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STUDIES OF STAPHYLOCOCCAL PRODUCTS ASSOCIATED WITH PATHOGENICITY

In vitro and *in vivo* studies of *smooth* and *rough* Colonial variants of *Staphylococcus aureus*.

STUDIES OF STAPHYLOCOCCAL PRODUCTS ASSOCIATED WITH PATHOGENICITY

In vitro and *in vivo* studies of *smooth* and *rough* colonial variants of *Staphylococcus aureus*

BY

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March, 1958.

A thesis submitted for the degree of Doctor of Medicine.

PAGES

PREFACE

The need for further knowledge regarding the manner in which staphylococcal infections are caused first claimed my attention several years ago when I was engaged in the treatment of such infections in the Western Infirmary, Glasgow. The interest then aroused led me to take up the study of bacteriology and in particular of the species Staphylococcus aureus. This thesis contains the results of a large proportion of the laboratory investigations which I have conducted over the last 5 years.

I wish to acknowledge with gratitude the amount of assistance I have had in carrying out this work. For strains of Staph.aureus I am indebted to Dr. P. Browning (Glasgow), Dr. S.T. Cowan (National Collection of Type Cultures), Dr. E.S. Duthie (Southampton), Prof. S.D. Elek (London) and Dr. J.A. Marks (Beckenham). For gifts of antisera I wish to thank Miss Molly Barr (Beckenham) and Dr. J. Marks (Cardiff) and for chemical compounds Dr. Crowther (Manchester) and Dr. Lominski (Glasgow). Specialised investigations beyond my own resources have been made available to me; Dr. R.E.O. Williams and Joan Rippon (Colindale, London) and Dr. Morag Timbury (Glasgow) have phage typed a number of strains; leucocidin production

has been measured by Dr. G.P. Gladstone (Oxford); Dr. E.B. Hendry (Glasgow) estimated blood protein and fibrinogen. In particular I would like to thank Dr. E.S. Duthie for gifts of very highly purified coagulase.

The first part of Section I (Variants of Staphylococcus aureus giving high and low yields of coagulase) has already been published. It describes one aspect of a wider investigation into factors influencing coagulase production, undertaken in collaboration with Dr. R.B. Morrison and Dr. I.R.W. Lominski. However, the first observation of the association between colonial morphology and coagulase production, and the isolation of all but one of the Rough variants, was made by myself; the information in Table 3 is to be attributed to my Colleagues. In Section II, I again had a collaborator, Dr. J.M. Johnstone. This work was initiated and carried out entirely by myself except for the parts dealing with histology.

All of the work on this thesis was done in the Department of Bacteriology of the University and Western Infirmary, Glasgow, and I am very grateful for my good fortune in finding there the facilities for this research and for the helpful advice and criticism of friendly colleagues.

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INTRODUCTION

(1) Choice of experimental model

Disease caused by pathogenic micro-organisms is today very much less common than it was 100 years ago. This change has been brought about in three ways: by eradication of reservoirs of infection through improved hygiene, by immunisation, and by chemotherapy.

Staphylococcus aureus differs from most pathogens in that it is so common on the skin and in the nose of most healthy people as to be considered a commensal. It is improbable then that any method will be found which will permanently exclude Staph.aureus from communities, thus obviating the possibility of infection. For a short time it seemed that chemotherapy would deal with staphylococcal infections, but several antibiotics initially active against Staph.aureus are gradually losing their effect on account of the emergence of resistant strains. The most outstanding example is that of penicillin; before 1945 it was unusual to find a resistant strain - by 1956 the proportion of these strains isolated from patients in hospitals was as high as 80 per cent. There are probably

many new chemotherapeutic agents to be found but this approach may fail in the same way.

Since exclusion is impossible and chemotherapy is in the process of failing, all that is left is the hope that new methods of immunisation can be devised, or else, that the non-specific resistance of the host may be increased. A prerequisite to either of these two measures is a better understanding of the mechanisms of pathogenicity in staphylococcal infection; the investigations described in this thesis were undertaken in the hope that they would constitute at least a contribution to this need. The study of variants differing among each other in the production of five toxic factors seemed to offer the chance of finding which if any of these factors were essential to the pathogenic action of the species.

In this type of work, it is a great advantage to be able to reproduce as nearly as possible the natural infection. With staphylococci, however, even in human volunteers, skin lesions can be produced only if the organisms are inoculated into the tissues and in numbers larger than those which can reasonably be expected to constitute the natural inoculum. The usual laboratory animals such as mice, rabbits and guinea-pigs are not

naturally susceptible. The study which forms the subject of this thesis was confined therefore to experimental infection of laboratory animals. Since a choice had to be made, mice were regarded as the most convenient because they are easy to work with, can be obtained in large numbers, and permit a low degree of variability of host resistance by the use of inbred strains. Experimental staphylococcal infections can be established in mice; in some important respects, such as the formation of localised abscesses and, in severe cases, septicaemia, these resemble human infections closely enough to provide a useful model.

(ii) The importance of variants among staphylococci

In the present work, variants of Staph.aureus were studied in an attempt to elucidate certain aspects of the in vivo and in vitro physiology of this species and not with a view to gaining information on the mechanism of variation. Probably the most frequently noticed instance of variation is in colonial morphology, but almost any function of the bacterial cell may be affected. The extensive review by Hadley (1937) gives an account of variation in morphology, biochemical activity, and virulence. In absence of a recent review much relevant information is

presented in the text-books, Topley and Wilson (4th edition, Chap. 9) and Lammana and Malette(Chap. 12).

The terms variant and mutant as applied to bacteria are often used as synonyms. In this thesis the term 'variant' has been preferred because it makes no inference about the mechanism of the genetical change+. In accordance with theory, characters vary independently and result from a mutation affecting a single gene. If, as it has been speculated, the staphylococcal cell contains between 1000 and 10,000 genes (Bryson, 1956), then no matter how important a variation may appear biologically, it represents only a minor change in the cellular organisation. In this sense bacterial variants are comparable and provide a tool for the separation of certain components and the investigation of their respective functions.

Variation in a single gene may result in more than one observable change, for instance the same variation in the pneumococcus accounts for the loss of capsule, change to rough morphology, and loss of virulence for mice. To the medical bacteriologist this type of variation, where morphological change is associated with change in virulence,

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+ New genotypes can arise other than by mutation, and genetical material may be transferred from one bacterial cell to another in a number of ways, e.g. by transforming principle (pneumococci) by phage transduction (salmonellas) and sexual recombination (coliforms).

is of prime interest. In general the colonial variation from smooth to rough (S-R) is accompanied by loss of virulence, though exceptions exist where the virulent form is more rough. Thus, study of variants has made possible the identification of bacterial components not only associated with but essential to pathogenic action, e.g. capsules in the anthrax bacillus, pneumococcus, Haemophilus influenzae, and Haemophilus pertussis; the type-specific M protein of Streptococcus pyogenes; and Vi and O antigens in species of the genus Salmonella.

It should be borne in mind, however, that no matter how important any of these components may be they probably represent only one of several factors which determine the capacity of the bacterium to cause disease. For example, it has been shown that the degree of virulence of Strep. pyogenes for laboratory animals does not depend on the amount of M protein possessed by the particular strain, and that M protein may be present in avirulent variants. Again, most of the characteristic lesions in diphtheria are reproduced experimentally by the injection of toxin and indeed the toxin is of overriding importance in the production of this disease. Nevertheless the famous strain of Corynebacterium diphtheriae, Park-Williams no. 8 renowned for its high yield of toxin in vitro, is only

poorly pathogenic. It follows then that it is not always possible to relate virulence to either the presence or the amount of any one bacterial product.

Staph.aureus is examined as often as other bacteria for diagnostic purposes; even so, variation is seldom observed. There are at least three reasons for this: (1) as a rule only two characters are considered (pigment and coagulase production); (2) the period during which cultures are observed, normally 24 hours, does not usually allow sufficient time for variants to appear; and (3) media most in use are unsuitable for the detection of variants. On the other hand, if a large number of characters is examined it becomes apparent that the species is heterogeneous, containing widely differing strains, and that variation is common. A glance at examples of variation in staphylococci suggests that variation may affect almost every observable character, e.g. colony size (Wise, 1956), penicillin resistance (Barber, 1947), colonial morphology (Bigger, Boland and O'Meara, 1937), hyaluronidase production (Rogers, 1953), haemolysin production (Rowntree, 1936; Elek and Levy, 1954), coagulase production (Smith, Morrison and Lommski, 1953), and virulence (Burnet 1930).

Of particular relevance to the present studies is the

work of Elek and Levy on alpha and beta haemolysin. These workers examined single-cell and single-colony cultures and found that, of four variants, three were stable in their haemolysin production, whereas one did not breed true. They concluded that alpha and beta haemolysin production are two independently controlled genetical characters and that all cells producing haemolysin do so to the same extent. Thus a culture giving high yields of haemolysin would do so because it contained almost 100 per cent of haemolysin producing variants, and one giving low yields a correspondingly lower proportion.

Another work to which reference must be made is that of Bigger, Boland and O'Meara (1927). They noticed that when old broth cultures were plated some of the colonies were irregular in outline, had a rough surface, and were viscid when touched with a platinum loop. These R variants were easily obtained from a number of strains. Subsequently, Hoffstadt and Youmans (1932) obtained from one strain of Staph. aureus no fewer than eight types of rough variant, one albus smooth variant, and two types of small-colony variant; the variants arose in cultures containing lithium chloride and in rabbits inoculated intravenously with the parent strain. Only one of their eight R variants

variants resembled the R variants of Bigger et al; such description and illustrations as they give of their other rough variants suggest that the term rough was hardly justifiable. The parent strain which was aureus and smooth was pathogenic for rabbits but two rough variants, an albus smooth and a small colony variant, were not.

The present study is confined to four staphylococcal products namely, coagulase and the three haemolysins: alpha, beta, and gamma. The choice was dictated by the consideration of the role of coagulase as an aggressin in establishing an infection (Smith, Hale and Smith, 1947); the general toxicity of alpha toxin; and, last but not least, because in the course of the work variants differing in precisely these factors became available. The omission of other staphylococcal products does not constitute a judgement upon the role that some of them may play in infection. I am aware how important hyaluronidase, leucocidins, and fibrinolysin may be. However, an attempt to correlate all staphylococcal products singly and in combination with pathogenicity would be entirely beyond the material means available.

In each section of this work a comparison is made between staphylococcal variants of smooth and rough colonial morphology. It has been suggested (Topley and Wilson, 4th. edition, page 323), that the letter S should no longer

denote smooth colonial morphology, but the colonial variant, whether smooth or rough, associated with virulence; R should be the symbol for the avirulent colonial type. In the first part of Section I (already published) S has been used to denote smooth colonial morphology and R, rough; no inference is made therein to the relative pathogenicity of the variants. To avoid confusion, the symbols S and R will not be used in other sections and the terms smooth and rough will refer to morphology only.

SECTION I

SMOOTH - ROUGH COLONIAL VARIATION AND COAGULASE PRODUCTION IN VITRO

The main contribution of this section is the demonstration of the association between colonial morphology and coagulase production. The recognition, isolation, and some properties of rough variants, other than of coagulase production, are also described.

Most of this part of the work has already been published in the Journal of Pathology and Bacteriology, 1952, vol. 64, pages 567-573 (Smith, Morrison and Lominski). In the preface of this thesis (page 1) the extent of my own contribution to this joint work is exactly defined. The second part of this section contains information based on the examination of more strains; it deals also with the chemical structure of compounds that may be used to assist in the demonstration of rough variants.

PAGES

PART ONE

PART TWO

ISOLATION OF VARIANTS OF *STAPHYLOCOCCUS AUREUS* GIVING HIGH AND LOW YIELDS OF COAGULASE

DOUGLAS D. SMITH,[+] ROBERT B. MORRISON[+] AND IWO LOMINSKI

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At present the recognition of pathogenic staphylococci *in vitro* largely depends on the result of the coagulase test; however, only a small proportion of positive strains give high yields of coagulase. Furthermore, laboratory strains often suddenly deteriorate and less often suddenly improve in respect of coagulase production, even when grown in the same batch of medium or in a chemically defined medium. In the course of work on the synthesis of coagulase by staphylococci we were often hampered by irregularities in the coagulase production of our strains, especially by the tendency of some of them to weaken in coagulase production; this led to a detailed investigation of our cultures.

The present study shows that in many strains of *Staphylococcus aureus*, variants can be found which differ in respect of coagulase production; and that high and low coagulase-yielding variants may be distinguished by

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+ During the course of this work D.D. Smith held the Coats and Perman Scholarships and R.B. Morrison a Carnegie Senior Scholarship.

their colonial appearance and characteristic growth in broth. The variants are not absolutely stable but may be kept by daily plating and selection of colonies for subculture. The proportion of high- and low-coagulase producers varies within one and the same strain, and this may well account for the spontaneous variations in coagulase production shown by cultures of Staph. aureus.

MATERIALS AND METHODS

Strains. We used ten strains of coagulase-positive Staph. aureus of human origin; they were selected because they gave high yields of coagulase. Two were one-year-old laboratory strains and the other eight were recently isolated.

Media. Three media were used: (1) horse-heart extract broth, (2) "Lablemco" broth agar, and (3) horse-heart digest-broth agar with 5 per cent. of oxalated horse blood. Medium no. 3 was used either unheated or heated at 80°C. for 10 minutes; the pH of the three media was between 7.2 and 7.5.

Plasma. Human plasma containing 0.6 per cent. sodium acid citrate and 0.4 per cent. glucose was used, care

being taken to use the same batch when coagulase production of variants was compared.

Estimation of coagulase. Four ml. of broth in 4 x ½in. test-tubes were inoculated and incubated at 37.5°C. for four days. These details are important because we found that the degree of aeration and the surface: volume ratio of the cultures influenced coagulase production. Two methods of coagulase estimation were used: (1) a clotting-time measurement, and (2) titration.

(1) Clotting-time measurement. 0.5 ml. of culture was added to 0.5 ml. of a 1 in 5 dilution of human plasma in 0.85 per cent. saline in a 4 x ½ in. test-tube. The tube was gently rocked through 45° from the vertical and the time required for clot to form was noted.

