistant penicillinase producing staphylococci, Barber (1947) found that it was impossible to differentiate between resistant and sensitive strains on grounds of morphology, cultural appearances, biochemical reactions or pathogenicity to rabbits.

Bondi & Dietz (1945) found that although there seemed to be relatively more coagulase producing strains among penicillinase producing staphylococci than among sensitive strains, they could show no general relationship and Howard (1954) found that phage pattern and sensitivity to penicillin show no relation with antigen line production, haemolysin pattern or virulence to mice.

Borchardt (1958), however, claims that his results in a study of staphylococci concerning phage typing, sensitivity pattern and virulence factors, show that there is some relationship between antibiotic resistance, phage typing and virulence.

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SECTION II.

PENICILLINASE PRODUCTION.

It is proposed in this section to examine twelve strains of staphylococci isolated from human lesions and regarded clinically as pathogenic. Clones from these strains will be examined for penicillinase activity and the distribution and stability of different degrees of activity shown. Some effects of penicillin and antipenicillinase on penicillinase production will be demonstrated. Descriptions of the methods used to estimate penicillinase production, to induce penicillinase production, and to produce antipenicillinase are included at the end of the section.

EXPERIMENT 1. PENICILLINASE PRODUCTION BY CLONES.

This experiment was designed to show that the penicillinase activity of a culture population is not homogeneous but varies from clone to clone.

Twelve staphylococcal strains isolated in pure culture from known human lesions were employed. None of the cases giving rise to these organisms had been treated with an antibiotic as far as could be ascertained.

The strains were grown in broth overnight at 37°C. aerobically, then plated out on nutrient agar, incubated at 37°C. overnight and examined for colonial variation. Twenty colonies were selected at random from each plate and sub-cultured in 20 c.c. 1% glucose broth overnight at 37°C. aerobically and each growth in broth tested for penicillinase production. A subculture of the original growth in broth was also tested as representing the total population of the strain.

The results are shown on Table 1.

The following points may be made -

(1) No significant colonial variation was remarked, e.g. the Rough:Smooth variation of Smith, Morrison & Lominski (1952) or the G. types of Wise (1956).

(2) The penicillinase activity showed a range of about four-fold from colony to colony.

(3) All colonies tested were found to produce some penicillinase.

(4) The penicillinase activity of the total population was similar to that of the most active penicillinase producers.

TABLE I

Penicillinase Production

STRAIN	Colonies chosen at random - arranged in order of activity																				Average	Total Population
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20		
A	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.3	0.4	0.4	0.45	0.5	0.5	0.6	0.6	0.6	0.75	0.8	0.9	1.0	0.48	0.9
B	0.4	0.4	0.45	0.45	0.5	0.6	0.65	0.7	0.8	0.9	1.0	1.0	1.25	1.25	1.3	1.3	1.4	1.4	1.4	1.5	0.93	1.1
C	0.2	0.2	0.2	0.2	0.3	0.3	0.3	0.4	0.4	0.4	0.5	0.5	0.55	0.6	0.6	0.7	0.8	0.8	0.85	0.85	0.48	0.8
D	0.4	0.4	0.45	0.45	0.45	0.5	0.5	0.5	0.5	0.6	0.6	0.6	0.6	0.65	0.7	0.7	0.7	0.7	0.75	0.75	0.55	0.6
E	0.2	0.2	0.2	0.2	0.25	0.25	0.3	0.3	0.3	0.4	0.4	0.5	0.55	0.6	0.8	0.9	0.9	1.0	1.0	1.0	0.51	0.9
F	0.3	0.35	0.35	0.4	0.4	0.4	0.5	0.6	0.6	0.7	0.75	0.75	0.8	0.85	0.85	0.95	1.0	1.1	1.2	1.2	0.7	1.2
G	0.5	0.5	0.5	0.6	0.6	0.6	0.65	0.65	0.75	0.75	0.8	0.8	0.85	0.9	0.9	1.0	1.0	1.05	1.1	1.2	0.78	1.1
H	0.5	0.5	0.6	0.6	0.7	0.75	0.9	1.0	1.1	1.1	1.15	1.3	1.4	1.5	1.55	1.6	1.7	1.75	1.8	1.8	1.17	1.6
J	0.2	0.25	0.3	0.35	0.35	0.35	0.4	0.4	0.4	0.4	0.4	0.4	0.45	0.5	0.55	0.6	0.6	0.6	0.65	0.65	0.44	0.6
K	0.85	0.9	0.9	1.0	1.1	1.2	1.25	1.35	1.45	1.45	1.5	1.55	1.6	1.7	1.7	1.75	1.85	1.9	2.0	2.0	1.45	1.8
L	0.3	0.35	0.35	0.35	0.4	0.4	0.4	0.45	0.45	0.5	0.5	0.5	0.5	0.55	0.65	0.7	0.7	0.8	0.8	0.8	0.52	0.7
M	0.4	0.4	0.45	0.45	0.45	0.5	0.55	0.55	0.55	0.6	0.6	0.6	0.6	0.7	0.75	0.75	0.8	0.9	0.95	1.3	0.64	0.8

Figures represent ml. of 0.005 N Iodine taken up per ml. of reaction mixture after 30 minutes.

EXPERIMENT 2. STABILITY OF CLONAL PENICILLINASE ACTIVITY.

This was devised to show as far as possible that the values obtained in the first experiment for the constitutive level of penicillinase activity of the various clones were stable and not due merely to temporary variation at the time of testing or to errors in technique.

The top, a middle and the lowest penicillinase producing clones are plated out on nutrient agar and six colonies from each sub-cultured in glucose broth and tested for penicillinase production as before.

The results are shown in Table 2.

It will be noted that the results with six colonies chosen at random from each plate are very similar to that from the original clone isolated. Their distribution as high, middle and low thirds is maintained and no overlap is seen in those clones tested such as Rogers (1953) found with hyaluronidase producing fractions although the range of activity (four-fold) with penicillinase production is much less than the 20-30 fold he discovered.

The results of experiments "1" and "2" may be summed up as follows. -

The constitutive level of penicillinase production in the absence of penicillin varies approximately four-fold, from colony to colony in the population of a strain. This is fairly constant and is not lost nor increased in the absence of penicillin, over a short period as demonstrated by one sub-culture, No claim is of course made,

that the level of activity remains constant permanently, but only that the results from each clone are reproducible after sub-culture.

[illegible]		Representative of Median Activity			Representative of Highest Activity		
Aver. of 6 Clones	Range	Orig. Clone	Aver. of 6 Clones	Range	Orig. Clone	Aver. of 6 Clones	Range
[illegible]	[illegible] -0.3	0.45	0.51	0.4 -0.6	1.0	1.19	0.85-1.2
0.42	[illegible] -0.5	0.9	0.88	0.8 -1.0	1.5	1.5	1.45-1.55
[illegible]	[illegible] -0.25	0.5	0.48	0.45-0.5	0.85	0.84	0.8 -0.9
[illegible]	[illegible]	0.5	0.51	0.5 -0.55	0.75	0.77	0.75-0.8
[illegible]	[illegible]	0.5	0.45	0.4 -0.5	1.0	1.06	1.0 -1.1
0.28	0.2 -0.3	0.7	0.68	0.6 -0.75	1.2	1.24	1.2 -1.3
0.47	0.45-0.5	0.8	0.86	0.8 -0.95	1.2	1.21	1.2 -1.25
0.51	0.5 -0.55	1.15	1.18	1.1 -1.25	1.8	1.81	1.75-1.85
0.2	[illegible]	0.45	0.42	0.4 -0.45	0.65	0.68	0.65-[illegible]
[illegible]	[illegible]-1.0	1.45	1.45	1.4 -1.5	2.0	2.05	2.0 -[illegible]
[illegible]	[illegible]	0.5	0.51	0.5 -0.55	0.8	0.82	0.8 -[illegible]
[illegible]	[illegible].5	0.6	0.57	0.5 -0.6	1.3	1.26	1.2 -[illegible]

[illegible] of 0.005 N Iodine taken up per ml. of reaction mixture [illegible]

TABLE 2

Penicillinase Activity of colonies subcultured from clones originally tested.

STRAIN	Representative of Lowest Activity			Representative of Medium Activity			Representative of Highest Activity		
	Orig. Clone	Aver. of 6 Clones	Range	Orig. Clone	Aver. of 6 Clones	Range	Orig. Clone	Aver. of 6 Clones	Range
A	0.25	0.26	0.2 -0.3	0.45	0.51	0.4 -0.6	1.0	1.19	0.85-1.2
B	0.4	0.42	0.4 -0.5	0.9	0.88	0.8 -1.0	1.5	1.5	1.45-1.55
C	0.2	0.21	0.2 -0.25	0.5	0.48	0.45-0.5	0.85	0.84	0.8 -0.9
D	0.4	0.4	- 0.4 -	0.5	0.51	0.5 -0.55	0.75	0.77	0.75-0.8
E	0.2	0.2	- 0.2 -	0.5	0.45	0.4 -0.5	1.0	1.06	1.0 -1.1
F	0.3	0.28	0.2 -0.3	0.7	0.68	0.6 -0.75	1.2	1.24	1.2 -1.3
G	0.5	0.47	0.45-0.5	0.8	0.86	0.8 -0.95	1.2	1.21	1.2 -1.25
H	0.5	0.51	0.5 -0.55	1.15	1.18	1.1 -1.25	1.8	1.81	1.75-1.85
J	0.2	0.2	- 0.2 -	0.45	0.42	0.4 -0.45	0.65	0.68	0.65-0.75
K	0.85	0.91	0.85-1.0	1.45	1.45	1.4 -1.5	2.0	2.05	2.0 -2.15
L	0.3	0.3	- 0.3 -	0.5	0.51	0.5 -0.55	0.8	0.82	0.8 -0.9
M	0.4	0.42	0.4 -0.5	0.6	0.57	0.5 -0.6	1.3	1.26	1.2 -1.3

The figures represent ml. of 0.005 N Iodine taken up per ml. of reaction mixture after 30 minutes.

EXPERIMENT 3. EFFECT OF PENICILLIN ON PENICILLINASE ACTIVITY.

This experiment was to show the effect of low concentrations of penicillin on the levels of penicillinase production found. Each original strain was plated on nutrient agar plates containing the following concentrations of crystalline penicillin G. 0.15, 0.3, 0.625, 1.25, 2.5 units/ml.

Growths of at least two colonies were obtained in ten strains on the agar containing 2.5 units/ml. of penicillin, and in the remaining two strains, on the agar containing 1.25 units/ml.

The colonies growing on the greatest concentration of agar were tested for penicillinase production and in six out of the twelve strains, chosen as giving a variety of activity, six colonies from the plates containing 0.625 and 0.15 units/ml. were similarly tested.

The results are shown in Table 3.

The results show that the penicillinase activity of the colonies growing on the highest concentration of penicillin was at least the equivalent of the activity of the most active clones isolated in Experiment "1" but was not much greater. The growth obtained on penicillin containing media bore some relation (although not an exact one) to the activity of penicillinase production.