(2) Titration. To 0.5 ml. of serial doubling dilutions of the culture in 0.85 per cent. saline was added 0.5 ml. of a 1 in 5 dilution of human plasma in merthiolated saline, the final concentration of merthiolate being 1 in 1000. The highest dilution of the cultures to show clot after 24 hours at 37.5°C. was noted and recorded as the coagulase "titre".

Isolation of variants

The method was originally devised in order to find out

whether the individual organisms in a culture of Staph. aureus were identical in regard to coagulase production. Accordingly, broth cultures were plated, and from a number of colonies, usually 24, broth cultures were again made. After 4 days' incubation, these were tested for coagulase by measurement of clotting times. When differences in clotting speed were found the cultures with the highest and lowest clotting times were again plated, colonies picked into broth and the broth-plate-broth cycle repeated several times with the aim of selecting the best and worst coagulase producers. At first the colonies were picked at random but we soon realised that the fast-clotting and the slow-clotting variants could be recognised by their characteristic growth in broth and colonial morphology, particularly on blood agar and heated-blood agar. The following association of coagulase production and growth characters was found: the fast-clotting variants gave granular growth in broth and colonies of varying degree of roughness, whereas the slow-clotting variants showed uniform turbidity in broth and gave smooth colonies, typical of Staph. aureus. Consequently growth characters became our guide in the selection of variants.

At this stage we recalled that Bigger, Boland and O'Meara (1927) had found that rough colonies of Staph. aureus may

readily be obtained by plating old broth cultures. As our fast-clotting variants were of the rough type and since we observed that rough variants may be detected in young broth cultures by the granular appearance of the broth and the presence of pellicle, we evolved the following method for the isolation of rough variants. Young broth cultures showing granularity, or broth cultures which had been allowed to stand for 1-8 weeks, were plated and checked for the presence of rough (R or fast-clotting) and smooth (S or slow-clotting) variants. These colonies served as starting material for broth cultures with which the experiments on coagulase production were carried out.

RESULTS

Description of variants

The experiments demonstrated the existence in our 10 strains of variants differing considerably in coagulase production and growth characters; a description of their distinguishing features is given below.

The fast-clotting variants giving high yields of coagulase showed a variable degree of roughness in their growth; in 24-hour broth cultures the liquid might be almost clear or more or less turbid but it always contained coarse granules visible to the naked eye. There was always a pellicle with early ring formation and a heavy deposit

which was viscid on shaking and had an irregular edge (Fig. 1). On solid media the colonial morphology showed a gradation in R characters. On blood agar or heated-blood agar the fast clotting variants of some strains were smaller, more opaque and more convex than the typical, smooth colony of Staph. aureus (Fig. 5); it was only when picked into broth that they revealed their rough character. When touched with a wire these colonies were coherent and could be shifted as a whole on the surface or lifted entire on the loop; they often had an irregular outline and dry appearance. On heated-blood agar the rough characters were more marked. Finally, four of our strains frequently gave typical R colonies (Fig. 2 and 3) resembling those described by Bigger, Boland and O'Meara. The rough characters became less marked with age and after 48 hours the fast-clotting might be indistinguishable from the slow-clotting variants. Haemolysis did not appear to be correlated with R$\longleftrightarrow$S variation. On occasion, after 48 hours' incubation, a slight clearing of heated-blood agar around the colonies was noted. Figs. 2-5 show the different colonial appearances associated with the fast-clotting and slow-clotting variants.

The slow-clotting variants giving low yields of coagulase showed growth of the smooth S type generally associated with Staph. aureus. In 24-48 hour broth cultures there was

J. PATH. BACT.—VOL. LXIV PLATE CXXXI

STAPH. AUREUS VARIANTS

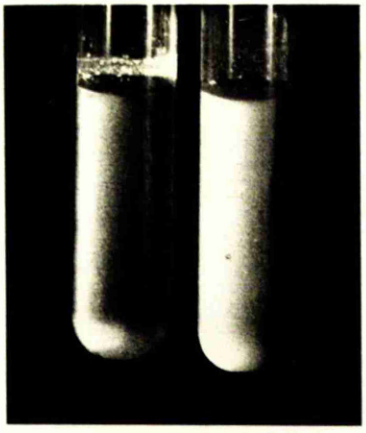


FIG. 1.—Broth cultures of, on left, R fast-clotting variant; on right, S slow-clotting variant. Natural size.

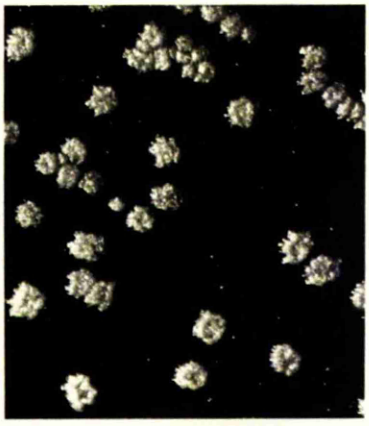


FIG. 2.—Colonies of R fast-clotting variants on heated-blood agar. ×*ca.* 5.

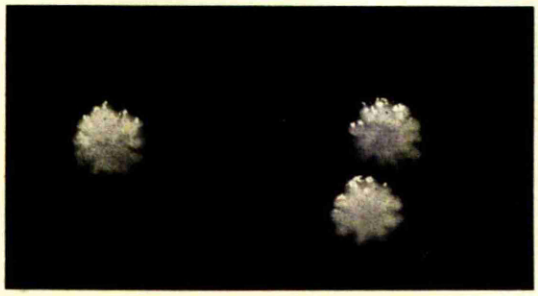


FIG. 3.—Colonies of R fast-clotting variants on heated-blood agar. ×*ca.* 10.

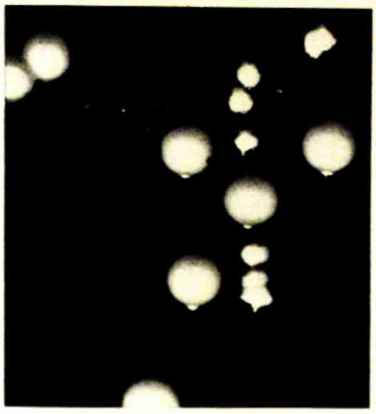


FIG. 4.—Colonies of R fast-clotting variants (small and irregular) and S slow-clotting variants (large and circular) on blood agar. ×*ca.* 5.

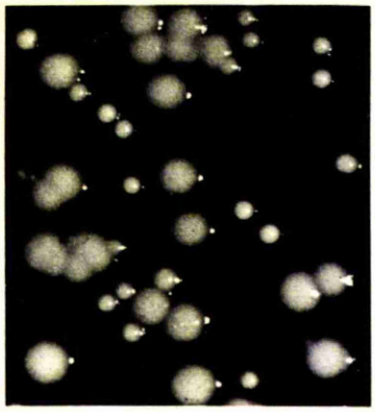


FIG. 5.—Colonies of fast-clotting variants (small and opaque) and slow-clotting variants (large). ×*ca.* 5.

uniform turbidity with a little smooth sediment which could easily be shaken up; we did not observe pellicle or ring formation in these young cultures (Fig. 1). On solid media 24-hour colonies were cirular, slightly convex, smooth, glistening and entire (Fig. 4 and 5). The clearing of heated-blood agar occasionally noted around the colonies of fast-clotting variants on 48-hour plates was much more frequent and extensive with the smooth variants.

From one strain a smooth variant was isolated which produced no coagulase after 72 hours' growth in broth. The results of phage-typing described below show that it was a variant and not an accidental contaminant.

The characters of the fast-clotting and slow-clotting variants and the association of R and S variation with coagulase production are summarised in tables I and II.

The question arose whether the difference in coagulase production between the two variants was due to a difference in growth. The results shown in table III indicate that the amount of coagulase produced by R as compared with the S variant is not proportionate to the difference in their growth.

On prolonged incubation the cell count of S variants sometimes rose and approached that of the R, but coagulase production lagged behind. Whenever older cultures of S

TABLE I

Growth characters of variants of Staph.aureus

Medium	R fast-clotting variant	S slow-clotting variant
Broth	Granular, with pellicle formation (fig. 1)	Uniform turbidity; n pellicle (fig. 1)
Heated-blood agar	Rough or irregular (fig. 2-4); or small, dull and opaque (small colonies of fig. 5); little or no chearing or discolouration of medium	Large, circular, glistening and smooth (large colonies of figs. 4 and 5); often discolouration and clearing of medium

TABLE II

Coagulase production of variants of Staph.aureus

Strain	Clotting time of original strain (minutes)	R or fast-clotting variant		S or slow-clotting variant	
		Clotting time (minutes)	Coagulase titre	Clotting time (minutes)	Coagulase titre
"5"	5	1	32,000	25	4
McC	15	1	32,000	25	256
McD	20	2	16,000	25	32
K	20	1	16,000	25	2
C	20	1	32,000	20	1,000
McA	20	1	16,000	25	2
W	20	1	32,000	16	2,000
M	20	1	16,000	16	1,000
Wils	20	1	8,000	25	2
Ken	20	2	16,000	25	32

The relation between clotting time and titre of coagulase varies with the strain.

The tire of coagulase was ascertained as described in the section on "Materials and methods". The figures are reciprocals of the highest culture dilution to show coagulase activity under the condition described.

In these three cultures inoculated from smooth colonies the relatively high production of coagulase was accompanied by the appearance of R variants.

TABLE III

Relation of coagulase production to dry cell weight in strain 5

Variant tested	Coagulase titre†	Dry weight of bacteria (g. per 100 ml.)
Rough	8000	0.0627
Smooth . . .	64	0.0382
Rough: smooth ratio	125:1	1.64:1

The figures in this table refer to 48-hour cultures grown in 40 ml. volumes with a large surface area.

The titre of coagulase was ascertained as described in the section on "Materials and methods". The figures are reciprocals of the highest culture dilution to show coagulase activity under the conditions described.

variants showed a rise in coagulase content, the broth had become granular and R variants could be found on plating.

Stability of variants

R variants produced S variants and vice versa, the rate of conversion differing from strain to strain. We always found that after one or more plate-broth-plate cycles, colonies of the opposite type appeared on the plate. With different strains, the number of such colonies ranged from 1 to 2 per plate to about 20 per cent. of the colonies present. On the whole, there seemed to be a greater tendency for S to be formed from R variants than the reverse. In three strains (C, W and M of table II) the tendency seemed to be in the opposite direction, and smooth colonies picked into broth often showed granular growth and gave high yields of coagulase. Plates spread from such broths revealed the presence of R colonies.

Changes in coagulase production within strains

As already mentioned, strains kept in the laboratory over a number of years often showed sudden changes in coagulase production. Our experiments demonstrated that, at the peak of coagulase production, strains yielded a high proportion of R fast-clotting variants and few or none during the phase when coagulase production was at its lowest level.

Observations with our strain 5 illustrate this point. An agar slope made from a typically granular broth culture was shown by plating to contain mainly rough variants. Subcultures made a few days later still gave granular growth in broth and a high yield of coagulase. Two months later, subcultures from the same slope gave smooth colonies on plates and uniform turbidity in broth with little coagulase production. The same change from R to S happened also with freeze-dried cultures of strain 5. Fourteen days after drying, subcultures gave rough colonies, granular growth in broth and a high yield of coagulase; but three months later they gave smooth colonies, uniform turbidity in broth and poor production of coagulase. The instability of these particular freeze-dried cultures may have been due to imperfect drying. For some strains which had considerably declined in coagulase production it was possible to restore this property; but only by repeated plating from old broth cultures were we able to isolate R fast-clotting colonies which on subculture gave high yields of coagulase.

To sum up, high yields of coagulase were always associated with the presence of R fast-clotting variants and low yields of coagulase with their absence.

Phage-typing

The instability of the variants strongly suggested that the starting material did not consist of a mixture of strains, but in order to check this point the variants of three strains were submitted for phage-typing. The results (table IV) confirm the view that, for each of the strains tested, the starting material indeed consisted of only a single strain.

Preservation of variants

Despite the instability of the variants it was possible to maintain them by daily platings on blood or heated-blood agar from colonies showing the characteristic morphology. Thus, over a period of four months the R fast-clotting variant of strain 5 was kept through 100 subcultures and the S slow-clotting variant through 50. It is important that subcultures should be made from plates not more than 24 hours old because, as already mentioned, after this time the colonies tend to lose their characteristic appearance. Also, on plating from old R colonies an increased proportion of S colonies was obtained.

TABLE IV

Phage-typing of the variants of 3 strains of Staph. aureus

Strain	Variant	Phage type
5	R (fast-clotting)	6/7/47/53/54
"	S (slow-clotting)	7/47/54
"	S (coagulase-negative)	6/7/47/54
McC	R (fast-clotting)	29/29A
"	S (slow-clotting)	29/29A
McD	R (fast-clotting)	53/76/77
"	S (slow-clotting)	53/75/76/77

These phage patterns are regarded as establishing close relationship between the variants within each particular strain.

DISCUSSION

The coagulase test used in the identification of pathogenic staphylococci is performed either on colonies from solid media or on broth cultures. In both instances a large population of organisms is tested. The problem therefore arises whether it is permissible to look on a positive result in this test as representative of the whole population; a few R fast-clotting variants might be present in a strain which consisted largely of coagulase-negative variants. On the other hand, a negative result need not exclude the possibility that the strain is capable of producing coagulase-positive variants; some basis for this exists in our finding of a coagulase-negative variant of strain 5. From our results there does not appear to be an unbridgeable gap between coagulase-positive and coagulase-negative staphylococci.

It seems unlikely that coagulase production and type of growth are the only characters in which the R and S variants differ. The intense clearing of heated-blood agar by S variants and preliminary results observed with a

chemically defined medium lead us to believe that the observed features reflect a basic metabolic difference between the two variants.

SUMMARY

1. From ten strains of Staphylococcus aureus, variants differing in respect of coagulase production and growth characters were isolated.

2. High yields of coagulase are associated with the R, and low yields with the S type of growth.

3. The variants are unstable, with a greater tendency for the R to become S than vice versa.

4. Changes in coagulase production observed in some of our strains may be related to the proportions of their R and S variants.

We wish to thank Drs Joan E. Rippon and R.E.O. Williams of the Central Public Health Laboratory, Colindale, for carrying out phage typing; Miss Joyce Edgar of the Blood Bank, Western Infirmary, Glasgow, for supplying us with specimens of plasma; Mr Frank Lonsdale of this Department

for his assistance in subculturing and selecting variants; and Mr David Colvin for the photographs. We are indebted to the Rankin Research Fund for a grant towards the expenses of this work.

REFERENCE

BIGGER, J.W., BOLAND, C.R. AND O'MEARA, R.A.Q. 1927. This Journal, xxx, 261.

PART TWO

FURTHER STUDIES ON ROUGH AND SMOOTH COLONIAL VARIANTS

The degree of roughness of rough variants ranges from colonies which are wrinkled and dry to those showing only slight irregularity of surface and outline; the difference depends not only on the strain but also, as has been shown, on the medium. A study of the influence of the medium on the morphology of these variants was undertaken both as a

matter of general interest and in an attempt to identify factors which would increase the roughness of rough variants and so facilitate their recognition.

The roughness of rough variants on nutrient agar is not marked, but in early investigations it was found that this appearance could be accentuated by the addition of blood, especially if the blood was heated at 80°C for 30 minutes. In more recent experiments, however, possibly due to an unidentified change in the basal nutrient agar, enrichment with whole blood was just as effective whether it was heated or not; it was also found that plasma was as active as whole blood and more active than washed red blood cells.

Roughness may also be increased, probably by a different mechanism, by the addition of certain chemicals to the medium. A substance known as Dispersol LN(ICI) was found to have an even greater effect on the morphology than blood (Figs. 6, 7 and 8). Dispersol LN does not inhibit the growth of Staph.aureus at a dilution of 1 in 800; it is a surface active agent and inhibits the swarming of Proteus (Lominski and Lendrum, 1942). The activity of this substance suggested the examination of other chemicals with

Rough and Smooth colonial variants grown on nutrient agar containing Dispersol LN.

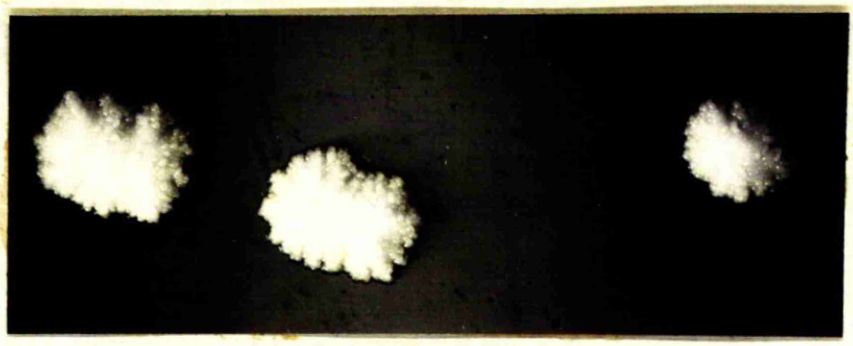


Fig.6. Rough variant of strain F after 48 hours growth. x2

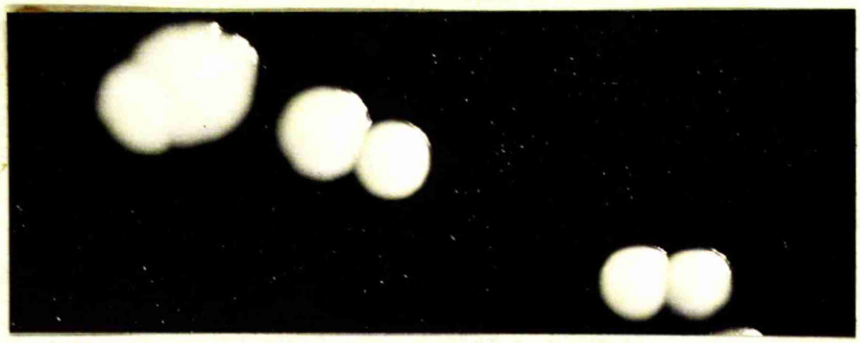


Fig.6. Smooth variant of strain F after 48 hours growth. x2

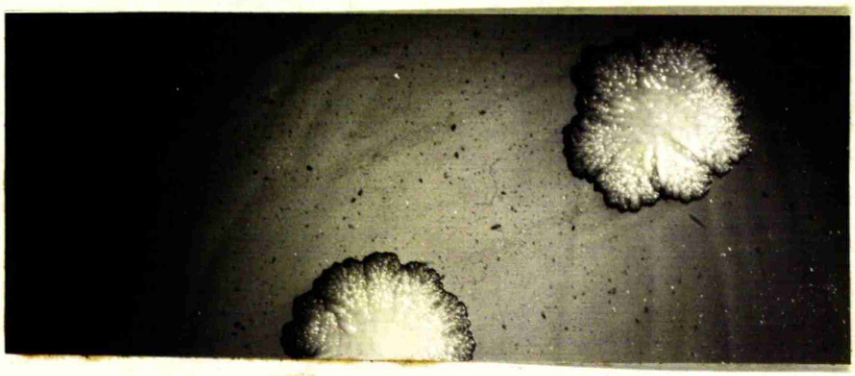


Fig.8. Rough variant of strain C13 after 48 hours growth. x2

surface tension lowering properties, but none of 10 anionic active agents, listed in table 5, or saponin, had any effect.

TABLE V

List of surface active agents tested for their effect on the colonial morphology of rough variants

Soaps R.COONa

1. Na caprylate
2. Na oleate
3. Na ricinoleate

Alkyl sulphates $R.O.SO_2ONa$

4. Na butyl sulphate
5. Na lauryl sulphate

Aryl and alkaryl sulphonates $Ar.SO_3.Na$

6. Alkylated aryl sulphonate (Santomerse D)
7. Complex stearyl alkyl sulphonate (Ultravon W)
8. Reduced form of 7 (Ultravon WA)
9. Aryl sulphonate (Invadine N)
10. as 9 (Invadine B)

These substances are some of those tested on strains of Proteus for antiswarming activity by Lominski and Lendrum (1942).

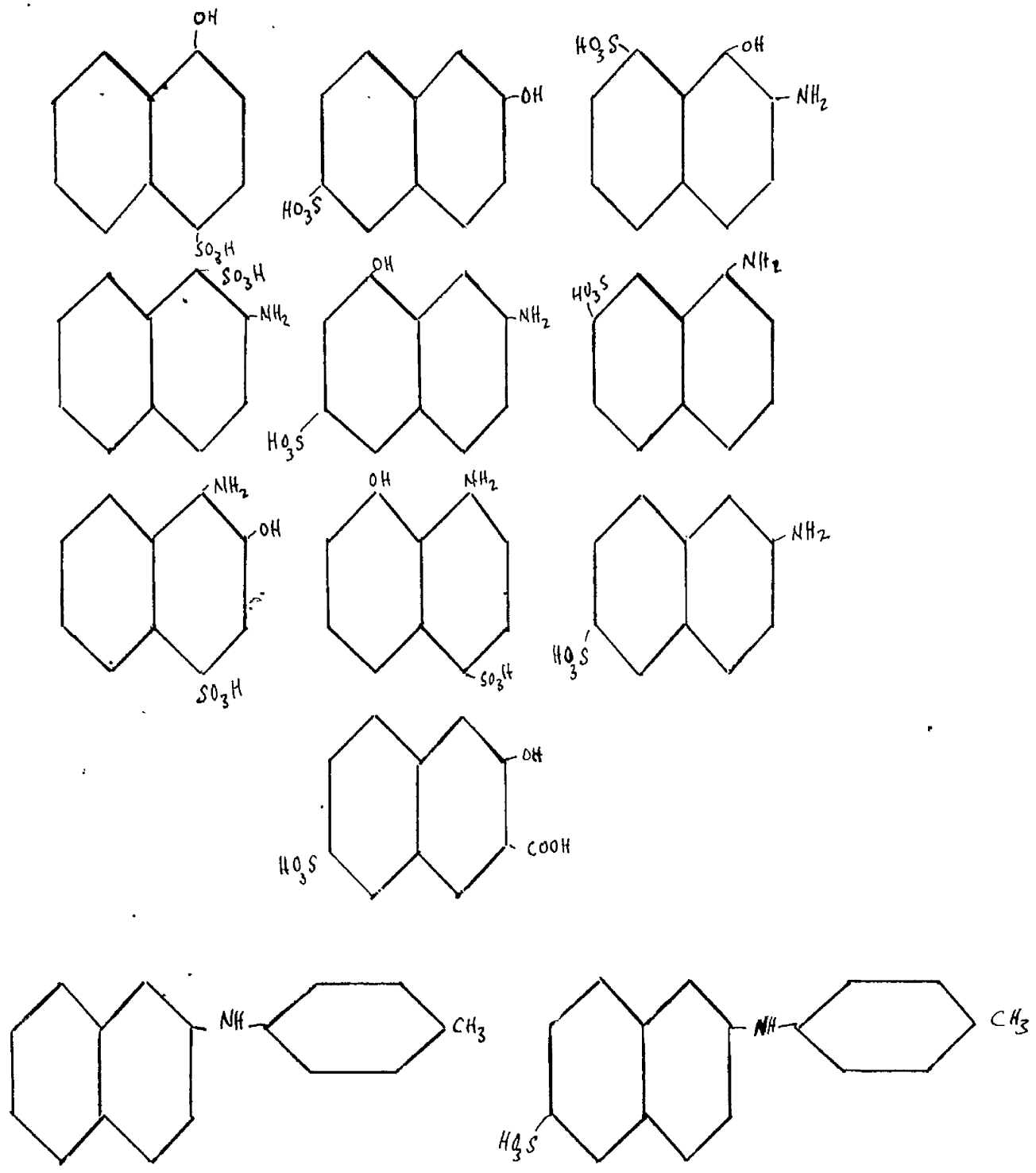
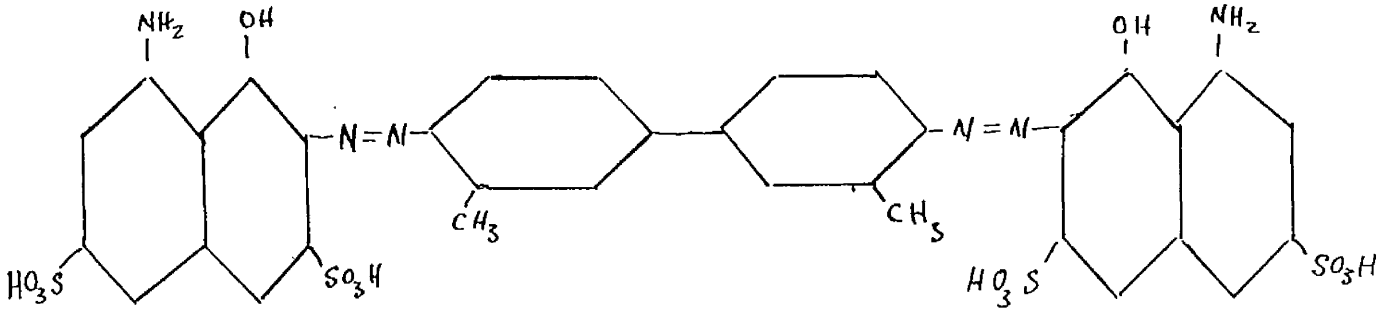
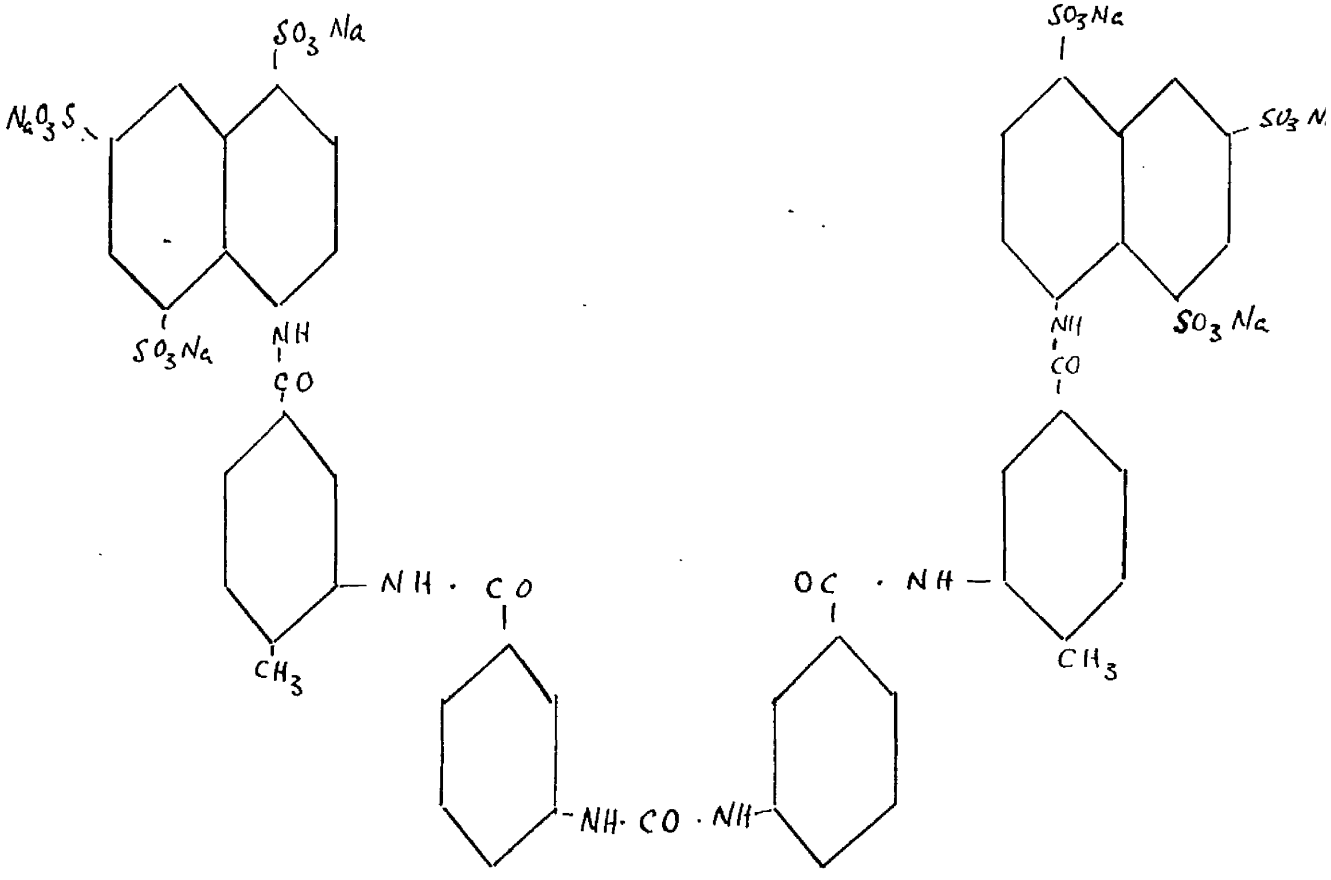


FIG.9. Structural formulae of compounds which did not increase the roughness of rough variants on solid media.