The colonies tested from the lower penicillin concentrations seemed to vary rather as did the original clones in Experiment "1" and not to fall too exactly into strengths of

TABLE 3

Penicillinase activity of clones from penicillin containing media.

STRAIN	Growth to Units/Ml.	Aver. Penase of top Clones	Penase Production by 6 clones from media containing 0.625 units/ml. penicillin						Penase Production by 6 clones from media containing 0.15 units/ml. penicillin					
A	2.5	1.3	0.3	0.3	0.5	0.95	1.0	1.1	0.4	0.5	0.6	0.6	0.7	0.7
B	2.5	1.65	0.5	0.5	0.7	0.7	1.0	1.0	0.5	0.6	0.75	0.85	1.0	1.0
C	2.5	1.1	0.5	0.55	0.75	0.9	0.9	1.1	0.3	0.5	0.5	0.7	0.8	0.85
D	1.25	1.0	0.4	0.45	0.45	0.55	0.6	0.6	0.3	0.45	0.45	0.5	0.5	0.5
E	1.25	1.0	-	-	-	-	-	-	-	-	-	-	-	-
F	2.5	1.5	-	-	-	-	-	-	-	-	-	-	-	-
G	2.5	1.25	-	-	-	-	-	-	-	-	-	-	-	-
H	2.5	2.5	0.7	0.7	0.9	1.2	1.4	1.5	0.5	0.5	0.7	1.1	1.5	1.8
J	2.5	1.3	-	-	-	-	-	-	-	-	-	-	-	-
K	2.5	2.1	1.0	1.1	1.25	1.3	1.35	1.5	1.0	1.0	1.05	1.15	1.2	1.3
L	2.5	1.6												
M	2.5	1.25												

Figures represent ml. of 0.005 N Iodine taken up per ml. of reaction mixture after 30 minutes.

penicillinase production equivalent to the concentrations of penicillin present.

It would seem that at the low concentrations of penicillin employed in Experiment "3" very little, if any, induction of penicillinase takes place. This would confirm the findings of Bondi et al (1954) who considered that staphylococcal penicillinase was a constitutive enzyme and varied only within narrow limits with changes in composition of the growth medium. Nevertheless, the fact that a few colonies survived on the medium containing 2.5 units of penicillin and that these were uniformly of relatively high penicillinase activity as compared with the majority of the population, would argue at least that the differential constitutive level within the population, allows some selection of those colonies basically best fitted to survive.

Needless to say, a rare mutant, of even greater penicillinase producing capacity, would be even more selectively favoured. Demerec (1945) considers that penicillin, while not influencing the rate of mutation, acts as a selective agent by preventing the growth of the more sensitive cells. These findings are, however, not an argument against adaptation of the enzyme activity by induction. Geronimus and Cohen (1957a) and (1957b) have shown that penicillinase is an adaptive enzyme both in vivo and in vitro.

An experiment to show induction of penicillinase by a penicillin substrate was now carried out.

EXPERIMENT 4. INDUCTION OF PENICILLINASE.

A set of four clones from each strain was chosen to undergo the induction procedure. These were a clone representing the lowest third of penicillinase activity, the middle third, and the highest third (L.A., M.A., and H.A.). In addition, a representative was chosen of the clones from the highest concentration of penicillin containing medium to give growth (H.P.).

Penicillinase production of these four clones from each of the twelve strains was induced and the results are shown on Table 4.

The results of this experiment show that all the clones from one original strain are inducible to a similar level of penicillinase activity. This agrees generally with the findings of Geronimus and Cohen (1957b) who showed that staphylococci isolated from penicillin treated mice and of a penicillinase activity 8-10 times greater than that of staphylococci from untreated mice, were inducible only to the same level as those from the untreated animals. This experiment shows further that differences in constitutive level of fractions of the total population are not reflected in differences in inducible level.

This would lead one to believe that little difference might exist in the sensitivities to penicillin and in the proportions of the population of the fractions which could withstand different concentrations of penicillin.

It was decided to investigate the sensitivity to penicillin of the various fractions of the population previously distinguished.

TABLE 4

Penicillinase Induction

STRAIN	BEFORE INDUCTION				AFTER INDUCTION				% Variation Before Induction	% Variation After Induction
	L.A.	M.A.	H.A.	H.P.	L.A.	M.A.	H.A.	H.P.		
A	0.25	0.45	1.0	1.3	4.0	3.8	4.2	4.4	140	14.6
B	0.4	0.9	1.5	1.65	6.9	6.3	7.8	7.1	115	21.4
C	0.2	0.5	0.85	1.1	2.9	3.3	2.9	3.1	152	6.4
D	0.4	0.5	0.75	1.0	7.7	7.6	7.2	7.7	93	6.2
E	0.2	0.5	1.0	1.0	5.3	5.9	6.1	5.7	119	14.0
F	0.3	0.7	1.2	1.5	8.6	8.3	8.3	8.5	130	4.0
G	0.5	0.8	1.2	1.25	4.8	5.1	5.0	4.9	80	6.0
H	0.5	1.15	1.8	2.5	8.2	7.3	7.1	7.5	129	14.6
J	0.2	0.45	0.65	1.3	3.4	3.8	3.7	3.8	170	11.0
K	0.85	1.45	2.0	2.1	11.4	12.9	11.9	12.7	78	12.2
L	0.3	0.5	0.8	1.6	3.2	3.5	3.2	3.4	162	9.0
M	0.4	0.6	1.3	1.25	6.9	7.3	7.4	7.5	95	8.1

Figures represent ml. of 0.005 N Iodine taken up per ml. of reaction mixture after 30 minutes.

EXPERIMENT 5. RELATIONSHIP of PENICILLIN RESISTANCE to PENICILLINASE PRODUCTION.

Tube serial dilutions in broth were employed to estimate the sensitivity to penicillin of clones representing the lowest third and the highest third in penicillinase activity before the induction procedure, and the highest third after induction. A final range of from 500 units/ml. of penicillin by doubling dilutions to 0.03 units/ml. was employed and three weights of inoculum were used - a heavy inoculum of about 10^9 organisms, a light inoculum of 10^4 organisms, and a very light inoculum of 10^2 organisms (as estimated by Brown's opacity tubes). Washed cells were employed to avoid carrying over any preformed penicillinase in the broth, although in fact, very little penicillinase activity can be demonstrated in such supernatants (Luria, 1946).

The results are shown in Table 5.

The results show that with a heavy inoculum there is a distinct relation of resistance to penicillin, with penicillinase production as assessed between one strain and another. However, no variation can be found within a strain, whatever the constitutive or induced level of penicillinase activity of the fractions concerned.

In the case of the light inoculum a different picture emerges - variation between strains is not marked but variation within each strain follows a pattern of low resistance in the case of the fraction with the lowest constitutive penicillinase activity, and higher resistance in the case of the fractions with higher constitutive and

TABLE 5

Tube Sensitivity Using Different Inocula

STRAIN	Heavy inoculum 10^9 organisms			Light inoculum 10^4 organisms			Very light inoculum 10^2 organisms		
	LA	HA	HAI	LA	HA	HAI	LA	HA	HAI
A	31.25	31.25	31.25	0.25	0.5	0.5	<0.03	<0.03	<0.03
B	62.5	62.5	62.5	0.5	1.0	2.0	"	"	"
C	62.5	62.5	62.5	0.5	1.0	1.0	"	"	"
D	15.6	31.25	15.6	0.5	0.5	1.0	"	"	"
E	31.25	31.25	31.25	0.5	1.0	1.0	"	"	"
F	125	125	125	0.5	2.0	2.0	"	"	"
G	62.5	62.5	62.5	0.5	1.0	1.0	"	"	"
H	31.25	31.25	31.25	0.5	2.0	2.0	"	"	"
J	31.25	31.25	31.25	0.25	1.0	1.0	"	"	"
K	250	250	250	0.5	1.0	2.0	"	"	"
L	31.25	31.25	31.25	0.5	1.0	1.0	"	"	"
M	125	250	250	0.5	1.0	1.0	"	"	"

LA = Lowest activity (penicillinase)
HA = Highest activity (penicillinase)
HAI = Highest activity after induction (penicillinase)

Figures represent the lowest concentration of penicillin inhibiting growth expressed in units/ml.

induced activity. These differences are not extreme, but they are consistent throughout and suggest that, as in the case of the growths obtained in solid media, there is a selective factor involved in the circumstances prevailing when a low concentration of penicillin comes in contact with small numbers of organisms.

With a very light inoculum of 10^2 organisms, sensitivity to penicillin appeared to be uniformly of a level of less than 0.03 units/ml.

EXPERIMENT 6. EFFECT OF ANTIPENICILLINASE.

In view of the small quantities of the antipenicillinase serum available only three strains were tested. A. G. and K. were chosen as providing a good variety of results as assessed in previous sensitivity experiments. Sensitivities were tested as before over an appropriately limited range and dilutions of the antipenicillinase rabbit serum were added to each tube. Normal rabbit serum and the pre-inoculation serum from the inoculated rabbit were used as controls.

Volumes used were - Rabbit serum 0.25 ml., organisms in broth, 0.25 ml. and penicillin in broth, 0.5 ml.

In the case of the heavy inoculum, 10^9 organisms, where the effect, in comparison with the massive amounts of penicillinase being produced, must have been very slight, no change whatever was demonstrated. This was also so in the case of the very light inoculum, 10^2 organisms,where no penicillinase activity was manifested.

The results in the case of the inoculum of 10^4 organisms are shown in Table 6.

The results in table 6 show that the antipenicillinase rabbit serum has an effect in decreasing the resistance of the organisms tested. Their resistance at this level of inoculum may then be presumed to be due to some extent at least to their production of penicillinase and their differences of resistance may well be due to differences in penicillinase activity.

TABLE 6

EFFECT OF ANTIPENICILLINASE ON LIGHT INOCULA

STRAIN	Clone with Lowest Penicillinase Activity		Clone with Highest Penicillinase Activity		Clone with Highest Penicillinase Activity Induced	
	Normal Rabbit Serum	"1 Unit" Antipenase Serum	Normal Rabbit Serum	"1 Unit" Antipenase Serum	Normal Rabbit Serum	"1 Unit" Antipenase Serum
A	0.25	0.06	0.5	0.06	0.5	0.12
G	0.25	0.06	0.5	0.12	0.5	0.06
K	0.5	0.06	0.5	0.06	1.0	0.12

Figures represent the lowest concentration of penicillin inhibiting growth in the presence and absence of anti-penicillinase 1 unit/ml.

ANALYSIS of RESULTS.

When a strain of staphylococcus aureus is isolated from a lesion in the absence of penicillin, it appears to consist of a variety of clones giving different degrees of penicillinase activity on initial testing. These clones show an even distribution of activity. They do not fall into easily distinguishable separate fractions of the total population. The activity is stable at least temporarily in the absence of penicillin and there is a variation over a range of from 2-fold to 5-fold within different strains with a slightly greater preponderance of clones of low activity than high. The penicillinase production of the total population of the strain is relatively close, however, to that of the most active clones in most cases, occasionally as high but never higher in the series tested.