Trypan Blue



Suramin

FIG.10 Structural formulae of two compounds which increased the roughness of rough variants

With the failure of surface tension lowering agents to increase roughness attention was turned to the chemical structure of Dispersol LN. From ICI it was learned that the chemical structure of this material was still unknown but that it is best described as the sodium salt of β-naphthalene sulphonic acid and formaldehyde. A number of naphthylamine and naphthol-sulphonic acids, whose formulae are shown below (Fig. 9), were tried without any success. At the suggestion of Mr. A.F. Crowther (Imperial Chemicals (Pharmaceuticals Ltd.) the effect of Suramin (Antrypol) a naphthylenesulphonic acid derivative, which is widely used as a trypanocidal drug was examined. Although less striking in its effect than Dispersol LN, it accentuated the roughness of the variants. The activity of Suramin in this respect prompted the testing of other naphthalene sulphonic acid derivatives. Two substances found active were Trypan Blue and Chlorazol Fast Pink, dyes with trypanocidal activity from which the colourless Suramin was evolved (Sexton, 1953) (Fig. 10).

To sum up these investigations, it appears that increased roughness of <u>rough</u> variants may be produced not by simple naphthol or naphthylamine sulphonic acids but by larger compounds derived from them.

Incidence of Rough Variants.

Rough variants are rarely seen on direct plating of freshly isolated strains. Of several hundred strains, either freshly isolated or old laboratory strains of human and animal origin, only 4 have shown rough variants on direct plating. About 50 per cent of freshly isolated strains readily gave rise to rough forms after 7 days' growth in broth, but persistent examination demonstrated them in a much higher proportion. The use of Dispersol LN has facilitated the recognition of rough - smooth variation in more than 30 strains apart from the 10 already described in the first part of this section.

Comparison of corresponding rough and smooth variants of the same strain has so far shown that the rough variant always gives a higher yield of coagulase; conversely very high yields are indicative of the presence of rough variants. One exception to this is the high coagulase-producing strain Newman (Duthie); this strain on blood-containing media shows a smooth colonial morphology and even on Dispersol LN media, rough forms appear only irregularly and do not breed true.

Phage Typing of Rough and Smooth Variants.

Phage-typing was used to ascertain whether smooth

and rough variants belonged to the same strain. In 9 out of 10 strains tested both variants showed the same or a very similar phage pattern; this type of variation was observed in strains of each phage group and in one strain which was untypable. In one of the 10 strains (A), however, rough and smooth variants had different phage patterns; one possible explanation of this is that the strain originally was not pure and represented a mixture of types.

TABLE VI

STRAIN	TYPE	GROUP
FB	untypable	
FR	"	
C16 S	42D	
C16 R	42D	
5614 S	3B/3C/71	II
" R	3B/3C/71	II
5385 S	29+	I
" R	29+	I
C13 S	42D/54/77	
C13 R	42D/54/77	
5546 S	29/6/54/75	I & III
" R	29/6/54/75	I & III
5372 S	7/42E/54/70/73	III
" R	54	2
5480 S	42E	III
5480 R	42E	III
K S	29	I
KR	29/42E/54/73	I
A S	52A	I
A R	6/7/42E/54/73	III

The table records the results of phage typing <u>smooth</u> and <u>rough</u> variants from each of 10 strains.

I am grateful to Dr. Morag Timbury of this department for the information contained in this table.

BIOCHEMICAL ACTIVITY OF ROUGH AND SMOOTH VARIANTS

In examining the effect of smooth - rough variation on biochemical activity it was found that the fermentation of commonly used sugars was unaffected. Not only did both variants attack the same sugars but appeared to do so at the same rate. The most outstanding difference in biochemical activity between rough and smooth variants was in the liquefaction of gelatin; in the strain examine stable rough variants did not liquefy gelatin in 10 days when cultured at about 20°C, whereas most smooth variants caused marked liquefaction by the 5th day.

Biochemical activities of 19 smooth and rough variants may be seen in table 7.

TABLE 7

Strain	Lactose	Glucose	Sucrose	Mannitol	Dulcitol	Litmus	Gelat
5R	+	+	+	+	0	A	0
5AS	+	+	+	+	0	AC	+
5ABS	+	+	+	+	0	AC	+
16R	+	+	+	+	0	AC	+
16S	+	+	+	+	0	AC	+
KR	0	+	+	+	0	0	0
KS	0	+	+	+	0	0	+
5385R	0	+	+	0	0	0	
5385S	0	+	+	0	0	0	
5602R	+	+	+	+	0	A	0
5602S	+	+	+	+	0	A	+
5546R	+	+	+	+	0	A	0
5546S	+	+	+	+	0	A	+
AR	+	+	+	+	0	A	0
HR	+	+	+	+	0	A	0
BoyR	+	+	+	+	0	AC	0
5372R	+	+	+	+	0	AC	0
Oxford	+	+	+	+	0	AC	+
CN 56	+	+	+	+	0	AC	+

Table recording the fermentation of 1 per cent sugar solutions in peptone water and action on litmus milk, of 9 smooth and 10 rough variants after 48 hours growth at 37 C after 10 days growth at 20 C gelatin cultures were examined for liquefaction.

Sugars: + = acid, 0 = no change

Litmus milk: A = acid, AC = acid clot, 0 = no change

Gelatin: + = liquefied, 0 = not liquefied

It may be mentioned in passing that some smooth variants produced no measurable coagulase in 4-day broth cultures; some of these coagulase-negative variants were found to form an enzyme capable of destroying coagulase. This enzyme and another closely related to it were described in considerable detail by Lominski, Morrison and Smith (1955).

In Section III differences in haemolysin production between smooth and rough variants are described.

SECTION II

STAPHLOCOAGULASE ACTIVITY IN VIVO

There is indirect evidence that coagulase acts as an aggressin by exerting its clotting action on plasma. Experiments demonstrating the clotting action of coagulase in vivo are described.

This work has been accepted for publication and is now in press: British Journal for Experimental Pathology, 1958, (Smith and Johnstone). The extent of my own contribution to this joint work is defined in the preface of this thesis (page 2).

PAGES

STAPHYLOCOAGULASE ACTIVITY IN VIVO

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The ability of certain staphylococci to clot plasma was demonstrated as early as 1908 (Much), but it was many years before it became recognised that coagulase production was peculiar to Staph.aureus, and therefore could be used as a criterion of pathogenicity (Cruickshank, 1937). At the present time the in vitro production of coagulase is the commonest and most important test used to differentiate pathogenic and non-pathogenic staphylococci.

Menkin and Walston (1935) made the first attempt to determine whether coagulase production was merely an in vitro character or whether it also played a part in the mechanism of disease. Unlike streptococcal lesions which tend to spread, those due to Staph.aureus are characteristically localised. Menkin and Walston suggested that this localisation might be due to a fibrin barrier resulting from coagulase action. They failed, however, to show in vivo clotting by coagulase and concluded that although the barrier existed, it was due to toxic action on the surrounding tissues. Subsequently Fisher (1936) also failed to find

evidence of coagulase action *in vivo*.

Strong presumptive evidence for the *in vivo* action of coagulase came from the work of Wilson Smith and his colleagues (Hale and Smith, 1945; Smith, Hale and Smith, 1947), who found that plasma clotted by coagulase inhibits phagocytosis of staphlococci. They also demonstrated that clotting of plasma *in vivo* might be a pre-requisite for the establishment of staphylococcal infection. In their experiments strains of staphylococci capable of clotting human but not guinea-pig plasma did not cause more than a transient lesion in guinea-pigs, but when the organisms were injected at a site into which human plasma had been previously injected, multiple abscesses developped. Lominski and Roberts (1946) showed that the serum of many healthy people contained a substance which prevented coagulase clotting *in vitro*. This inhibitory substance was absent from patients who had recently suffered from a major staphylococcal infection, such as acute osteomyelitis. Sera with a high anticoagulase titre protected rabbits against experimental infection (Lominski, 1949). Coagulase inhibition by human serum was subsequently demonstrated by a number of other workers (Kaplan and Spink, 1948; Rammelkamp, Badger, Dingle, Feller and Hodges, 1949; Tager and Hales, 1948a). Finally it was found that animals actively

immunised against coagulase were also protected from infection (Boake, 1956).

In the face of all this evidence there can be little doubt that coagulase plays a part in staphylococcal infection, but there is no direct evidence that it does so by exerting its clotting action. The nearest approach to this is possibly the work of Tager (1954), who found that after the injection of massive doses of coagulase rabbits died with lowered fibrinogen levels in the blood.

We believe that the present work briefly reported earlier (Smith and Johnstone, 1956) provides direct evidence that coagulase may produce clotting in vivo. This clotting action was judged both by the estimation of rabbit blood fibrinogen levels before and after intravenous coagulase injections, and by the histological demonstration of intravascular fibrin deposits in rabbits after these injections.

MATERIALS AND METHODS

Coagulase. Three preparations of coagulase were used. Coagulases 1 and 2 were dried, purified preparations obtained from the well-known coagulase-producing strain Newman: they contained no alpha toxin, being non-haemolytic for rabbit red cells and producing no dermonecrosis in rabbits. Both were very potent: coagulase 1 at a dilution of 1 in 500,000

clotted human plasma diluted 1 in 10 in 1 hour; coagulase 2 was approximately four times more active. Although highly purified these preparations were not pure and contained only approximately 5 per cent of coagulase (Duthie, personal communication).

Coagulase 3 was a crude preparation from our own high coagulase-producing strain 5R. It was the supernatant of 4-day broth cultures and clotted human plasma at a dilution of 1 in 2000 in 1 hour. This preparation was used for *in vitro* titrations only.

Each of these coagulase preparations clotted not only human but also mouse and rabbit plasma. As measured by clotting times the potency against rabbit and human plasma was the same, but against mouse plasma it was about 16 times lower.

Coagulase injection. Each dose of purified coagulase was dissolved in 5 ml. of saline and slowly injected (10-20 minutes) into the ear vein of rabbits. In mice the coagulase, dissolved in 0.5 ml. of saline, was injected into the tail vein.

Animals. Chinchilla rabbits, weighing between 2 and 4 kilos, and the Porton Swiss White strain of mice were used.

Rabbits immunised against coagulase. Animals were immunised with a coagulase similar to coagulase 5; it was the

supernatant of 4-day fluid cultures of strain 5R. In addition to coagulase it also contained gamma lysin. Initially 0.2 ml. of this preparation was injected intracutaneously, followed at weekly intervals by 2 subcutaneous, and 3 intravenous injections, each of about 0.75 ml. The animals were bled 10 days after the last injection and the serum examined for its anti-coagulase titre. The preparation contained gamma lysin and intracutaneous injection produced skin necrosis: it was used as the immunisation was originally undertaken to obtain gamma antisera.

Titration of Coagulase Inhibition of Rabbit Sera. The titration was that devised by Lominski and Roberts (1946). Constant amounts of coagulase, together with doubling dilutions of the serum being tested, were incubated at 37°C for 90 minutes; thereafter human plasma was added to a final dilution of 1 in 10, and the highest inhibiting dilution of serum was recorded. Readings were taken at the time the control clotted.

Estimation of blood proteins. Fibrinogen was estimated by the biuret method described by Barry, Feeney and Geoghegan (1955), heparin was found to be preferable to oxalate as anticoagulant. The method of Gornall, Bardawill and David (1949) was used for the estimation of albumin and globulin.

Histological Demonstration of Fibrin. Rabbits died within 30 minutes of the intravenous injection of large doses of coagulase, i.e. 10 mg. of coagulase 1 or 5 mg. of coagulase 2, or were killed at 30 minutes after the injection of smaller doses. After preliminary fixation in 10% formol saline the organs were fixed for 24 hours in corrosive formol. Paraffin sections were prepared and stained by haemalum and eosin, Gram's method, Masson's trichrome, Picro-Mallory, eosin phloxine and tartrazine, and Liebs' phosphotungstic acid haematoxylin.

RESULTS

The intravenous injection of coagulase in rabbits resulted in a marked and very rapid fall in fibrinogen, with widespread intravascular clotting, affecting particularly the lungs.

Effect of Coagulase on Blood Fibrinogen Levels: Blood fibrinogen levels before and after the intravenous injection of coagulase 1 are shown in Table 8. The slow administration of small amounts had little general effect on the animals, but, if given quickly, they became very agitated and developed leg weakness for a short period. After the administration of a large amount the animals rapidly became extremely excited before collapsing: violent spasmodic leg movements and gasping respirations preceded death. In all animals

the puncture wounds in the ears bled profusely and bleeding was difficult to control.

After injection the blood fibrinogen level fell very rapidly, reaching its lowest point at about 30 minutes, then it quickly rose until by 48 hours it had reached approximately twice the original value. The level slowly returned to normal in 6 to 8 days (Table 8).