It is felt that a difference as great as five times in the basic constitutive level of penicillinase production might be significant in certain concentrations of penicillin such as are liable to be met with in the normal environment of the organism. The greater penicillinase producers would tend to be relatively selected by the more probable destruction of the initially less active clones.

A further most important factor in resistance to penicillin is the adaptation of the organism's penicillinase activity to the presence of penicillin in the substrate. This has been shown to occur in all fractions of all strains tested to levels of as much as 16 times the original activity.

All fractions of each of the strains tested were inducible to the same level whatever the original constitutive level of the fraction was, but there were considerable variations from strain to strain.

In the presence of low concentrations of penicillin in solid media a definite selection was shown of strains showing relatively high constitutive levels of activity with minimal degrees of induction apparent. The colonies growing on the highest concentrations of penicillin either have been selected from their less resistant fellows purely on their constitutive levels of penicillinase activity or they have been selected by their ability to answer the challenge of a small concentration of penicillin in the substrate by faster adaptation and induction of penicillinase activity even if only to a very small degree.

In the presence of high concentrations of penicillin, either in the course of the induction procedure or in fluid media, when in large numbers the staphylococcal cells are capable by adaptation of producing large quantities of penicillinase and resisting the presence of large amounts of penicillin, When present in smaller numbers, however, they are shown to behave more selectively and their resistance to penicillin depends again apparently more on their constitutive level of activity. In very small numbers they are completely sensitive.

The degree of resistance of the staphylococcal cell to penicillin at least at moderate levels has been shown to depend on penicillinase production by demonstrating the

effect on it of antipenicillinase.

It would seem then, that in a mass reaction staphylococci are capable in vitro of inducement of penicillinase activity in response to penicillin and that the weakest penicillinase producers within a strain are as capable of this as the strongest. In more individual circumstances such as obtain on a solid medium or perhaps in the natural human host, this mass phenomenon may not apply and individual clones may well be selected for their initial ability to produce penicillinase before induction can take place, rather than their ability to increase markedly their penicillinase activity in response to a penicillin containing environment.

If this is so, then the estimation of the relative pathogenicity of the fractions of the total population of the strain, capable of greater or less constitutive penicillinase activity must be of importance. Important also, must be any change in pathogenicity which results from or is coincidental with induction of penicillinase activity.

The next section of this thesis is concerned with these relative degrees of pathogenicity.

METHOD of ESTIMATION of PENICILLINASE ACTIVITY.

The method used is based on that of Tucker (1954). This was evolved from the description by Henry & Housewright (1947) of a manometric method for the assay of penicillinase, which was based on the formation of penicilloic acid from penicillin in the presence of this enzyme. Tucker found that a convenient way of following this reaction without the use of a manometric apparatus is to estimate the penicilloic acid iodometrically.

The uptake of iodine is not stoichiometric, 6-9 equivalents being absorbed per mol of penicilloic acid according to conditions, but the author found that with a standard procedure reproducible results were obtained and this claim has been confirmed. Perrett (1954), who advocates a similar iodometric method, considers that 8.3 atoms of iodine are bound per molecule of penicillin destroyed. He assumes also that the iodine absorbing substance formed is penicilloic acid. The uptake of iodine by the reaction mixture is measured over a short period - namely 30 minutes, as it is the power of the staphylococcus to produce penicillinase quickly in abundance that is its strength in resisting the action of penicillin. Perrett emphasises the importance of pH and temperature in the iodometric assay of penicillinase and shows that maximum destruction of penicillin takes place at pH 6.5 - 7.0 and temperature of about 40°C.

Washed cells are used throughout. Bondi et al (1954) consider that practically all the enzyme activity is found within the cells of the staphylococcus in contrast with the extracellular activity of the enzyme produced by B. cereus (Pollock 1950).

The staphylococcus is grown in 20 ml. of 1% glucose broth for 18-24 hours aerobically at 37°C. The cells are washed twice and then, after standardising against Browne's opacity tubes, resuspended in 9 ml. of 0.025 M. phosphate buffer at pH 7.0 in a universal container and shaken for 10 minutes with a mechanical shaker in a water bath at 37°C.

1.0 ml. of a solution of crystalline penicillin G. (M.W. 356.4 and assaying 1675 units/mg.) in a strength of 15,000 units/ml. in 0.025 M. phosphate buffer was added and shaking continued for 3 minutes, A 1.0 ml. amount was taken off 30 minutes after adding the penicillin, and immediately transferred to 5 ml. of 0.005 N. Iodine and titration carried out with 0.005 N. Thiosulphate, using 0.5% starch as indicator.

The uptake of iodine per ml. of reaction mixture after a period of 30 minutes was taken as a measure of the enzyme activity. A control tube containing penicillin 1,500 units/ml. without enzyme is included. When penicillinase activity is greater than is supported by the 1,500 units/ml. of the substrate, dilution of the enzyme suspension is made and the results corrected appropriately.

As this test depends on the activity of the number of

cells present in an overnight growth some preliminary tests were carried out to determine what variations in opacity of the growths existed as between strains and within strains. The results showed very small variations between strains and insignificant variation within strains.

MEDTHOD of INDUCTION of PENICILLINASE.

The method used was based on that of Geronimus and Cohen (1957a) who showed that it was possible to permit an appreciable increase in penicillinase activity by induction in a buffer containing amounts of a digest broth medium which allowed only very limited growth to take place.

The medium used in induction was based on this and was 10% of a 1% glucose digest broth containing 0.075% of a 0.25 M pH 7.0 phosphate buffer. Oxine 8.3×10^{-4} M.8 hydroxy-quinoline added to a reaction mixture after exposure to penicillin stopped further increase in activity promptly without altering the activity already present. If added to the cells before exposure to penicillin, it virtually eliminated any increase in penicillinase activity.

A culture of each organism is set up in 20 ml. of 1% glucose broth and incubated overnight at 37°C. The cells are washed three times and resuspended in 12 c.c. of 0.25 M. phosphate buffer at pH 7.0. The suspension is shaken in a water bath at 37°C. for 15 minutes and then 5 ml. are added to each of two 5 ml. quantities of 10%, 1% glucose broth in water and each is shaken at 37°C. in the water bath for 15 minutes. 0.1 ml. of Penicillin (10,000 units) is added to one bottle and the other receives 0.1 ml. of distilled water and acts as a control. Both bottles are shaken in the water bath at 37°C. for two hours. The cells are washed in 8.3×10^{-4} M. oxine and resuspended in 3.5 c.c. of oxine to stop further induction.

METHOD of PRODUCING ANTIPENICILLINASE.

Antipenicillinase (Perlstein & Liebmann, 1945a, 1945b) was used to try to investigate further the penicillinase production of staphylococci in a penicillin substrate, and the effect of the enzyme on the sensitivity of the organism. "Wellcome" commercial penicillinase inactivating 100,000 units of penicillin/ml. was obtained. This preparation is a sterile filtrate of a strain of Bacillus licheniformis containing 0.25% phenol. This was injected subcutaneously into a rabbit as described by Housewright & Henry (1946), in 0.5 ml. volumes at 4 day intervals for 8 weeks. Blood was withdrawn from the rabbit's ear before the first injection, and again a week after the last injection. Housewright & Henry found that normal undiluted rabbit serum had an inhibitory effect on their test organisms and so diluted serum was used both in tests and controls. The antipenicillinase serum was first assayed using the Oxford staphylococcus as test organism and showed a rather disappointing activity equivalent only to the concentration of penicillinase/ml. required to inactivate 4 units of penicillin. This was called 4 units of antipenicillinase.

The presence of this activity, however, did not mean that sufficient similarity existed between the commercial penicillinase and staphylococcal penicillinase for an antibody to one to affect the other. Manson, Pollock & Tridgell (1954) found that Bacillus subtilis penicillinase was significantly different from Bacillus cereus penicillinase

in the value of various factors and was quite distinct immunologically. Housewright & Henry (1946) however had found that antipenicillinase produced by the injection of penicillinase elaborated by a type of Bacillus cereus inactivated penicillinase produced by a type of Staphylococcus aureus and argued that the penicillinase from these two sources must be immunologically. similar.

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SECTION 111.

CRITERIA OF PATHOGENICITY.

INTRODUCTION.

In this section a number of tests, in vitro and in vivo, will be applied to some of the clones isolated in the previous section and shown to have different degrees of penicillinase activity within one strain. The object of this is first to show whether a fraction of the total population of a strain likely to be favoured in the presence of penicillin, is any more or less pathogenic than the remainder of the population, and second, to show whether after penicillinase activity has been induced to a much higher level than the original constitutive one, the cells are any more or less pathogenic than before.

Three fractions, therefore have been chosen from each of the twelve strains. They represent the lowest third in penicillinase activity, the highest third and the highest third after the induction procedure has taken place. (L.A., H.A., and H.A.I.)

No variation within these 36 population fractions is considered in this section. It is well appreciated that they themselves may consist of fractions of high and low activity as regards the manufacture of any toxin or enzyme but it is felt that they must be considered now, purely as total populations. It is the total effect either in vivo or in vitro of all of any fractions of the population produced by any given clone which is significant, the population produced should be considered as a whole.

The various criteria of pathogenicity are now considered separately in this section.

(1) COAGULASE.

(a) Value as a criterion of pathogenicity.

The value of the criterion of coagulase production as an indication of the pathogenicity is almost universally accepted, but must still be open to certain doubts.

Elek (1959) considers that coagulase production provides a line of cleavage between potentially pathogenic organisms and those of similar morphology but not possessing the power to produce disease. He considers that in this connection whether the property is of fundamental importance to the organism for its attack on the host is of secondary interest. He thinks, however, that coagulase may have a part in the initiation of the lesion or may protect the lesion already formed.

Numerous experiments have been carried out designed to show differences in the action of coagulase positive and coagulase negative staphylococci or differences in the action of the defence mechanism of the host upon the organism. Few of these experiments actually deal with the action of coagulase; most in fact, deal rather with associated differences which may or may not be due to coagulase production at all.

Smith & Dubos (1956a) investigated the belief that coagulase facilitates in some way the establishment in the host of staphylococci, either by rendering them more resistant to phagocytosis or by protecting them against the bactericidal effect of serum. They inoculated mice intravenously with staphylococci, both coagulase positive and negative, and followed this by viable counts at various times on various

organs. They considered that initially nearly all staphylococci, whether coagulase positive or negative, were rapidly killed. In fact, differences associated with coagulase production did not become apparent until a few days after infection when an increase in the numbers of staphylococci surviving after the initial phase began to be noted, particularly in animals inoculated with coagulase positive staphylococci. This seemed to show that no effect of coagulase in initiating a lesion takes place, but in fact the organisms used were incapable of coagulating mouse plasma so that the results may not be universally applicable.

Spink and Vivino (1942), however, say that while they do not wish to imply that coagulase per se is the factor responsible for the difference in the antibacterial action of blood against coagulase positive and coagulase negative strains, they have found that coagulase positive strains resist the bactericidal action of human defibrinated blood, whereas coagulase negative strains are killed in large numbers.

Ekstedt and Nungester (1955) confirm that coagulase positive strains of staphylococci in undiluted human serum grow well, while coagulase negative strains are inhibited, and they found no correlation of this differential growth with any of the other common metabolic activities of the organisms. They further found that extracts of culture filtrates from coagulase positive strains, rich in coagulase, when added to normal human serum, allowed the profuse growth of coagulase negative strains in it, while similar concentrates from coagulase negative strains had no effect.

Erstedt (1956) using 35 strains of staphylococci, confirmed the finding that the presence of coagulase could be correlated with increased growth in pooled human serum, but admits that the possibility must be recognised that the actual factor responsible for the growth of the staphylococcus in serum is not coagulase, but a substance associated with it.

The suggestion that coagulase protects an already formed lesion by forming a fibrin barrier around it is dismissed by Elek (1959). He points out that such a barrier is found in inflammation caused by other organisms and even by croton oil and clearly cannot be caused by coagulase but must follow tissue damage. Also, he points out that the injection of crude coagulase does not lead to the formation of a fibrin barrier (although a toxic filtrate of staphylococci will do so).

Hale and Smith (1945) found that fowls, rats and mice whose blood contains no accessory factor required for the coagulation of plasma by staphylocoagulase are particularly resistant to experimental infection. They showed that the phagocytosis of coagulase positive staphylococci is inhibited in the presence of coagulable plasma, that the initial protection afforded to the organism is specific and is not dependent upon a physical alteration of the medium, for the phagocytosis of other organisms present at the same time is unaffected.

They found, also, that the addition of a little coagulase activator to the mixture will inhibit the phagocytosis of staphylococci in the plasmas of certain species not normally

coagulable. They also considered that the agglutination of both organisms and leucocytes which resulted from the coagulase activity conferred a further protection on the staphylococci.

Hale and Smith summed up the defensive role of coagulase as conferring upon the organism a resistance against the hosts first line of defence, thus enabling it to elaborate its toxic products.

Smith, Hale and Smith (1947) using intratesticular injections of staphylococci in guinea pigs showed that the degree of testicular involvement found was greater with strains capable of clotting guinea pig plasma. They found by giving mice intraperitoneal and intravenous injections of staphylococci with various plasmas and sera, that there was greater mortality with human than with guinea pig plasma and greater mortality with plasma than with serum. They consider that the initial infectivity of staphylococci is largely dependent upon their power of coagulase production - so that without this power, the other virulence factors are probably of little avail. At later stages it may still aid the further multiplication of the organism in defiance of the ensuing phagocyte response and may determine the outcome when infected emboli are transported to other sites.

On the other side, however, Rogers and Tompsett (1952) point the danger of presuming that because of close and consistent association with pathogenicity coagulase can be presumed in itself to be a causal factor. They have shown that another factor very closely associated with coagulase

is important in the early stages of infection. This factor causes the survival of the organisms after phagocytosis has taken place and is associated with evidence of destruction of the leucocytes.

Tager and Hale (1947) showed that there were many more strains of staphylococci with a high titre of coagulase among cultures obtained from the body surfaces than among those from inflammatory lesions, which does not point to an association of coagulase production and pathogenicity.

Lack (1956), who reviews a considerable number of factors associated with pathogenicity, says that he doubts whether coagulase is an important factor in pathogenicity, but he suspects that the association of coagulase production with greater resistance to neutrophil lysozyme is important.

It would seem reasonable to assume that if the actual ability of coagulase to clot plasma is a determinant of virulence, that evidence of its clotting plasma in vivo should be available.

Smith and Dubos (1956a) stated that coagulase had not yet been shown to be active in vivo even in those animal species whose blood it can coagulate in vitro. They considered that no convincing evidence had come to light of a mechanism whereby coagulase could affect causally the fate of staphylococci in the tissues. They regarded coagulase as an index rather than as a determinant of virulence.

Various authors have published accounts of lack of success in producing actual in vivo clotting by coagulase. Fisher (1936a) was unable to produce intravascular clotting

either clinically, grossly or microscopically in rabbits injected with massive doses of potent cultures and filtrates.

Menkin and Walston (1935) were unable to produce lymphatic blockage in rabbits by the intracutaneous injection of active cell free coagulase.

However, Tager (1954) found that the injection of rabbits with very large doses of coagulase occasionally led to a fall in the blood fibrinogen and the death of the animal.

Other evidence is given by Lominski (1949) who found that rabbits could be protected from fatal staphylococcal infection by a substance inhibitory to a staphylocoagulase. He found this inhibitory substance (Lominski and Roberts, 1946) was present in significantly greater incidence in a control population than in a population suffering from staphylococcal infection.

Boake (1956) found that he could actively immunise rabbits with coagulase and that this made them more resistant to intravenous challenge with coagulase positive, but not coagulase negative staphylococci. In a further experiment he showed that when mice were injected intraperitoneally with coagulase positive staphylococci suspended in a clotting system, that serum from rabbits immunised with coagulase diminished the virulence of these staphylococci, provided it was allowed to come in contact with them for a period before the addition of the clotting system.

Finally, Smith and Johnstone (1956) succeeded in demonstrating in vivo coagulation. They injected purified

coagulase of high activity intravenously into rabbits. The preparation which was free from demonstrable alpha toxin killed the animals apparently by blockage of the pulmonary vasculature with extensive fibrin deposits in the capillaries and small arteries. This was also found in the liver, kidneys and adrenals.

The evidence concerning staphylocoagulase as a determining factor in the pathogenicity of the staphylococcus is therefore, not a little contradictory. However, there seems little doubt that coagulase, if not in itself an outstandingly important factor, is generally accepted as being so closely associated with virulence for its presence to be acceptable as a criterion of it. Whether or not it can be accepted quantitatively as an estimation of the degree of pathogenicity of an organism is, of course, another matter. Doubts must be considerable in view of its uncertain causal relationship with pathogenicity and in view of the doubtful validity of quantitative estimation of coagulase production - this will be discussed in the next sub-section.

(b) Method of estimation.

In deciding what method to use to estimate coagulase production, consideration had to be given to the very numerous factors involved and the many technical difficulties of such an estimation. Lack and Wailling (1954) say that even a rough quantitative estimation of coagulase is beset with difficulties. All organisms were first tested for the bound coagulase of Duthie (1954a) by the method of Cadness-Graves et al (1943) and were then tested by the method of Fisk (1940)

for free coagulase, both purely qualitative tests. These tests are those recommended by Williams and Harper (1946) after consideration of eleven methods of estimation of free coagulase and two methods of estimation of bound coagulase.

A quantitative estimation of coagulase however, presented further problems. It was decided that the antigenically distinct "bound coagulase", thought by Duthie to be bound to the cell wall and to act directly on the fibrinogen of certain animals causing clumping of the staphylococcal cells, would not be considered. No satisfactory quantitative test has been devised and free coagulase is much more generally accepted as being associated with pathogenicity.

Smith and Hale (1944) consider that while free coagulase is inert as far as the conversion of fibrinogen to fibrin is concerned, it is, however, the precursor of a thrombin like substance which can convert the fibrinogens af all species they tested into fibrin clot. They found that the formation of the thrombin like substance requires the presence of an activator, which is present in adequate quantity in some plasmas, but deficient or completely lacking in others. They consider the reaction analogous to normal thrombin formation from prothrombin by the agency of thrombokinase except that calcium is not required. Rubinstein (1958) concluded that prothrombin was identical to the coagulase reacting factor and that the thrombin-like substance produced attacked fibrinogen at some site other than that attacked in normal blood clotting to produce a material somewhat different from ordinary fibrin.

Another factor was first introduced by Lominski and Roberts (1946) who found that some plasmas when undiluted failed to clot in the presence of coagulase even though they clotted well when diluted and concluded that this was due to the presence of an inhibitory factor. They found that this inhibitory substance was present in 212 out of 348 sera, while Tager (1956) found more than 50% of human plasmas inhibitory. Further sources of difficulty are mentioned by Smith, Morrison and Lominski (1952) who found that unstable variants could arise, some the "R" variants being fast clotting, high titre and some "S" variants, slow clotting and low titre. They considered that changes in coagulase production observed in some of their strains could be related to the proportions of R. and S. variants present at the time of testing. Elek (1959) mentions the possible destruction of coagulase either by proteinases of the staphylococcus itself or those present in animal plasma. Lominski, Smith and Morrison (1953) found that smooth variants produced a coagulase destroying factor (C.D.F.) and that supernatants of slow clotting on non-clotting growths generally contained this factor. They assumed it to be a proteolytic enzyme and thought that it too might help to explain irregularities of coagulase production. Lominski, Morrison and Smith (1955) carried this work further when they showed that factors obtained from certain "S" variants can both increase the potency of, and destroy the coagulese produced by "R" variants of the organism.

Other factors still must be taken into account, such as the presence in test plasma of antibodies to coagulase, or deterioration in the accessory factor after refrigeration for a long period (Elek 1959). The presence of $C.O._2$ in the incubation of the culture (Gillespie, Devenish and Cowan, 1939) and even the size of the tubes used may alter the production of coagulase.

The optimum length of incubation of a culture before estimation of coagulase is made is doubtful. The various opinions of its role in vivo make it difficult to decide this on clinical grounds. Duthie (1954b) considers that free staphylocoagulase is formed early in the lag phase and is released continuously mainly at the first division in heavily seeded cultures. He also advocates the addition of serum albumen to the medium to enhance the production of both free and bound coagulase. Rogers (1954) considers that coagulase appears after inoculation without lag additional to that in growth but increases at a slower rate than growth until it ceases to increase before the growth stops.

The method then requires, of course, standardised conditions of culture to avoid variations in coagulase production, it requires a suitable plasma to avoid absence of accessory factor and the presence of inhibitory factor. By using a fluid culture representing the whole population of the strain the selection of variants will be minimised. It may be assumed that the coagulase production measured this way is the outcome of the factors enhancing and potentiating it and the factors inhibiting and destroying it.

This would surely resemble, apart from the effect of defensive mechanisms of the host, the coagulase production in vivo. If the coagulase production estimated is proportional to the percentage of high producing variants over low producing variants then this can still be acceptable as an index of the coagulase production of the culture as a whole.

False results owing to destruction of fibrin by fibrinolysin or proteinases can be excluded by a strict regime of observation of the tubes in the test. The methods actually used are based on those of Smith, Morrison and Lominski (1952).

10 ml. of broth in 1 oz. bottles were inoculated and incubated at 37°C. for four days.

Two methods were employed. -

(1) Clotting time measurement. 0.5 ml. of culture was added to 0.5 ml. of a 1/5 dilution of human plasma in 0.85% saline in a 4 x ½" test tube. The tube was gently rocked through 45° from the vertical and the time taken for a clot to form was noted. Times were measured at every 10 minutes unless one tube of a group had formed a clot, in which case more frequent measurements were made.