Blood samples were also examined electrophoretically and by this means the fibrinogen values obtained chemically were confirmed and it was also shown that fibrinogen alone was being estimated. Further, electrophoresis of the plasma after chemical removal of fibrinogen confirmed that the fibrinogen was being estimated completely.

A sample of coagulase 1, inactivated by autoclaving for 60 minutes, was found to have no *in vitro* clotting power. 6 mg. were injected intravenously with no apparent effect on the rabbit and its fibrinogen level was not altered significantly (Table 8).

Effect of Coagulase on Plasma Protein: In four animals the total plasma proteins were estimated, after injection of coagulase, daily for 8 days. The total protein value fell rapidly, with the fibrinogen, after intravenous coagulase 1 injection, but the fall was much greater (0.8 to 1.4 g./100 ml.) than could be accounted for by removal of fibrinogen

TABLE 8

Effect of coagulase I, on rabbit blood fibrinogen levels when injected intravenously

Rabbit number	Coagulase 1 (mg.)	Fibrinogen Level (mg/100 ml.) ZERO	30 MINS	1 DAY	2 DAYS	3 DAYS	4 DAYS	6 DAYS	Remarks
F2	2	270	95	400	460	360	370	265	Mild Reaction
F6	4.5	270	40	320	500	410	435	330	No Apparent
		+ (7.2)	(6.3)	(5.8)	-	(5.8)	(5.8)	(6.0)	Effect
F4	5	300	60	410	-	550	-	-	No Apparent Effect
D30	5	270	110	410	670	525	-	290	Severe Reaction
D31	10	185	25						Death
W6	10	480	18						Death
E54	6 Autoclaved	330	305						No Effect Autoclaved Coagulase 1.

These figures illustrated the changes in total protein value (mg./100 ml.) following intravenous coagulase injection.

+ Figs. in brackets are the total protein values (gms./100 ml.).

alone. Thereafter the total protein value slowly returned towards the original level. The albumin values remained constant and the fall was largely accounted for by a general decrease in globulins, particularly in the gamma globulin fraction.

Inhibition of Coagulase in vitro: The coagulase inhibitory titre of the serum of five normal rabbits ranged from 1 in 16 to 1 in 64, but in immune rabbits it was 1 in 1,000 or more. This inhibition refers to titrations against both coagulase 2 and the homologous coagulase 3.

Inhibition of Coagulase in vovo: Graded doses of coagulase 2 were injected intravenously into rabbits and it was found that the fall in fibrinogen 30 minutes after the injection was roughly proportional to the amount of coagulase injected. With 0.125 mg. of this preparation in normal rabbits approximately 60-70% of fibrinogen was removed from the blood in 30 minutes. However, when the same amount was injected into actively immunised rabbits whose sera inhibited coagulase the quantity of fibrinogen removed was very much less (15%, 29% and 48%).

Histological evidence of intravascular clotting: No macroscopic abnormality was seen in the organs of rabbits killed by, or after, the intravenous injection of coagulase. Microscopically, however, numerous and widespread intravascular

lesions were evident. The lungs were the most severely affected organs and, when the injections were given very rapidly and death occurred within 5 or 10 minutes, few lesions were seen in other viscera. If the coagulase was administered more slowly (15-20 mins.) the intravascular lesions were readily seen in other organs such as the kidneys and liver but were never as numerous as in the lungs.

The lesions consisted of intravascular deposits giving the typical staining reactions of fibrin. These deposits occluded capillaries and arterioles mainly, and, to a lesser extent, small and moderate sized arteries. The deposits were formed of multiple strands of fibrin arranged parallel to one another and to the long axis of the vessel which they occluded (Fig. 11). They were entirely intravascular, occasionally enmeshing small numbers of red cells: no evidence of damage to the vessel walls was seen.

In the lungs an enormous number of fibrin deposits occluded the majority of the vessels of capillary size (Fig. 12), and death appeared to be due to acute right heart failure following mechanical blockage of the pulmonary vasculature.

Effect of Coagulase on Mice: Doses of up to 2 mg. of the very potent coagulase 2 were injected intravenously into pairs of mice. After one hour one of each pair was killed.

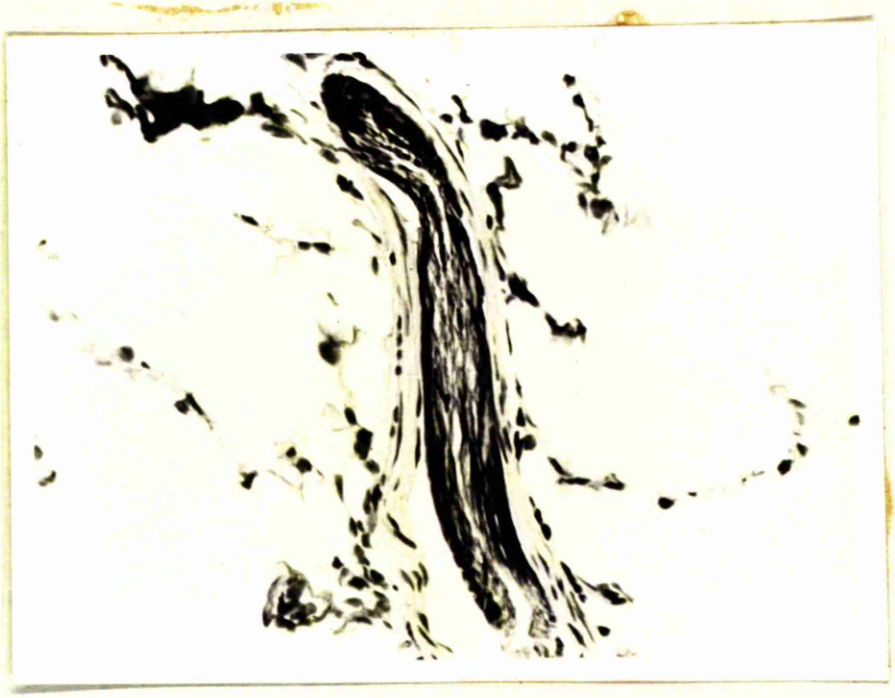


Figure 11: After intravenous coagulase injection the resulting fibrin deposits are formed of multiple strands, ranged parallel to one another and to the axis of the occluded vessel.

GRAM X 380

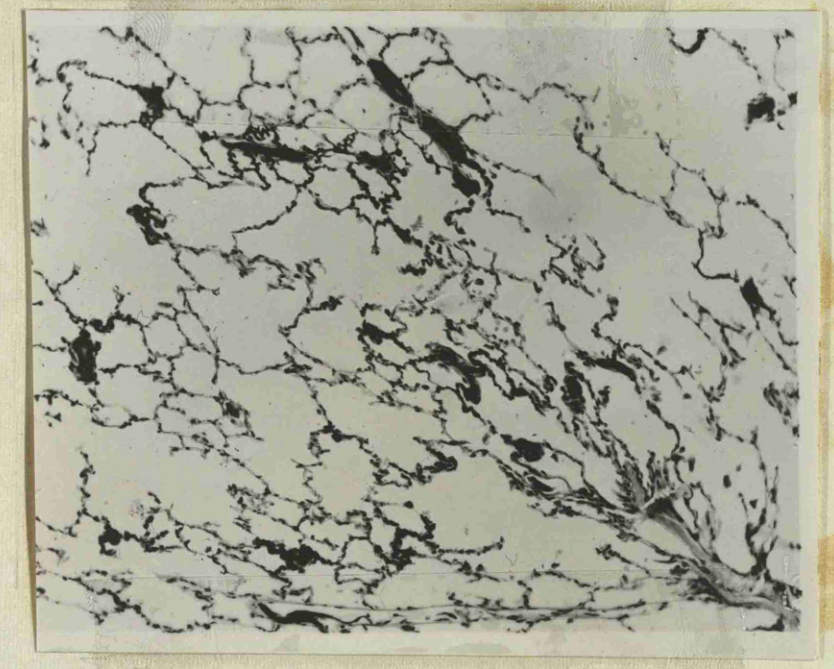


Figure 12: This representative lung field from a rabbit killed by intravenous injection of coagulase shows occlusion of the majority of capillaries and vessles by dark fibrin deposits.

MASSON'S TRICHROME X 105

The remaining mice survived apparently unaffected by the injection and no evidence of undue bleeding was noted. Microscopical examination of the organs of the mice which were killed failed to show any evidence of intravascular clotting, such as was seen in the rabbit, or any other abnormality.

DISCUSSION

The present experiments give direct evidence that coagulase causes clotting *in vivo*. The failure of some of the previous attempts to show this action may be related to the unsuitable route by which coagulase was introduced, i.e. subcutaneous, intracutaneous and intrapleural (Menkin and Walston, 1935; Fisher, 1936) and to the low potency of the coagulase used. Although Fisher used the intravenous route the coagulase injected was comparatively weak. Lack and Wailling (1954) injected intra-pleurally into rabbits plasma along with a potent purified coagulase similar to that used in the present work. They attributed their failure to show clotting *in vivo* to the action of staphylokinase present in the preparation of coagulase itself.

Since coagulase will clot heparinised or oxalated blood *in vitro*, it might have been expected that blood drawn shortly after the injection of massive doses of coagulase would clot despite the presence of an anticoagulant. The

fact that it did not do so suggests the absence of active coagulase; its dilution, neutralisation by antibodies, and fixation in the tissues may account for this.

In mice no histological changes, such as were found in rabbits, followed the intravenous injection of coagulase; the explanation of this lies probably in the relative deficiency of coagulase-reacting factor of mouse blood, approximately one hundredth that of rabbit blood, according to Tager and Hales (1948b).

The coagulase preparations used, although purified, were not pure. The possibility however, that some fraction other than coagulase was responsible for the in vivo clotting action is remote. Coagulase has a known in vitro clotting activity, and therefore it seems reasonable to assume that both the fall in fibrinogen level and the intravascular fibrin deposits are the results of the action of coagulase itself.

SUMMARY

Potent purified coagulase injected intravenously into rabbits produced fibrinogen depletion and intravascular clotting.

The fibrinogen level fell precipitously after injection, then rose rapidly, reaching twice the original level at

48 hours, and returned to normal in a week. The fall in fibrinogen was much less in rabbits immunised against coagulase.

No significant fall in fibrinogen was observed when coagulase, inactivated by autoclaving, was injected into normal rabbits.

Following intravenous coagulase injection into rabbits multiple fibrin deposits occluded many capillaries and small vessels: they were most marked in the lungs but occurred also in the liver and kidneys.

In mice, no changes were observed after the intravenous injection of coagulase.

We are indebted to Dr. E.S. Duthie (Royal South Hants and Southampton Hospital) for the generous gift of purified coagulase and to Dr. E.B. Hendry (Western Infirmary, Glasgow) and his staff for all the biochemical estimations.

BARRY, A.P., FEENEY, J.K., and GEOGHEGAN, F.J. (1955) Brit.med.J., 11, 12.

BOAKE, W.C. (1956) J.Immunol., 76, 89.

CRUICKSHANK, R. (1937) J.Path.Bact., 45, 295.

FISHER, A.M. (1936) Bull.Johns Hopk.Hosp., 59, 393.

GORNALL, A.G., BARDAWILL, C.J., and DAVID, M.M. (1949) J.biol.Chem., 177, 751.

HALE, J.H. and SMITH, W. (1945) Brit.J.exp.Path., 26, 209.

KAPLAN, M.H. and SPINK, W.W. (1948) Blood, 3, 575.

LACK, C.H. and WAILLING, D.G. (1954) J.Path.Bact., 68, 431.

LOMINSKI, I. and ROBERTS, G.B.S. (1946) Ibid., 58, 187.

MENKIN, V. and WALSTON, H.D. (1935) Proc.Soc.exp.Biol. (N.Y.), 32, 1259.

MUCH, H. (1908) Biochem.Z., 14, 143.

RAMMELKAMP, C.H., BADGER, G.F., DINGLE, J.H., FELLER, AE., and HODGES, R.G. (1949) Proc.Soc.exp.Biol., (N.Y.), 72, 210.

SMITH, D.D. and JOHNSTONE, J.M. (1956) Nature, 178, 982.

SMITH, W., HALE, J.H. and SMITH, M.M. (1947) Brit.J.exp.Path., 28, 57.

TAGER, M. (1954) Bull.N.Y.Acad.Med., 30, 475.

Idem and HALES, H.B. (1948a) J.Immunol., 60, 1.

(1948b) Yale J.Biol.Med., 21, 91.

SECTION III

SMOOTH - ROUGH VARIATION OF STAPHYLOCOCCUS AUREUS AND ALPHA AND GAMMA LYSIN PRODUCTION IN VITRO

PAGES

INTRODUCTION

The finding that smooth and rough colonial variants differed in coagulase production prompted a comparison of their haemolytic activities.

It would be difficult to survey in detail all the work on staphylococcal haemolysins; for an up to date account reference may be made to the most recent edition of Topley and Wilson (Fourth edition pages, 707-711) which also contains the relevant bibliography of the subject. Staphylococcal haemolysins have attracted attention ever since the experiments of Burnet (1929), which showed that an apparently single antigenic substance was responsible for the dermonecrotic and lethal action in rabbits, and also for the haemolysis of rabbit RBC. Burnet's substance (alpha toxin or alpha lysin), was followed by the discovery of two more antigenically distinct haemolysins, beta (Glenny and Stevens, 1935), and gamma (Smith and Price, 1938b), both also toxic for rabbits. A fourth haemolysin, named delta by Williams and Harper (1947) completes the list; according to Marks (1951) this appears to be identical with the alpha 2 of Morgan and Graydon (1956). It differs from the others in being non-antigenic and alcohol-soluble; although of low toxicity. Recent work by Gladstone and van Heyningen (1957), shows that

it acts as a leucocidin.

Little attention has been paid in the past to the effect of variation on haemolysin production. It is well known that strains differ greatly in the amount of alpha lysin produced (e.g. Burnet 1930; Parish and Clark, 1932; Christie, North and Parkin, 1946; Marks, 1951; Elek and Levy, 1954) and in the pattern of haemolysins produced (Glenny and Stevens, 1935; Bryce and Stevens, 1936; Elek and Levy, 1950). One of the earliest references to variation in haemolysin production is that of Burnet (1930) who noticed that albus variants, corresponding to those described by Bigger, Boland and O'Meara (1927), unlike the aureus strains from which they derived, gave high yields of alpha lysin in the absence of additional CO_2. Rountree (1936), and more recently Elek and Levy (1954), drew attention to frequency of variation in haemolysin production among strains of Staph.aureus.