(2) Titration. To 0.5 ml. of serial doubling dilutions of the culture in 0.85% saline was added 0.5 ml. of 1/5 dilution of human plasma in merthiolated saline - the final concentration of merthiolate being 1 : 1000. The highest dilution of the cultures to show clot after 24 hours at 37°C. was recorded as the coagulase titre.

The chosen three fractions of the population of each strain were tested for coagulase production as described. The results are recorded in Table 7 and will be discussed at the end of the section.

TABLE 7

COAGULASE ACTIVITY						
STRAIN	Coagulase activity assessed by clotting time. ACTIVITY = 100/t in minutes			Coagulase titre.		
	LA	HA	HAI	LA	HA	HAI
A	2.0	2.2	2.0	8	8	8
B	2.5	2.5	2.5	8	8	4
C	0.74	0.74	0.71	32	32	32
D	2.0	1.66	2.0	16	16	16
E	0.83	0.77	0.77	8	8	8
F	1.66	1.66	1.33	4	4	4
G	5.0	4.0	4.0	16	16	16
H	1.25	1.66	1.4	8	8	4
J	0.74	0.74	0.74	4	4	4
K	0.74	0.71	0.74	64	128	128
L	1.4	1.66	1.66	16	8	16
M	0.69	0.74	0.69	2	4	4

Figures for coagulase activity as assessed by time represent activity = 100/time in minutes.

Figures for coagulase titre are the reciprocal of the greatest dilution of the enzyme sample giving coagulation in 24 hrs.

(2) HAEMOLYSINS.

(a) Value as a criterion of pathogenicity.

In considering the pathogenicity of staphylococci, it would be impossible to ignore the group of haemolytic exotoxins as being likely to be implicated in establishing the virulence of the organism. Consideration was given to four haemolysins - alpha, beta, gamma and delta. Elek and Levy (1954) point out that there is great variation in actual lysin production. They consider that what may be termed the natural state of coagulase positive staphylococci is that in which each cell possesses the ability to produce all the haemolysins. Any other pattern observed represents a mutational loss of character, and forward and backward mutations in temporary equilibrium account for the pattern observed in a given strain. The haemolytic pattern found in such a strain depends on its mutational state, in vivo and in vitro.

1. ALPHA HAEMOLYSIN, (Glenny and Stevens, 1935) has long been recognised as being associated with pathogenicity. This was mentioned by Cruickshank (1937) who observed that this exotoxin is produced by staphylococci that are pathogenic to man, while McFarlan (1938) also referred to the connection between Alpha haemolysin, coagulase production, and pathogenicity. More recently Elek and Levy (1950b) found that alpha and delta lysins were frequently associated with strains of coagulase positive staphylococci from human lesions.

Christie, North and Parkin (1946) in an investigation into 1,027 strains of staphylococci from human, animal and other sources, found a definite relationship between the elaboration of alpha toxin and mouse pathogenicity. In fact, so impressed were they with the value of alpha haemolysin production as an indication of virulence that they considered further tests necessary only where the degree of haemolysis is less than that given by the usual pathogenic strains. Another author who rates very highly the value of this criterion is Marks (1952) who states that since all alpha toxigenic strains are coagulase positive, the coagulase test is superfluous, pathogenicity being adequately determined by alpha toxin production. Marks considers that the production of alpha toxin is a more accurate and convenient criterion of the pathogenicity of staphylococci than is the coagulase test. Selbie and Simon (1952), in an article relating virulence to mice with certain in vitro properties of the staphylococcus came to the conclusion that the most important of these in vitro properties in this respect was alpha haemolysin production. Dobias, Ballo and Kemenyvari (1959) describe a mthod of demonstrating alpha toxin in pus and conclude that there is a relation between the titre of the toxin in the pus and the clinical severity of the disease in empyema patients. They consider that the determination of this toxin in pus can be used as a method of rapid diagnosis. Authors who stress the importance of alpha haemolysin production include Gillespie, Devenish and Cowan as long ago as 1939, who found complete correlation with coagulase

activity, and Brown as recently as 1960, who suggests that the virulence of a staphylococcal strain can be most conveniently estimated by its alpha haemolysin production.

Several other authors while agreeing on the association of alpha haemolysin production with pathogenicity, do not regard the relationship as so absolute or definite. Jackson, Dowling and Lepper (1955) in a comparison of alpha haemolysin production with the coagulase test and clinical observations of virulence, agreed that alpha toxigenicity occurs principally among coagulase positive strains but considered that the quantities of coagulase and alpha haemolysin produced by the strain need have no correlation and found, in fact, that only 82% of the coagulase positive staphylococci produced alpha haemolysin at all. Gillespie and Simpson (1948) found that of 168 coagulase positive staphylococci only 162 were alpha toxigenic but agreed on the general close relation with the coagulase production and the clinical pathogenicity of the strains examined. Jackson, Dowling and Lepper concluded that the determination of alpha haemolysin titre is a useful adjunct to the coagulase test in identifying pathogenic strains in vitro. They found a greater proportion of strains produced alpha toxin, the lower in the respiratory tract was the source of the organisms, but Tager and Hale (1947) found no significant difference in alpha haemolysin titre in 242 strains from different sources. Williams and Harper (1947) found that 93% of their coagulase positive strains were alpha toxigenic and Schwabacher et al (1945) found 91% of her coagulase positive strains produced alpha

Elek and Levy (1950) found 4% of their series appeared non alpha toxigenic but found that on sub-culture alpha toxin is produced in a large population. Elek (1959) points out that the confirmation of the existence of pathogenic strains which fail to produce alpha lysin would invalidate the assumption that alpha lysin is a sine qua non of pathogenicity, but that these findings refer to estimations from single colonies. He has found that when a strain producing all three haemolysins (alpha, beta and delta) is examined, it is evident that a continuous to and fro mutation exists and the findings in fluid cultures represent the products of the various clones.

Elek considers that the frequency with which the lysin is produced by strains obtained from lesions is sufficiently high to be of some practical value, although its use as an indicator of virulence is neither as convenient nor as reliable as coagulase testing.

The actual action of alpha haemolysin in a human lesion is not clear but Brown, Prichard and Quillam (1959) observed a slowly developing contracture of isolated preparations of rabbit jejunum and guinea pig ileum and this was prevented by alpha antitoxin. Many workers believe that alpha haemolysin is identical with the lethal toxin and the dermonecrotoxin.

2. BETA HAEMOLYSIN - appears to be associated quite markedly with strains of coagulase positive staphylococci obtained from animal sources. Elek (1959) found that from 59 pathogenic staphylococci isolated from animal sources,

88% produced beta haemolysin, either alone or in combination with another haemolysin, whereas of 200 coagulase positive strains of human origin only 11% produced beta haemolysin and all of these also produced alpha and delta lysins. It was therefore felt that little value, as regards the estimation of pathogenicity of an organism in human infection, could be placed on this test. In fact, a qualitative test for the production of this lysin on sheep blood agar was made in the course of testing for the presence of other apparently more vital lysins. None of the strains tested appeared to produce any beta lysin. It is interesting to note that all but one produced some fibrinolysin. Christie and Wilson (1941) and Rountree (1947) found these two products to be mutually exclusive although Rountree could show no inhibitory effects. Lack and Wailling (1954), however, found that 8.2% of their strains produced both beta lysin and fibrinolysin.

3. GAMMA HAEMOLYSIN is defined by Jackson, Dowling and Lepper (1955) as being similar to alpha lysin but immunologically distinct. No attempt was made to test separately for production of this lysin.

4. DELTA HAEMOLYSIN (Williams and Harper, 1947) was confirmed by Marks and Vaughan (1950). Jackson and Little (1956) are quoted by Elek (1959) as considering it to be identical with the leucocidin acting on human leucocytes. These authors, (Jackson and Little 1957) found the association of delta lysin with human pathogenicity at least as close as that of alpha lysin. Elek and Levy (1950b) considered

that along with alpha lysin, delta lysin production was frequently associated with coagulase positive human strains. Gladstone and Van Heyningen (1957) enumerated three leucocidins, that of Neisser and Wechsberg (1901), that of Panton and Valentine (1932) and a third leucocidin which has a lytic action unlike that of the P.V. leucocidin. This the authors have called a "leucolysin" and they consider it identical with delta lysin. They found this leucolysin produced by large numbers of coagulase positive staphylococci and by some coagulase negative strains. This last finding, if their contention that the leucolysin is delta lysin is correct, disagrees with Elek and Levy (1950b) who found that no coagulase negative strains produced delta lysin.

Various authors give different figures in their series of strains examined, for delta lysin, and arising out of the difference in their figures, tend to different views of its correlation with pathogenicity. Lack and Wailling (1954) found that 50% of their strains from cases of osteomyelitis produced delta lysin and did not consider that the lysin was one of the staphylococcal products most closely associated with pathogenicity.

Elek (1959) found in a series of 359 strains that 97% of coagulase positive human pathogens produced delta lysin as against 96% producing alpha lysin.

It was decided therefore to estimate the alpha and delta lysin production of the various organisms.

METHODS and RESULTS.

1. Alpha Haemolysin.

The method chosen was a tube titration although it is appreciated that this has disadvantages as compared with a plate method. Elek and Levy (1954) consider the plate method a more sensitive indicator, allowing a haemolysin masked at one point by the action of another, to diffuse out further and reveal its presence. In the tube all cells are simultaneously subjected to the action of whatever combination of haemolysins is present and serial dilution will still result in the same relative proportions acting together on the substrate. Further disadvantages are due to interactions between haemolysins, and bacterial variations. They consider that quantitative measurements are unreliable.

In spite of these arguments it is felt that the tube test offers the only possible satisfactory quantitative estimation of haemolysin production although the plate method may offer a better overall picture of qualitative haemolysin production. Whether the haemolysis and differentiation shown on the plate give a better correlation with a total effect of the haemolysin production in vivo or not, is open to doubt. It might be argued that if any interaction between two or more haemolysins is going to take place, it would be worthwhile to measure in some way the consequent haemolytic effect. As the tests envisaged using rabbit and sheep cells are in any case entirely artificial as regards the human host, it is felt that the tube test is as reasonable and

acceptable a criterion as any. It has further advantages in this series. It gives a clear and unequivocal end point of haemolysis with serial dilutions of a supernatant. It will not be affected by beta haemolysin as this does not appear to be produced by any of the strains, nor will it be affected be delta lysin if grown in broth as this is not produced in fluid cultures (Williams and Harper, 1947, Marks and Vaughan, 1950). The method is based on that of Howard (1954). Cultures are made in 20 ml. of digest broth, incubated in 30% CO_2 and 70% air for 48 hours at 37°C. After centrifugation at 3,000 rev./min. for 30 minutes the supernatant is set up in doubling dilutions with 0.85% saline plus equal volumes 2.5% rabbit red cells in 0.85% saline - total volume of 1 ml./tube. "Burroughs Wellcome" staphylococcal antitoxin was used as a control, 0.02 ml. in the lowest dilution tube.

The tubes are incubated for 1 hour in a 37°C. water bath. The reciprocal of the highest dilution showing at least 50% haemolysis is taken as the haemolytic titre.

The results are as shown in Table 8.

TABLE 8

HAEMOLYSIN ACTIVITY						
STRAIN	Alpha-haemolysin titre			Delta-haemolysin titre		
	LA	HA	HAI	LA	HA	HAI
A	64	128	64	4	4	4
B	256	256	512	16	16	16
C	64	64	64	4	4	4
D	32	32	64	-	-	-
E	16	16	16	-	-	-
F	128	64	128	8	4	8
G	64	64	64	-	-	-
H	128	128	128	16	16	16
J	128	128	128	32	32	32
K	256	256	256	16	16	16
L	32	16	16	-	-	-
M	512	512	512	32	16	16

Figures represent the reciprocal of the highest dilution showing at least 50% haemolysis.

2. Delta Haemolysin.

The method used to estimate delta lysin was based on that evolved by Marks and Vaughan (1950). Sheets of cellophane cut to fit petri dishes were sterilised in the autoclave with a little water to keep them moist. The sheets were placed aseptically on the surface of freshly poured undried nutrient agar plates and then inoculated with three drops of a broth culture distributed over the whole surface with a spreader. The plates were incubated at 37°C. for 48 hours in an atmosphere of 30% $C.O._2$ in air. The organisms were then washed off the cellophane with 2 ml. of sterile normal saline. The suspension of organisms was allowed to stand at 4°C. overnight after which they were standardised against Brown's tubes, the cells removed by centrifugation and doubling dilutions of the supernatants obtained were carried out in 1 ml. volumes of normal saline. To these was added 0.25 ml. of 2.0% suspension of thrice washed horse cells. Readings were made after 1 hour incubation in a water bath at 37°C. The reciprocal of the highest dilution showing at least 50% haemolysis is taken as the haemolytic titre.

The results are shown in Table 8.

FIBRINOLYSIN.

The action of fibrinolysin (Fisher 1936b) in promoting infection by a staphylococcus is not clear. By its nature it would appear possible that it could allow the spread of a staphylococcal lesion once established, by destroying the fibrin barrier produced around it. Whether or not this fibrin barrier protects the stáphylococcus from the host, more than it protects the host from the staphylococcus is a matter of conjecture.

Blair (1952) mentions that it has been suggested that in cases of staphylococcal phlebitis, fragments of infected thrombus may be liberated into the circulation by fibrinolysin.

Christie and Wilson (1941) found that 92 out of 99 coagulase positive human strains produced fibrinolysin, whereas only 4 out of 43 coagulase positive animal strains did so. None out of 42 human and only 1 out of 14 animal, coagulase negative strains did so. They noted a relationship between the production of beta toxin (largely by animal strains) and the absence of fibrinolysin. They used a method of spot inoculation of plasma agar plates to demonstrate fibrinolysis.

Rountree (1947) also found the relationship between beta haemolysin and fibrinolysin, but noted that the presence of beta toxin produced in mixed inocula, with a fibrinolytic strain did not inhibit fibrinolysis on fibrinogen agar nor was there any absorption of beta toxin by heat precipitated fibrinogen.

Christie, North and Parkin (1946) also using spot inoculation of plasma agar plates, found that 651 out of 685 coagulase positive strains tested were fibrinolytic. They concluded that fibrinolysin production is an indication of pathogenicity although all pathogenic strains are not fibrinolytic.

Lack (1956) used a similar plate method and found that by adding soya bean trypsin inhibitor to the medium he could separate the organisms tested into those whose proteolytic activity was due to activation of plasmin - the staphylokinase group which were inhibited, and those whose activity was due to their own protease, which were uninhibited.

Lack and Wailling (1954) found that 75% of staphylococci from cases of osteomyelitis produced fibrinolysin and quote staphylokinase with coagulase and alpha haemolysin production as appearing to be most closely associated with virulence.

Method.

The method adopted with the staphylococci, nearly all of which produced zones of clearing on heated plasma agar plates (Christie, North and Parkin, 1946) was based on the tube titration described by Christie, Graydon and Woods (1945). Staphylococcus "F" did not give a zone of clearing.

Strains of fibrinolytic staphylococci were grown in 100 ml. meat extract broth in flasks for 48 hours at 37°C. in 20% CO_2 in air. The broth was clarified by centrifuging

and 0.02% merthiolate was added to prevent further growth of organisms. Oxalated human plasma was heated for five minutes at 55°C. and the precipitated fibrinogen removed by centrifuging and resuspended in a volume of 0.02% merthiolated saline equal to that of the discarded supernatant fluid. Serial doubling dilutions of the fibrinolytic solution were made with the merthiolated saline and 0.8 ml. of each was added to a small tube with 0.2 ml. of the fibrinogen suspension. The tubes were placed in a water bath at 37°C. overnight. The results were read for lysis of the precipitated fibrinogen. The greatest dilution giving complete lysis of the flecks of fibrinogen was accepted as being the end point. The results were expressed as the reciprocal of this dilution.

The lysis was fairly easy to read in the low dilutions but the end points were less clear beyond 1/80. The results which can be seen in Table 9 fluctuated with no significant pattern at all levels as assessed between the different clones from the same original strain.

HYALURONIDASE.

The association of hyaluronidase production with pathogenicity has been discussed by a number of authors. Duran-Reynals (1933) first mentions a "certain spreading factor" existing in bacteria, with the outstanding property of inducing an increase in tissue permeability. He considers that the invasion of the skin by the staphylococcus depends on the presence of this substance and goes so far as to suggest that this enhancing substance, elaborated locally by organisms, passess into the circulating blood and by bringing about a general increase of tissue permeability, may act to enhance local infections elsewhere.

A number of arguments for the importance of hyaluronidase production by Cl. Welchii were at first advanced by McClean, Rogers and Williams(1943) but further work by McClean and Rogers (1943) and work by Evans (1943a and 1943b) tended to minimise this and Humphrey (1944) working with a staphylococci concluded that hyaluronidase is not a major factor in determining the virulence of staphylococci.

Schwabacher et al (1945) after examining 814 strains of staphylococci and micrococci from healthy carrier sites and clinical infections, suggest that virulence is associated to some extent with hyaluronidase production but consider that the actual part played by hyaluronidase in determining the virulence of a strain of staphylococcus is not clear.

Lack and Wailling (1954) confirm the association of hyaluronidase with virulence in a study of the factors possibly contributing to pathogenicity in 435 strains of

staphylococci. They found that all strains produced coagulase and all produced hyaluronidase, although 26 produced very little. They could, however, find no correlation between high hyaluronidase production and any particular spectrum of toxins elaborated.

There seem to be sufficient grounds at least for regarding a quantitative estimation of the enzyme as of value and even though a true increase in morbidity due to it is in some doubt, it would seem that an enzyme which has the power of increasing invasiveness and spread of an organism cannot but be regarded as important.

Method. Two methods were considered for the quantitative estimation of hyaluronidase, the turbidimetric assay of Tolksdorf et al (1949) and the mucin clot prevention test of McClean, Rogers and Williams (1943). The mucin clot prevention test was preferred for its simplicity. It depends upon the destruction by hyaluronidase of the capacity of a protein - hyaluronic acid complex to form a typical mucin clot on the addition of acetic acid.

A stock solution of potassium hyaluronate of 0.2% is made in distilled water and stored at 4°C. with a few drops of chloroform as preservative. The serum used is sterile rabbit serum, in accordance with the suggestion of the authors that the serum should be from a healthy animal which has not been immunised with any antigen that might contain hyaluronidase . A drop of chloroform is added as a preservative and the serum stored at 4°C. A serum substrate mixture is made on the day of the test in the proportions

of 1 vol. hyaluronate solution, 1 vol. 1/10 dilution of serum in 0.85% saline and 2 vol. of distilled water. The enzyme samples consist of the supernatants, after centrifuging, of overnight growths of the organisms in 20 ml. broth at 37°C. These are diluted with distilled water in serial dilutions in a final volume of 0.5 ml., a control tube containing 0.5 ml. distilled water. 1 ml. of the serum substrate mixture is added to each tube including the control, contents mixed, placed in a water bath at 37°C. for 20 minutes and then removed to the refrigerator in ice cold water for 5 minutes to stop the enzyme action. One drop of 5% acetic acid is added to each tube and the tubes shaken gently until precipitation and clots develop.

Results are read as

- ve. No precipitation or flecks but slightly misty.

± ve. A few flakes but no deposit.

\+ ve. Numerous flecks or deposit.

The results are expressed as the reciprocal of the highest dilution of the enzyme preparation which prevents complete clot formation, i.e. the tubes read as ± ve.

The results of estimation using the representative clones of the lowest, highest and highest induced fractions of each strain population are shown in Table 9.

TABLE 9

STRAIN	HYALURONIDASE PRODUCTION			DEOXYRIBONUCLEASE PRODUCTION			FIBRINOLYSIN PRODUCTION			LIPASE	PHOS-PHATASE
	LA	HA	HAI	LA	HA	HAI	LA	HA	HAI		
A	48	48	48	5	5	5	40	20	20	++	+
B	48	48	48	7	6	7	20	20	20	++	+
C	24	24	24	6	6	6	160	160	80	+	+
D	24	24	24	8	9	9	80	160	160	-	+
E	12	12	12	9	9	9	40	40	20	-	+
F	96	96	96	8	8	7	<5	<5	<5	+	+
G	48	48	48	9	10	10	320	320	160	+	+
H	48	48	48	8	8	8	40	160	160	+	+
J	6	6	6	9	9	9	40	20	40	++	+
K	96	96	48	6	7	6	20	40	40	+	+
L	24	24	24	7	7	7	40	40	40	+	+
M	96	96	96	9	9	9	40	40	20	++	+

Hyaluronidase: Figures represent reciprocal of greatest dilution preventing mucin clot.

Deoxyribonuclease: Figures represent reciprocal of greatest dilution giving a zone, expressed as a power of 10.

Fibrinolysin: Figures represent reciprocal of greatest dilution giving complete lysis of fibrinogen.

DEOXYRIBONUCLEASE.

In the case of this enzyme no claims are made that it plays any part in determining the pathogenicity of a staphylococcal strain, but it is included merely as an indication of the general enzymic activity of the organism. A clear statistical correlation with coagulase production is shown by Di Salvo (1958), who found that 200 coagulase positive staphylococci were all deoxyribonuclease producers, while 104 coagulase negative strains were not. An interesting point has been made by Gillisen (1959) who has suggested that when a sensitive staphylococcus is rendered resistant to penicillin (presumably in vitro induced resistance not dependent upon penicillinase production) its deoxyribonuclease acid content relative to total nitrogen content is increased. This paper is largely discounted in a review by Garrod (1960) but the author claims that he has previously found that a brief treatment with deoxyribonuclease reduces the proportion of penicillin resistant cells in a population of staphylococci.