In the present work attention was at first focussed on alpha lysin. Alpha lysin is generally considered to be linked with pathogenicity: it is the most potent of staphylococcal toxins, antisera to it provide a degree of protection against experimental infection (Burnet, 1929; Downie, 1936), and a very high proportion of strains isolated from human lesions produce it (Bryce and Rountree, 1936;

Schwabacher, Cunliffe, Williams and Harper, 1945; Elek and Levy, 1950). It has even been suggested by Marks (1952) that it should replace coagulase as the most important criterion of pathogenicity, since all or nearly all strains from human lesions produce alpha lysin; the proportion of strains from carriers which produce it is much lower.

Gamma-lysin production is frequently associated with that of alpha lysin, optimum conditions for production are similar (Smith and Price, 1938b), and both are estimated by their action on rabbit RBC. In the course of the present work gamma lysin relatively free of other lysins became available, so the opportunity was taken to examine some of its properties.

Much less attention was paid to beta and delta-lysin production. Where beta lysin was produced, it was measured quantitatively, delta lysin was measured only qualitatively.

MATERIALS AND METHODS

Strains. Sixteen strains of human and animal origin were used; they included 'Newman' (the high coagulase-producing strain of Duthie) CN 56 (Burroughs-Wellcome) also known as Wood 46, a high alpha toxin producer, strain Y2 (NCTC 5664) used by Smith and Price (1938b) and later by Marks (1951) for the production of gamma lysin, and strains C13 and C16 (Elek) two alpha-lysin producers; of the remaining twelve strains some had been cultured *in vitro* for more than a year and some were newly isolated. A total number of 14

rough colonial variants and 15 smooth variants were obtained (see Section I); from some strains more than one haemolytic type of smooth variant was obtained.

Haemolysin preparation. Haemolysins were prepared by the method devised by Burnet (1930) for the production of alpha lysin and used later for the production of beta-lysin (Bryce and Rountree, 1936), and gamma lysin (Smith and Price, 1938b). Petri dishes containing meat extract with agar at a concentration of 0.85 per cent were heavily inoculated with organisms from 24 hour agar slope cultures suspended in broth, and incubated in a mixture of air and 20 per cent CO_2 for 24 hours. The Petri dishes were emptied into a large beaker and the agar broken up; saline 2 ml. per Petri dish was added; after the culture had stood for 4 hours at 10 C, the fluid was removed by straining through several thicknesses of cheese cloth and the cells were removed by centrifugation. The preparation was stored frozen at -20 C.

Alternatively haemolysin was prepared by inoculating 5 ml. amounts of meat extract broth in 4 x $\frac{1}{2}$" test-tubes. After incubation at 37 C for 3 to 5 days the cells were removed by centrifugation, and the supernatant containing the haemolysin was stored by freezing at -20 C. The preparations from broth cultures although more easily obtained,

had lower titres than those obtained by Burnet's method.

Preparations of Monovalent alpha and gamma antisera. Since both alpha and ga ma haemolysins were measured by the lysis of rabbit RBC the extent of lysis in titrations of mixtures containing both would be determined by which ever one is in excess. By obtaining antisera which neutralised only one of these lysins it was possible to determine the titre of each haemlysin in the presence of the other.

Gamma antiserum was prepared by immunising rabbits against gamma lysin prepared from strains producing little or no alpha lysin. 0.2 ml. of lysin from strain 5R was injected intracutaneously into Chinchilla rabbits, followed at weekly intervals by 2 subcutaneous and 3 intravenous injections, each of about 0.75 ml. The animals were bled 14 days after the last injection and the serum examined for its gamma antibody content. 1 ml. of this serum neutralised 2,000 MHD of gamma lysin but did not neutralise 10 MHD of alpha lysin.

Alpha antiserum was prepared by removing the gamma antibodies from a serum which contained antibodies to both alpha and gamma lysin. The serum used for this purpose was EX.1644 (Burroughs Wellcome) which was found to contain in each ml. sufficient antibody to neutralise 160,000 MHD of alpha and 5,000 of ga ma lysin. Gamma lysin containing 500 MHD per ml. was added until flocculation occurred; after allowing

the mixture to stand for 18 hours at 4°C the floccules were removed by centrifugation. It was found that about 6,500 MHD gamma lysin had to be added to each ml. of serum EX 1644 in order to obtain a serum of which 1 ml. neutralised 8,000 MHD of alpha lysin and only 16 of gamma lysin. The ratio of alpha/gamma had been increased from 32/1 to 500/1. For practical purposes, especially as it was used diluted, this serum can be referred to as monovalent.

Beta antiserum. For the present investigations the alpha antiserum also served as beta antiserum, since serum EX 1644 contained not only alpha and gamma but also beta antibody. The beta antiserum required was one which neutralised beta but not gamma lysin. The content of alpha antibody was immaterial since the haemolysin preparation against which it was used contained practically no alpha lysin.

Identification of alpha and gamma lysins by Plate Method. The method was that described by Gillespie and Simpson (1948) with the modification that monovalent antisera were used in addition to the usual polyvalent sera containing both alpha and gamma antibody. Nutrient agar plates containing 5 per cent washed rabbit RBC were prepared and on to them was laid a strip of filter paper $\frac{1}{2}$" wide soaked in (1) alpha monovalent serum, (2) gamma monovalent serum, or (3) alpha and gamma antiserum (serum EX 1644). When

dry, plates were inoculated by streaking out variants at right angles to the strips of paper. After incubating at 37°C for 24 hours in a mixture of air and 20 per cent CO_2 examination for lysis was made.

Estimation of alpha and gamma lysin by the dilution method

Serial doubling dilutions of haemolysin preparations were made in 10 per cent broth in saline (Burnet, 1931) and to this was added three times washed rabbit RBC in saline to make a 1 per cent suspension. In each tube was a total volume of 1 ml. consisting of 0.25 ml. lysin dilution, 0.25 ml. saline or antiserum, and 0.5 ml. of RBC. In each tube Thiomersalate (B.D.H.) was present at a concentration of 1 in 10,000. Readings of haemolysis were made after 1 hour at 37°C.

A titration of haemolysin against rabbit RBC alone does not distinguish between alpha and gamma lysin when both are present in a preparation. In the present work there were preparations in which

a) Alpha was greater than gamma

b) Gamma was greater than alpha

c) Alpha and ga ma were equal

In order to determine the titre of gamma or alpha it was necessary to neutralise the lysin not being estimated by using

the appropriate antiserum. This was done by introducing into each tube of a titration, sufficient of either of the two monovalent antisera to neutralise the maximum amount of lysin shown to be present in a titration containing no serum.

With the preparations used, haemolytic activity was completely abolished when alpha and gamma lysins were both neutralised. No lysis which could be attributed to delta lysin was found.

RESULTS

In preliminary experiments smooth and rough colonial variants of two strains only, 5 and F, were examined for haemolysin production.

HAEMOLYSIS OF RABBIT RED BLOOD CELLS

Assessment by the plate technique. Both the smooth and rough variants of the two strains gave zones of haemolysis on rabbit RBC agar plates, but they differed in the extent and manner in which they lysed the RBC. Smooth variants caused a wide zone of hazy lysis with a narrow clear zone within it; with the rough variants the zone of lysis was narrow and clear. In each case the lysis was inhibited by a therapeutic, refined staphylococcal antiserum, RA 362A, of the

type recommended by Gillespie and Simpson (1948) and by Elek and Levy (1950).

Assessment by Tube titration. Tube titrations of smooth and rough lysins (prepared by Burnet's method or by growth in broth), against rabbit RBC showed quantitative and qualitative differences.

Smooth lysins showed two zones, the first consisting of the lower dilutions in which haemolysis was complete, succeeded by a zone in which the amount of lysis gradually diminished with increasing dilution. The appearance of the rough titration was different; there was only one zone, in which haemolysis was complete, and it ceased abruptly. The zone of complete lysis (100 per cent lysis) with smooth variants occurred to the same titre as the lysis of the rough variants; the titre of the additional zone of incomplete lysis (more than 50 per cent lysis), of the smooth variants was about 16 times higher.

Comparing the appearances of the plate tests with the tube titrations, it seems possible to look on the zone of partial lysis in the titration of the smooth preparations as corresponding to the zone of hazy lysis in the plates.

Another difference in the titrations was the rate at which lysis occurred. The progress of rough lysis is much faster than that of the S lysis (Table 9) even when the titre of the smooth lysin is much higher than that of the rough lysin.

TABLE 9

Time (mins)	Smooth lysin		Rough lysin	
	50% lysis	100% lysis	50% lysis	100% lysis
$2\frac{1}{2}$	0	-	16	-
5	4	-	64	-
$7\frac{1}{2}$	32	4	128	64
10	64	4	"	"
$12\frac{1}{2}$	128	32	256	128
15	256	"	"	"
$17\frac{1}{2}$	"	"	"	"
20	"	128	"	"
$22\frac{1}{2}$	500	"	"	"
25	"	"	"	"
$27\frac{1}{2}$	1,000	"	"	"
30	"	256	"	256
35	"	"	500	"
45	2,000	"	"	2
60	4,000	500	500	500

Table 1 records the rate of progress of lysis of rabbit RBC at 23°C caused by a preparation from a smooth and a rough variant.

The figures show the reciprocal of the titres causing 50% lysis and 100% lysis.

IDENTIFICATION OF HAEMOLYSINS

Identification of haemolysins using a polyvalent antiserum

Preliminary examination of smooth and rough haemolysins was undertaken using the refined serum RA 363A, which like other sera prepared against alpha lysin contains not only alpha antibody but also gamma antibody (Smith and Price, 1938b Marks, 1951). As in the plate tests the undiluted serum neutralised the lytic action of both smooth and rough haemolysin preparations in tube tests; however, it was noticed that at higher dilutions, while still inhibiting the smooth haemolysins, it failed to neutralise those of the rough variants. In terms of MHD, the serum neutralised eight times more smooth than rough haemolysin; the two haemolysins thus appeared antigenically distinct.

Smooth haemolysin preparations. Both the appearance (hazy haemolysis) on the plate and the high titre in the tube titrations suggested that the haemolysin from the smooth variants was alpha lysin. The identity of the smooth haemolysin with alpha lysin was shown when it was found that the serum neutralised the haemolysins of 5A$_{\sigma}$ and FABS to the same extent as that of Wood 46 which is generally regarded as the standard alpha lysin.

It was subsequently shown, however, that Wood 46, contrary to the finding of Marks (1951), also produces gamma lysin, if only to a titre sixteen times less than that

of alpha lysin. Since 5AS and FABS preparations also contain gamma lysin in the same proportion to alpha as those of Wood 46 and since the serum used contained enough gamma antibody to neutralise it, the conclusion that the two smooth variants produce mainly alpha lysin remains valid.

Rough Haemolysin Preparations. It was assumed at this stage that the haemolysin of the rough variants, not neutralised to the same extent as the smooth haemolysins by serum RA 362A, and therefore antigenically distinct, was gamma lysin. Again, neutralisation tests showed that, at least predominantly, the lysis of rabbit RBC by variants 5R and FR was indeed due to gamma lysin. For a standard of comparison gamma lysin was prepared from strain Y2, (used for this purpose, first by Smith and Price (1938b) and subsequently by Marks (1951). At the same dilution serum RA 362A neutralised the same number of MHD of the haemolysins from the rough variants of strains 5 and F, and of the standard gamma lysin from strain Y2.

As in the case of the smooth variants, it was later found, and evidence will be presented, that the rough variants produce both alpha and gamma lysins.

Identification of haemolysins using monovalent antisera

Antisera. Glenny and Stevens (1935) showed that, of two haemolysins (alpha and beta) present in a mixture, the titre of either of them can be determined after neutralisation

of the other component with the appropriate antiserum. With a modified technique, Marks (1951) found gamma lysin in 20 of 23 strains although they produced more alpha than gamma lysin. He used a serum adjusted so that in one reacting dose there was, in addition to excess of alpha antibody, 1 unit of gamma antibody. The amount of gamma lysin in a preparation was expressed in terms of the smallest volume not neutralised by 1 unit of antiserum.

In the present experiments two monovalent antisera were used, one containing alpha the other gamma antibody (for details see Materials and Methods page 65). These sera may be referred to again at this point. Monovalent alpha antiserum was obtained from serum EX 1644 by removing from it gamma antibody by flocculation with gamma lysins from the rough variants 5R and FR. Such serum lost its capacity to neutralise the standard gamma lysin from Y2. Similarly, flocculation with the known gamma lysin of strain Y2 removed from the same serum the antibodies for the haemolysins of 5R and FR. These experiments provided a monovalent alpha antiserum, although in practice it was found that after flocculation there still remained a small amount of gamma antibody, but alpha antibody was in great excess. The experiments also demonstrated the antigenic identity of gamma lysin with the dominant component of 5R and

FR haemolysin.

Monovalent gamma antiserum was obtained by immunising a rabbit against haemolysin from the rough variant of strain 5. The serum neutralised gamma lysin of strain Y2, but not alpha lysin of Wood 46. The absence of alpha antibody in the serum prepared against this haemolysin is rather surprising, as 5R produces some alpha lysin (see page). A possible explanation is the small yield of alpha lysin by this variant in vitro; the alpha titre is usually 1/64 which is about 128 times less than that of a similarly prepared haemolysin from Wood 46. It is probable that more prolonged and efficient immunisation would have led to the appearance of alpha antibodies.

Identification of alpha and gamma lysins by plate method

In the plate test for staphylococcal haemolysins (Gillespie and Simpson, 1948; Elek and Levy, 1950) it is customary to use antisera which contain both alpha and gamma antibody. In the present experiments monovalent alpha and gamma antisera were used instead.