Method. The method used is that of Di Salvo (1958). 2 mg./ml. of Deoxyribonucleic acid and 0.8 mg./ml. of Calcium chloride are incorporated in nutrient agar plates. Spot inoculations are made with drops of overnight growth of the organisms in broth and the plates are incubated overnight at 37°C. and then flooded with Normal hydrochloric acid, when a clear zone in the otherwise opaque plate indicates deoxyribonuclease activity. An attempt was made to transfer the constituents of this test to a tube test with a view to using dilutions of supernatants of the organisms in broth

and devising a quantitative test, but no distinctions between clearing and opacity could be seen in the tubes. An attempt to use supernatant dilutions in a plate technique was more successful, the supernatant dilutions being dropped into a cup cut in the agar with a cork borer, and also into Oxford cups. The cups cut in the agar are preferred. The test is carried out by the original technique only using 10-fold dilutions in broth of the centrifuged supernatants of over-night growths in broth. Two plates are used with six cups to a plate, 10-fold dilutions from 1 to 10^{10}, and a control cup. No definite end points could be established at less than 10-fold dilutions. Results were very high and are shown on Table 9 expressed arbitrarily as the power of the dilution of the supernatant.

LIPASE.

As in the case of deoxyribonuclease no claims have been advanced suggesting that lipase production has any correlation with the pathogenicity of a strain of staphylococcus. Christie and Graydon (1941) used blood agar plates containing cream, and nutrient agar plates containing butter fat to identify exo-lipase producers and concluded that there was, in fact, no correlation between lipase production and pathogenicity. Davies (1954) gives a method for the demonstration of exo-lipase production using nutrient fat agar containing horse fat, and a method of quantitative estimation using nutrient fat Nile blue sulphate agar. By these methods he found that 85% of staphylococcus aureus and 75% of staphylococcus albus were lipolytic.

Method. Staphylococci were tested for lipase production by streaking on nutrient fat agar, but attempts to produce growth on the nutrient fat agar plus 2 ml. of 0.1% (w/v) Nile blue sulphate per cent were unsuccessful. The Nile blue sulphate appeared to inhibit growth. Culture filtrates prepared as advised by Davies (1954) failed in every case to produce either clearing of the nutrient fat agar or the colour changes mentioned in the Nile blue sulphate agar.

Lipase estimation was perforce based on a qualitative test, namely the presence of a zone of clearing on nutrient fat agar round a spot inoculation of the organism. Results were read after 48 hours as follows.-

- = no clearing.

\+ = faint clearing.

++ = marked clearing.

The results are shown in Table 9.

[illegible] identification [illegible] phosphatase production in [illegible] phenolphthalein [illegible] method for the [illegible] free phenolphthalein by the action of phosphatase. [illegible] work to find the optimum [illegible] production found that 12/51 staphylococci they tested did not produce phosphatase, but unfortunately gave no details of the other biochemical activities of their strains.

Method. The qualitative method of Barber and Kuper (1951) was used as follows. -

A sterile solution of 0.01 M. sodium phenolphthalein phosphate is added to melted nutrient agar at pH 7.4 in the proportions of 1 part phenolphthalein to 49 parts of agar. The organisms to be tested are spot inoculated on the medium and phosphatase positive colonies liberate free phenolphthalein and become bright pink when held over an ammonia bottle.

The results are shown on Table 9.

All the organisms tested produced phosphatase as shown by this method.

PHOSPHATASE.

Barber and Kuper (1951) found a complete identification between coagulase production and phosphatase production in staphylococci. They used spot inocula on a phenolphthalein phosphate agar and tested by contact with ammonia for the liberation of free phenolphthalein by the action of phosphatase.

Tonelli and Marinelli (1958) in work to find the optimum pH for phosphatase production found that 12/31 staphylococci they tested did not produce phosphatase, but unfortunately give no details of the other biochemical activities of their strains.

Method. The qualitative method of Barber and Kuper (1951) is used as follows. -

A sterile solution of 0.01 M. sodium phenolphthalein diphosphate is added to melted nutrient agar at pH 7.4 in the proportions of 1 part phenolphthalein to 49 parts of agar. The organisms to be tested are spot inoculated on the medium and phosphatase positive colonies liberate free phenolphthalein and become bright pink when held over an ammonia bottle.

The results are shown on Table 9.

All the organisms tested produced phosphatase as shown by this method.

ANTIGEN-ANTIBODY LINE PRODUCTION.

The use of an agar diffusion technique allowing a double diffusion gradient was advocated by Elek (1948). It permits a separation although not always an identification of a large number of distinct diffusible antigens by setting an organism against an antitoxin and producing multiple lines in the agar medium used. Elek and Levy (1950a) found that the average number of distinct antigens found in strains isolated from various lesions was six, while the strain Wood 46, the homologous strain with the antitoxin, produced ten lines. They applied this technique also (Elek and Levy, 1950b) to haemolysin production by incorporating various animals' red cells in the agar.

The findings by this method show the multiplicity of the diffusing products of the pyogenic staphylococcus. Their differentiation in the agar reveals far greater complexity of the diffusible antigens of staphylococci than previously suspected and puts out of court much of the serological evidence purporting to support the unity theory (Elek, 1959).

Some authors have related the number of lines manifested by this technique with other factors. Anderson (1956) found a distinct correlation of the complexity of the haemolysin pattern with alpha haemolysin production, hyaluronidase production, pathogenicity to mice (using the intraperitoneal method of Christie, North and Parkin, 1946) and with the severity of the original clinical infection. He failed to find any correlation with fibrinolysin, pigment or coagulase production.

Howard (1954) found that the number of antigen antibody flocculation lines formed by strains of staphylococcus aureus is directly related to the relative virulence for mice using the intramuscular method of Selbie and Simon (1952). He found strains giving a complex haemolysin pattern produce significantly more lines than strains giving a simple pattern or than non-haemolytic strains, and that they have a higher alpha toxin titre. Recently Brown (1960) has confirmed these findings as regards the correlation with alpha haemolysin production and complexity of haemolysin production and has found some correlation too with the site and type of infection.

Method. The method is based on that of Elek (1948). The medium consists of infusion broth to which 0.2% of potassium dihydrogen phosphate and 0.03% of magnesium sulphate are added, both salts being used crystalline. The pH is adjusted to 6.8 and the broth is clarified. Plates of 10 ml. are poured and a sterile filter paper strip dipped in Burroughs Wellcome staphylococcal antitoxin 1250 units/ml. is laid across the surface and allowed to sink into the medium while it is still fluid. The plate is dried in the incubator and used the same day. The organisms are streaked, one loopful from an overnight broth culture, across the centre of the antitoxin strip at right angles to it, one streak on each plate. The plate is incubated at 37°C. and examined at 24 and 48 hours. A positive reaction is denoted by the appearance of fine white lines, forming an arrowhead within half an inch of the filter paper and pointing towards it.

The cultures representing the lowest and highest thirds and the highest third after induction, from each strain were tested by this method. The results are shown on Table 10.

[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	2	2	2
[illegible]	2	2	2
[illegible]	2	2	2
[illegible]	4	4	4
[illegible]	3	2	3
[illegible]	[illegible]	5	5
[illegible]	[illegible]	5	5
K	[illegible]	6	6
L	[illegible]	3	[illegible]
M	[illegible]	6	[illegible]

[illegible] of antigen- [illegible] induction.

TABLE 10

ANTIGEN-ANTIBODY LINE PRODUCTION

STRAIN	LOWEST PENICILLINASE ACTIVITY	HIGHEST PENICILLINASE ACTIVITY	HIGHEST PENICILLINASE ACTIVITY AFTER INDUCTION
A	4	4	4
B	6	5	6
C	2	2	2
D	2	2	2
E	2	2	2
F	4	4	4
G	3	2	3
H	5	5	5
J	5	5	5
K	6	6	6
L	2	2	2
M	6	6	6

Figures represent numbers of Antigen-antibody lines after A8 hrs. incubation.

MOUSE INOCULATION.

Obviously no estimation of the pathogenicity of an organism would be complete without some assay of its effect in vivo, and for this a suitable laboratory animal is required. Of the commonly available ones rabbits were rejected as vastly expensive, guinea pigs as notoriously insusceptible and mice were consequently preferred. The site and method of inoculation and the dosage remained to be decided. The methods considered were, intravenous, intraperitoneal, intramuscular and intranasal. Mice are generally accepted as being extremely resistant to staphylococci by the intraperitoneal route (Gorrill, 1951). Several authors (Miller, 1934, Buttle, 1937, Browing, 1940, Browning and Calver, 1947, Fisher, 1959) have mentioned the use of 5% mucin injected with the staphylococcal cells as enhancing the action of the staphylococci. This enhancement is held by Lambert and Richley (1952) to be due to the anticomplementary effect of mucin extract due to its heparin content. Christie, North and Parkin (1946) used intraperitoneal inoculation without mucin as a method of estimating pathogenicity to mice but used doses of as much as 4×10^9 organisms to secure 100% mortality with pathogenic strains of staphylococci. Miller (1934) found that the gastric mucin commercially available was variable in its action and used a special granular mucin obtained from the Wilson Laboratories in Chicago, as did Browning and Calver (1947). Fisher and Thompson (1956) used 1 ml. of overnight broth culture estimated as containing $1 - 2 \times 10^9$ organisms to achieve a large percentage morbidity

without gastric mucin. They found some correlation between virulence to mice by this method and clinical pathogenicity and postulated the formation of a highly toxic product in the peritoneal cavity which they thought might be of considerable significance from the stand-point of human infection. It was felt, however, that this method, requiring either a special mucin or very large number of organisms to be effective, was not ideal and the other methods were considered.

Intravenous inoculation into the tail veins of the mouse is a well established means of estimating pathogenicity. Gorrill (1951) found that 4×10^7 Staphylococcal organisms gave 100% mortality, 4×10^6 gave 40 - 50% and 4×10^5 gave no deaths after fourteen days in his series. Smith (1956) gave mice 0.1 ml. of an overnight culture of staphylococcus aureus intravenously. He showed some relation between virulence and the ability of the organisms to multiply in the mouse kidney, while McCune, Dineen and Batten (1956), giving 0.1 ml. of overnight broth culture intravenously, found a marked degree of multiplication in the kidney and even abscess formation. This method appeared more suitable as requiring much smaller numbers of organisms on the average to achieve something approaching a 50% mortality and requiring no exotic materials.

Selbie and Simon (1952) advocated a method which they considered gave an infection in the mouse more closely resembling the natural infection of man. This was the intra-muscular injection of the test organism into the posterior aspect of the mouse's thigh. They then measured the resultant

swelling of the inoculated thigh at intervals as compared with the normal thigh. In their experiments they grew their test organisms in air, and in CO_2 and air and found that the resultant absence or presence of alpha haemolysin in the inocula had little effect on the lesions produced. Despite this they considered that the principal role in determining the size of the lesions produced is played by alpha haemolysin. This causes damage to the tissues in the early stages of the lesion and is also of importance in maintaining the infection. A further contribution to the initial size of the lesion is made by coagulase, which the authors think probably inhibits phagocytosis and so promotes the invasion of the tissues by staphylococci, but which afterwards they believe determines a more rapid regression of the infection by promoting the formation of fibrin barriers. Fibrinolysin, which has the effect of breaking down fibrin barriers, is also considered to be of importance. These authors then, found a correlation between coagulase, haemolysin, fibrinolysin, and virulence to mice by the intramuscular method. They found no correlation with hyaluronidase production colour, gelatin liquefaction, mannitol fermentation or resistance to penicillin.