As already mentioned the two smooth variants cause two zones of lysis on rabbit RBC agar plates; an outer hazy and an inner clear zone; monovalent gamma antiserum neutralised the inner clear zone but not the outer hazy zone. A polyvalent serum, such as RA 362A of high alpha and gamma antibody content, caused a marked neutralisation of both

zones. Occasionally with these two variants and some others which were tested, there remained a narrow zone of uninhibited lysis, almost certainly due to delta lysin (Williams and Harper, 1947; Elek and Levy, 1950).

The two rough variants caused a single clear or slightly hazy zone inhibited by gamma but not by alpha antiserum. As will be shown by the tube method, alpha lysin was also produced, but its presence would not readily be detected since the amount produced was very small. Other rough variants caused two zones of lysis which could be separately inhibited by the two antisera, suggesting that they produced appreciable amounts of both lysins, (see figs. 13, 14 and 15).

The findings of these plates were confirmed and extended by tube titrations which will now be described.

Titrations of haemolysins in presence of monovalent antisera

Smooth and rough variants, isolated from 8 strains were examined for alpha and gamma lysin using monovalent antisera. The principle applied was to neutralise the dominant lysin by the corresponding monovalent serum and to identify the residual lysin with the other serum. When lysis was not reduced by either of the monovalent sera singly but neutralisation was complete with both sera used together, it was taken that alpha and gamma lysins were present to the same titre.

Plate tests for alpha and gamma lysins on nutrient agar plates containing washed rabbit RBC, using polyvalent and monovalent antisera.

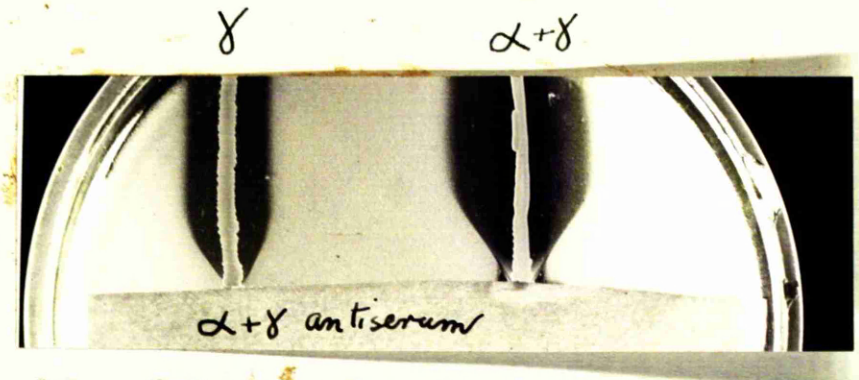


Fig. 13. Polyvalent antiserum containing alpha and gamma antibodies neutralises the hazy zone of the alpha gamma strain and the single zone of the gamma producing strain. The small amount of uninhibited lysis caused by the alpha gamma strain is presumed to be due to delta lysin.

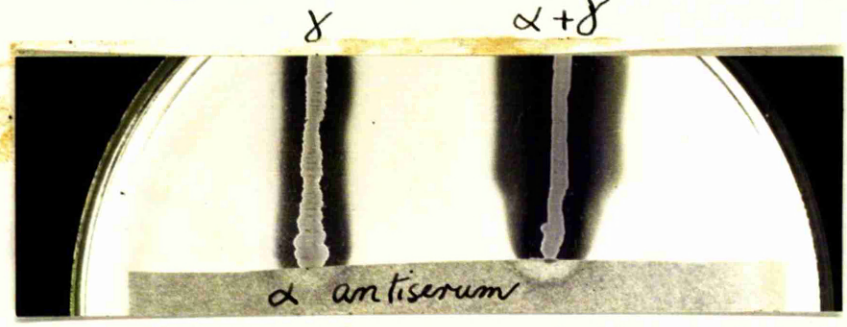


Fig.14. Alpha antiserum neutralises the outer zone of lysis caused by the alpha gamma producing strain, but not the inner clear zone nor the lysin caused by the gamma lysin producing strain.

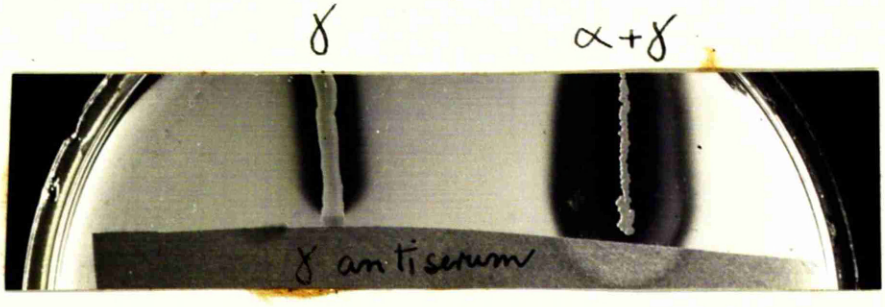


Fig. 15. Gamma antiserum neutralises lysis of the gamma lysin producing strain, but only the internal clear zone of the gamma strain.

Smooth haemolysin preparation. The preparations of the smooth variants were titrated and their alpha and gamma titres are shown in Table 10. All but one were found to produce both lysins; the ratio of alpha to gamma, as may be seen, was between 32/1 and 8/1, except in the preparation AS where the ratio was 1/1. One strain only, with a comparatively low alpha titre produced no detectable gamma lysin.

It may be noted that the variant 5AS is of the type described by Burnet (1930), namely it is an albus and gives high yields of alpha lysin in the absence of additional CO_2.

Table 10

Variant	alpha lysin titre (reciprocal)	gamma lysin titre (reciprocal)
(Smooth)		
FABS	8000	256
5AS	16000	500
C13S	8000	500
C16S	8000	500
5385S	4000	128
5546S	4000	128
5602S	128	16
AS	500	500
(Rough)		
FR	8	1000
5R	64	500
C13R	500	500
C16R	1000	256
5385R	128	256
5546R	128	256
5602R	32	128
AR	32	500

Table 10 records the production of alpha and gamma lysins by smooth and rough variants.

Rough haemolysin preparations. The alpha and gamma lysin titres of 8 rough preparations are listed in table 10. It may be seen from the table that all rough variants are strikingly similar in the amount of gamma lysin produced, the maximum difference being as from 1 to 4. There are marked differences in alpha lysin production which ranges from traces in variant FR to high titres in C13R and C16R. The alpha to gamma ratio ranges from 4/1 to 1/128.

Summing up these experiments, rough and smooth variants both produce alpha and gamma lysin; in smooth variants alpha lysin tends to predominate, whereas in the rough variants the major lysin is more often gamma lysin.

COMPARISON OF PROPERTIES OF GAMMA LYSIN FROM ROUGH VARIANTS WITH THAT FROM THE STANDARD STRAIN Y2

It was thought interesting to ascertain whether gamma lysin from rough variants, identical antigenically with that from the standard strain, corresponded in other properties. For this purpose a preparation from 5R was used.

Gamma lysin from 5R was found heat labile, being destroyed in less than 20 minutes when heated to 56°C at pH 7.5. It was almost completely lost on filtration through Seitz or kieselguhr filters. Its haemolytic activity was destroyed by 0.5 per cent formaldehyde in 24 hour. In each of these respects it corresponds with the gamma lysin described

by Smith and Price (1938b).

INHIBITION OF GAMMA LYSIN BY BETA LYSIN

It is well known that the lytic action of alpha lysin on sheep RBC is inhibited by beta lysin (Christie and Graydon, 1941; Elek and Levy, 1954). Since gamma lysin closely resembles alpha lysin, except antigenically, it was thought interesting to find out whether the similarity extends to inhibition by beta lysin. It was especially tempting on account of an observation made while comparing gamma lysin of 5R with that of strain Y2. As already described (see page 72) the two variants show an antigenic similarity which allowed the predmoninant lysin of 5R to be identified as gamma lysin; however, whereas gamma lysin of 5R lyses sheep RBC after 1 hour at 37°C, the lysin of the standard Y2 did not. Of the two only Y2 produces beta lysin; it seemed therefore, a likely explanation that beta inhibited the action of gamma lysin with regard to sheep RBC. The experiments described below appear to substantiate this hypothesis.

Inhibition of gamma lysin by beta lysin was shown in two ways: directly, by adding beta lysin to gamma lysin and observing inhibition, and indirectly, by neutralising with a monovalent beta antiserum, beta lysin in a mixture of beta and gamma lysins and observing the action of gamma lysin.

Effect of beta lysin on the lysis of sheep RBC by gamma lysin

Plate tests. Grown at 37°C variants producing only gamma lysin cause a narrow zone of lysis of sheep RBC neutralised by gamma monovalent serum. Variants which produce both gamma and beta lysin cause not lysis, but a broad zone of discoloration characteristic of the action of beta lysin on sheep cells in plates at 37°C (Naidu, 1934; Bryce and Rountree, 1936; Christie and Graydon, 1941; Elek and Levy, 1950); lysis due to beta lysin occurs only on cooling. The technique used to domonstrate the inhibition of gamma lysin is the same as that used by Elek and Levy (1954) to demonstrate the inhibition of alpha by beta lysin. A pure gamma variant was stroked out on a plate at right angles to a beta gamma variant; the zone of gamma lysis was inhibited where it passed through the zone of discoloration of the beta lysin (see figure 16).

Tube titrations. Two gamma lysin preparations (variants 5R and Boy R) titrated against sheep cells at 37°C caused lysis at a dilution of 1 in 32; lysis was abolished by gamma antiserum. These two preparations were again titrated, but to each dilution was added 0.25 ml. of beta lysin, prepared by heating to 56°C for 25 minutes a preparation of Y2 to remove the gamma lysin (Smith and Price, 1938a). In

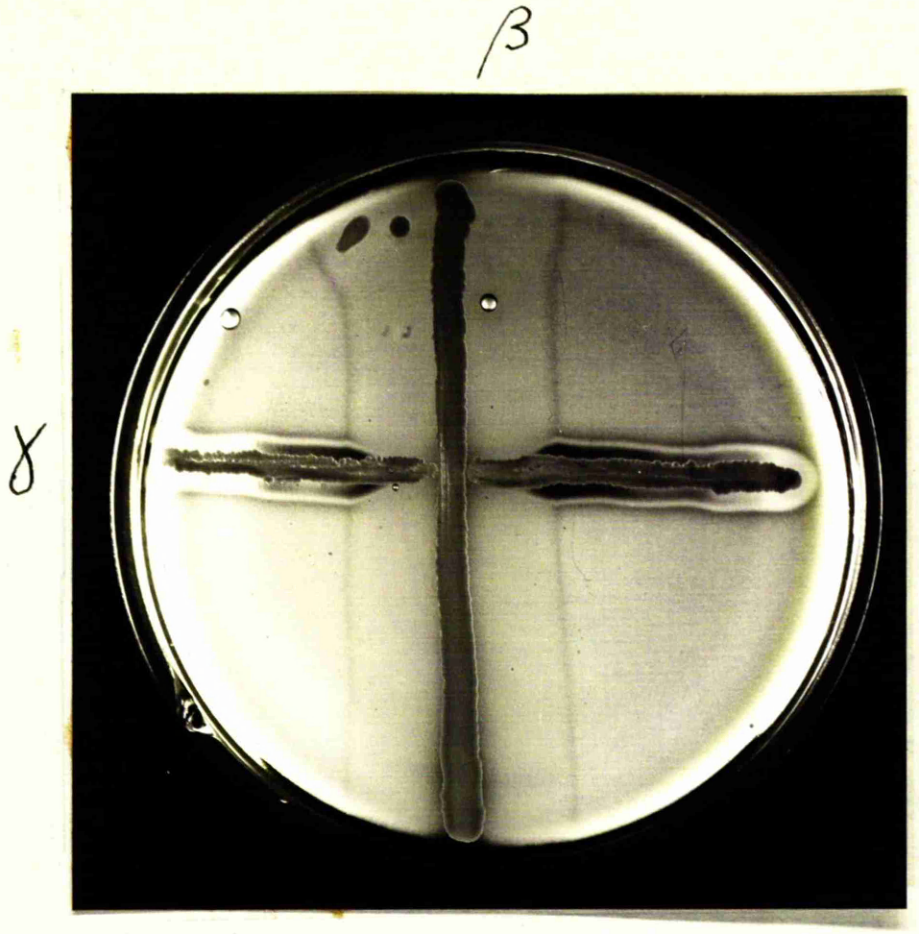


Fig. 16. Gamma lysis of sheep RBC inhibited by beta lysin.

the presence of beta lysin gamma lysin did not lyse sheep RBC.

Demonstration of gamma lysin activity by neutralisation of beta lysin with monovalent antiserum

Plate tests. As just described rough variants producing both gamma and beta lysin cause only a zone beta discoloration at 37°C. Five such variants were stroked out at right angles to a filter strip containing monovalent beta antiserum. Where the effect of the beta lysin was neutralised, a zone lysis appeared.

Tube titration. A preparation containing both beta and gamma lysin (variant FR) was titrated against sheep RBC; there was no lysis after 1 hour at 37°C. In a parallel titration each tube contained 0.25 ml. of monovalent beta antiserum, prepared by flocculating out gamma antibody from serum EX. 1644 which contains a moderately high content of beta antibody; lysis occurred to a titre of 1 in 32, but there was no lysis in a titration to which had been added both gamma and beta antibody. The monovalent beta serum alone caused no lysis.

This last experiment has already been performed by Smith and Price (1938b) with the strain Y2, a beta-gamma strain. They found that it lysed sheep RBC when beta lysin was neutralised by a monovalent beta antiserum. On the other hand Marks (1951), who did not neutralise beta lysin,

reported strain Y2 preparations as having no action on sheep RBC at 37°C.

The present experiments not only confirm the findings of Smith and Price and Marks, but also account for the discrepancy of their results. The direct experiment in which gamma lysin failed to attack sheep RBC when beta lysin was also present gives a clear explanation of the phenomenon. The explanation is that, like alpha lysin, gamma lysin is inhibited by beta lysin.