The intranasal route was discarded as being difficult to control quantitatively and because of the resistance of mice to challenge this route (Gorrill, 1951)

It was decided then to use the intravenous and the intramuscular routes. It was felt that in each a different emphasis might be placed in the relative values of the various toxins and enzymes elaborated by the organisms.

Method - Intramuscular.

The method is based on that of Selbie and Simon (1952). Albino mice weighing approximately 20G. were used. These were from a strain bred and maintained over some years at the Victoria Sectoral Laboratory, Glasgow. For each organism three mice were inoculated intramuscularly in the posterior aspect of the left thigh with 0.2 ml. of an overnight growth of the organism in digest broth. The mice were examined at 1, 2, 4 and 6 days after inoculation, when measurement to the nearest millimetre was made of the transverse diameter of the thigh. By subtracting the width of the untreated thigh (found to be 7-8 mm. in every case) a differential figure was given. It was found that ulceration and abscess formation interfered with measurement after the sixth day (Selbie and Simon found this after seven days), so measurement was discontinued at this point. In a few cases, particularly those showing the greatest swelling, abscesses appeared about the sixth day. In these cases no measurement was taken, but the mouse concerned was counted as giving the measurement secured on the fourth day. Only four mice were involved, all at the sixth day. It was felt that these few cases should not be allowed to invalidate the sixth day readings.

The three cultures, representing the lowest and highest thirds and the highest third after induction, from each strain were tested by this method. The results are shown in Table 11.

The figures are the total of the differences between the diameters of the normal and the inoculated thighs of the three mice used, at four readings, expressed in millimetres. As the diameters of the normal thighs were very uniform throughout (7-8 mm.) no further attention was paid to the normal thigh figures.

TABLE 11

PATHOGENICITY TO MICE INTRAMUSCULAR AND INTRAVENOUS

STRAIN	Lowest Penicillinase Activity		Highest Penicillinase Activity		Highest Penicillinase Activity after Induction	
	Total thigh differences of mice in mm.	LD_{50} x 10^6	Total thigh differences of mice in mm.	LD_{50} x 10^6	Total thigh differences of mice in mm.	LD_{50} x 10^6
A	46	-	52	-	47	-
B	69	-	72	-	77	-
C	61	-	62	-	62	-
D	27	-	28	-	31	-
E	24	-	26	-	22	-
F	67	-	64	-	68	-
G	40	323.6	36	379.6	39	379.6
H	73	68.5	74	112.5	70	42.6
J	70	8.6	71	10.7	75	10.7
K	83	7.8	76	8.8	76	7.8
L	40	38.5	46	42.4	41	31.9
M	83	7.8	80	9.1	79	10.6

Figures in columns 1, 3 and 5 represent total differences between diameters of thighs of 3 normal mice and 3 inoculated mice.

Figures in columns 2, 4 and 6 represent LD_{50} in millions of organisms.

Method - Intravenous.

After some preliminary investigation which showed that the LD_{50} of the majority of the strains seemed to lie at about the 10 - 100 x 10^6 level by the intravenous method, the following method was adopted. The organism to be tested was grown overnight in broth, washed three times in and resuspended in meat extract broth to an opacity of Brown's opacity tube No. 3, i.e. approximately 3 x 6 x 10^8 organisms/ml. Dilutions were then made serially in meat extract broth to 1/2, 1/8, 1/32, and 1/128 and 0.5 c.c of these dilutions was injected, i.e. approximately 450,000,000 - 7,000,000 organisms. Mice used were similar to those used in the intramuscular method. No dietary restrictions were made before or after inoculation to avoid any variation in susceptibility to staphylococcal infections (Smith and Dubos, 1956b).

The three cultures representing the lowest and highest thirds, and the highest third after induction, from the six strains G. H. J. K. L. and M. were tested by this method. Only six strains were tested as it seemed from the results that nothing was likely to be gained from sacrificing the further large number of mice required to complete the twelve strains.

The range of dosage of organisms covered the variations in the six strains tested. The LD_{50} was calculated in each case by the method of Reed and Muench (1938). The results are shown in Table 11 in millions of organisms.

ANALYSIS of RESULTS.

(1) Variations in pathogenicity related to penicillinase production.

The organisms tested consisted of three fractions from each of twelve strains of staphylococci. These fractions were distinguished and chosen for one feature only, namely their different basic constitutive level of penicillinase activity. Two fractions were chosen as representing the least active clones in penicillinase production and the most active clones in penicillinase production. A further culture to be tested for pathogenicity represented the most active penicillinase producers after undergoing penicillinase induction. These are referred to in the tables throughout as L.A = lowest activity, H.A = highest activity and H.A.I. = highest activity induced.

When the results obtained in each of the tests of pathogenicity are compared, no variations of any significance can be found occurring among these fractions within the strains. In every strain the results obtained for each fraction of the population showed a remarkable consistency with no trend to greater or lesser activity.

Regarding the strains as a whole, no correlation whatsoever could be established between the penicillinase activity of the organisms and their pathogenicity as estimated by the tests described.

It may therefore be categorically stated as a result of the findings of this thesis that no correlation has been demonstrated between the penicillinase activity of the

staphylococci examined, both as total populations and as separate clones, and their pathogenicity.

(2) <u>Variations among strains in different biochemical activities.</u>

For convenience at this point all the results obtained in tests for pathogenicity and biochemical activity are brought together in Table 12. Apart from the antigen-line production, which is recorded as the number of lines actually produced, the figures shown are relative estimations of activity, based on the results in previous tables, as follows. - "0" represents no activity and "1", "2" and "3" represent relative degrees of activity. Phosphatase production is arbitrarily shown as "1" in each case. The figures of course represent the activity of the strain as a whole, combining the results obtained from the fractions tested. The last column represents the overall average relative penicillinase activity as represented by the average of the figures in Table 1.

Before mentioning the relationships existing generally among these criteria of virulence, it is interesting to notice the results of the coagulase estimation by two methods, and the mouse pathogenicity estimation by two methods.

In the case of coagulase production it will be readily seen by reference to Table "7" and to Table "12" that there is no apparent relationship between coagulase activity as measured by the time taken to produce coagulation and by the titre to which coagulase activity can be taken.

In the case of mouse pathogenicity, only six strains can be used for comparison. Examination of Table "11" and Table "12" shows a similarity in trend although individual strains do not vary exactly together.

To estimate overall correlation of toxic products generally, it was decided to use the antigen line production as a basis of comparison as it could be accepted at least as showing the multiplicity of biochemical activity of the strains.

It will be readily appreciated from a study of Table 12 that there is a distinct correlation between antigen line production and the production of alpha haemolysin and delta haemolysin, and the pathogenicity to mice by both methods.

No correlation can be demonstrated between the group mentioned above and hyaluronidase, fibrinolysin, deoxyribo-nuclease, lipase, phosphatase or coagulase production.

TABLE 12

STRAIN	ANTIGEN LINES	MOUSE IV	MOUSE IM	COAG. (TIME)	COAG. (TITRE)	ALPHA LYSIN	DELTA LYSIN	HYAL-URONIDASE	FIBRIN-OLYSIN	D.N. ASE	LIP-ASE	PHOS-PHAT-ISE	PENIC-ILLIN-ASE
A	4	-	2	2	1	2	1	2	1	1	2	1	1
B	6	-	3	2	1	3	2	2	1	2	2	1	2
C	2	-	3	1	3	2	1	2	3	1	1	1	1
D	2	-	1	2	2	1	0	2	3	2	0	1	1
E	2	-	1	1	1	1	0	1	2	3	0	1	1
F	4	-	3	2	1	2	1	3	0	2	1	1	2
G	3	1	2	3	2	2	0	2	3	3	1	1	2
H	5	2	3	2	1	3	2	2	2	2	1	1	3
J	5	3	3	1	1	3	3	1	2	3	2	1	1
K	6	3	3	1	3	3	2	3	2	1	1	1	3
L	2	2	2	2	2	1	0	2	2	2	1	1	1
M	6	3	3	1	1	3	3	3	2	3	2	1	1

Figures represent relative activity.

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SUMMARY

SECTION 1.

This section takes the form of an introductory discussion. Some of the features of the resistance of staphylococci to penicillin due to penicillinase production and some environmental factors which may affect this resistance are reviewed. A brief mention is made of the new penicillin B.R.L. 1241, and finally some general aspects of the problem of estimating the pathogenicity of staphylococci are considered.

SECTION 11.

This section gives an account of some experiments in the estimation of penicillinase activity both constitutive and inducible. The following points are made from the results.

1. Constitutive penicillinase activity within a strain varies from clone to clone.

2. In the absence of penicillin in the substrate the level of activity of each clone is relatively stable.

3. On solid media containing small concentrations of penicillin, very little if any induction of penicillinase activity takes place, but there is evidence that there is some selection of clones of higher constitutive penicillinase activity.

4. All clones of whatever constitutive level of penicillinase activity are inducible by large concentrations of penicillin to the same high level of activity.

5. Sensitivity to penicillin by the tube method seems to depend, with a large inoculum, on the total penicillinase inducibility of the cells. With a light inoculum it seems to depend more on the constitutive level of the penicillinase activity. With a very small inoculum, complete sensitivity comparable with the Oxford staphylococcus, is the rule.

6. It is suggested that in the human host, some relative selection may take place at certain penicillin concentrations of organisms with higher basic constitutive levels of penicillinase activity, without any great degree of penicillinase induction taking place.

7. The effect of antipenicillinase rabbit serum is shown in reducing the resistance of penicillinase producing staphylococci in certain concentrations of penicillin and with certain weights of inoculum.

SECTION 111.

This section is concerned with the examination of a selection of clones isolated from each strain used in the previous section. These clones are distinguished only by their various degrees of penicillinase activity. Clones of low activity (L.A.) and high activity (H.A.) before induction, and the clones of high activity after induction (H.A.1.) are used. The pathogenicity and general biochemical activity of these clones is estimated using the following criteria, - coagulase, alpha haemolysin, delta haemolysin, fibrinolysin, hyaluronidase, deoxyribonuclease, lipase, phosphatase and antigen line production and pathogenicity to mice by intra-

muscular and intravenous inoculation.

The following conclusions are reached -

1. No correlation is demonstrated between pathogenicity, general biochemical activity and penicillinase production, either as between the total populations of different strains or between fractions of the population within strains.

2. There is distinct correlation shown between antigen line, alpha haemolysin and delta haemolysin production and mouse pathogenicity as estimated quantitatively.

3. There is no correlation shown between any of the group mentioned above and coagulase, hyaluronidase, fibrinolysin, lipase or deoxyribonuclease production as estimated quantitatively.

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