STABILITY OF VARIANTS

All the variants remained stable in haemolysin production over a period of two years, when subcultured from plate to plate. The stability was tested in two ways. Firstly by plating on nutrient agar containing rabbit or sheep RBC and noting the extent of the zone of lysis round the individual colonies. Numerous such examination did not reveal differences within the cultures with regard either to the type of cells lysed or the extent of the zones. The second method was to measure the alpha and gamma lysin production of the variants by titrating preparations obtained at intervals over two years. No variation in either alpha or gamma lysin production was observed. Beta lysin production was not so often tested but here again no instability was observed.

As will be described below (page88) broth cultures of the

variants were not stable and gave rise to variants whose haemolytic activities differed from the parent.

HAEMOLYSIN PATTERN

Table 11 shows the haemolysin production of smooth and rough variants; alpha, beta, and gamma lysins have been measured quantitatively, delta lysin qualitatively. In addition to strains already included in table 10 are a number which are represented by only one variant (smooth or rough). Table 11 also contains variants of strain 5 and F which have not been mentioned hitherto. They will be discussed in some detail in the next chapter.

Table 11

	Haemolysins (reciprocals of titres)			(plate tests)
	alpha	gamma	beta	delta
FABS	8000	256	8000	+
FBS	128	32	128	0
FR	8	1000	4000	0
FRS	0	0	16	0
5AS	16000	500	0	+
5ABS	16000	256	8000	+
5AR	500	500	0	0
5R	64	500	0	0
5RS	0	0	32	0
C13 S	8000	500	0	+
C13 R	500	500	0	+
C16 S	8000	500	0	+
C16 R	1000	500	0	+
5385 S	4000	128	0	+
5385 R	64	256	0	+
5546 S	4000	128	0	-
5546 R	128	256	0	-
5602 S	128	16	0	+
5602 R	32	128	0	+
AS	500	500	0	+
AR	32	500	0	+
S variants from which no R variant was obtained				
CN 56	8000	500	0	+
G	4000	128	0	+
491	2000	64	0	+
R variants for which no haemolytic S variant was available				
3917	8	256	2000	+
Y2	8	256	2000	0
Boy R	16	500	0	0
HR	16	1000	2000	0
Newman	500	500	0	+

Table 3 recordes the alpha beta and gamma titres of preparations from 15 smooth and 14 rough variants. Delta lysin, as shown by the lytic effect of variants grown on agar containing washed horse RBC, is also shown.

The conclusion reached from the comparison of the pairs of smooth and rough variants from the same strain is that smooth to rough variation results in a total or partial loss of alpha lysin. The examination of strains represented by either a smooth or rough variant on the whole shows the same trend.

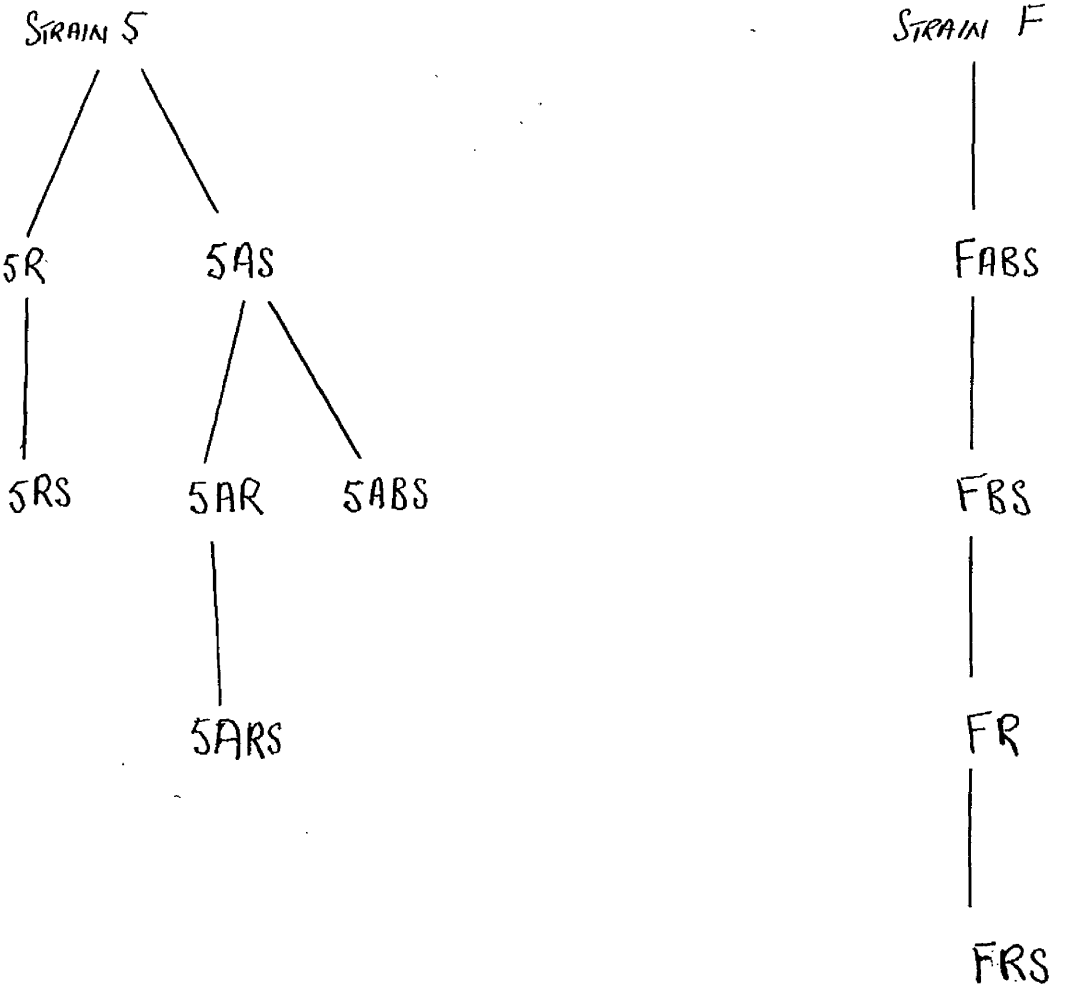
Information is too scanty to draw any conclusions regarding the effect of smooth to rough variation on beta or delta lysin production. Both smooth and rough variants may produce beta lysin to a high titre.

It is interesting to note that the classical alpha lysin producing strain, Wood 46, is smooth; like other smooth variants giving high yields of alpha lysin, it also produces gamma lysin. In conformity with the findings of the present work it is also of interest that the classical gamma lysin producer, strain Y2, is rough.

Another strain which should be commented on is strain Newman. Although usually smooth in morphology it is occasionally rough on solid media containing Dispersol LN (ICI) and shares with many of the rough variants the property of giving high yields of coagulase in vitro. As in morphology so in haemolysin production it does not fall into an extreme group, and produces both alpha and gamma lysin to the same titre.

DIFFERENCES IN HAEMOLYSIN PRODUCTION BETWEEN MORPHOLOGICALLY IDENTICAL VARIANTS OF THE SAME STRAIN

As may be seen from table 11 more than two variants, that is, a smooth and a rough were obtained from the two strains 5 and F. The following is a short account of the isolation of these variants. Diagram 1 shows the origin of the variants.



Strain 5 is an old laboratory strain; it gave rise to a rough variant, 5R, characterised by very high yields of coagulase (see Section I). A short time later an albus smooth variant, 5AS, was isolated which not only gives low yields of coagulase, but produces an enzyme which actually destroys coagulase (Lominski, Morrison and Smith, 1953). At the time these variants were isolated, interest centred on coagulase production and colonial morphology, and no examination was made of the haemolytic properties of the parent strain which is no longer available. When, as has been described, marked differences were found in the haemolysin production of 5R and 5AS, these variants themselves were examined for further variation. In broth cultures of 5R smooth variants, termed 5RS appeared within a few hours; these differed from 5AS in being non-haemolytic. From broth cultures of 5AS two variants have been isolated. Variant 5ABS resembles its parent in morphology and in high alpha and gamma lysin production, but in addition gives high yields of beta lysin. The other variant is 5AR, which differs from the original 5R in producing alpha lysin to about the same titre as gamma lysin (although it produces more coagulase than 5AS, it yields much less than 5R).

Strain F was originally isolated by Dr. Foggie from a

lamb dying of septicaemia, and had been passaged frequently through mice, for which it is very virulent, by Dr. Paul Browning. When this strain was received in this laboratory it contained two variants. One was aureus smooth, non-haemolytic on horse, rabbit and sheep RBC and non-pathogenic for mice; the other was an albus smooth variant, FABS very active in haemolysin production and highly virulent for mice. In young broth cultures there appeared the variant, FBS, relatively poor in haemolytic activity; from broth cultures 2 to 3 weeks old, not only FBS, but also the rough variant FR, was isolated. FR was isolated from broth cultures of FBS which were only 4 days old. Since old broth cultures of FABS contain both FBS and FR, and since 4 day old cultures of FBS contain FR but no FABS, it has been assumed that the rough variant arises from the poorly haemolytic FBS. As in the case of 5R, FR readily gives rise to non-haemolytic smooth variants FRS.

The variants just described confirm the conclusion already reached, namely, that smooth - rough variation is accompanied by a loss of alpha lysin; the loss in the case of 5AS - 5AR is partial, in FBS - FR almost complete. It also appears that smooth - rough variation may be accompanied by an increase in gamma lysin production as FBS - FR (see also 5602S - 5602R in table 11).

The converse variation rough - smooth requires some comment. The two variants referred to 5RS and FRS, and another 5 smooth variants (not listed) from other rough variants are almost completely non-haemolytic. Only preliminary studies have been made of such variants but it seems that the variation rough - smooth is accompanied not by a gain but by a further loss in haemolytic activity.

DISCUSSION

Elek and Levy (1954) found considerable differences in alpha lysin production between strains, due, they suggest, to the numbers of alpha lysin producing variants present in the strains. In their experiments an alpha positive variant from a weak strain produced the same amount of alpha lysin as an alpha positive variant from a 'strong' strain, so that the major factor determining differences in alpha lysin production appeared to be the proportion of alpha lysin producers.

The present experiments reveal the existence of another mechanism whereby cultures may differ in alpha lysin production, i.e. variants intrinsically differing in alpha lysin yield. The experiments have shown that variants may differ by as much as 4000 times in this respect, that they are stable and that when tested for alpha lysin their cultures are entirely composed of members producing the same amount of alpha lysin. Thus, while not denying that cultures of two strains differing in alpha lysin production may do so by differing in the number of variants producing this lysin, there is little doubt that differences between two cultures may depend equally well on the degree to which each individual of the whole population produces alpha lysin.

The present findings resemble rather those of Rogers

(1953) with regard to hyaluronidase production. Rogers found that cultures of newly isolated strains contained a number of variants differing in the amount of hyaluronidase produced. These variants bred true, each giving rise to cultures containing individuals all of which produced the same amount of hyaluronidase as the variant from which it derived.

The presence of gamma antibody in sera prepared against the alpha lysin of strain Wood 46 requires comment; it led Marks to suspect that gamma lysin was produced by this strain in addition to alpha lysin, but he failed to detect it. That Marks did not find gamma lysin may possibly be attributed to the fact that, as shown in the present experiments, its titre in proportion to alpha lysin is small about 1 to 16. It may be that gamma lysin was present in an even lower proportion, because during the present work a definite impression was gained that gamma lysin is less stable than alpha lysin. Despite the discrepancy between the results of Marks and the present work with regard to this strain, the finding strengthens his contention that gamma lysin is widespread among alpha toxigenic strains.

For the detection of alpha lysin production in freshly isolated strains plate tests have been used by several workers, (Williams and Harper, 1947; Gillespie and Simpson, 1948; Elek and Levy, 1950, 1954; Howard, 1954); the

serum employed for specific neutralisation of alpha lysin contained in fact, not only alpha but gamma antibody. It is possible, but unlikely, that gamma lysin could have been mistaken for alpha lysin in these tests for the following reasons. Freshly isolated strains are almost certain to be smooth and, if the conclusions of the present work are valid generally, would produce alpha lysin to at least the same titre as gamma lysin. Only rough variants isolated from old broth cultures have been found to yield gamma lysin in excess of alpha lysin and only these could have led to confusion.

It is generally been assumed hitherto, that alpha lysin acting on rabbit RBC in plates, caused a wide zone of haemolysis with a hazy margin (Gillespie and Simpson, 1948; Elek and Levy, 1950; Marks and Vaughan, 1950). The present work has shown that strains producing alpha lysin also produce gamma lysin; since neutralisation of the inner hazy zone by gamma monovalent serum results in a wide hazy zone with no clear inner zone it is no longer possible to attribute more than the hazy lysis to alpha lysin. The clear inner zone is caused by gamma and also by delta lysin. Since gamma is antigenic, and delta is not, specific neutralisation allows the lysins to be distinguished.

SUMMARY

It was discovered that the rabbit RBC haemolysin produced by the smooth and rough variants of two strains were antigenically distinct; the smooth variants produced, at least predominantly, alpha lysin and the rough gamma lysin.

Since both alpha and gamma lysins are estimated by their lytic action on rabbit RBC, the titre of a mixture of these two lysins is determined by the one in excess. Two monovalent antisera were prepared, one neutralising alpha but not gamma, the other gamma but not alpha. By plate tests using these two monovalent antisera it was possible to distinguish alpha and gamma lysin production by strains. By tube titrations the alpha and gamma titres of preparations from 15 smooth and 14 rough variants were assessed.

Rough and smooth variants both produced alpha and gamma lysins; in preparations from smooth variants, alpha lysin tends to predominate, whereas with rough variants the major lysin is more often gamma lysin.

Associated with the colonial variation smooth - rough there is a loss of alpha lysin production; in the rough variants of 2 of 8 strains there was also an increase in gamma lysin production. The reverse morphological variation, viz., rough - smooth is associated with an almost complete loss in haemolytic activity.

Gamma lysin resembles alpha lysin in being lytic for rabbit RBC, dermonecrotic and lethal. It has been demonstrated that the gamma lysin also resembles alpha lysin in that its lytic action for sheep RBC is inhibited by beta lysin.

SECTION IV

COAGULASE AND TOXIN PRODUCTION OF STAPHYLOCOCCUS AUREUS IN RELATION TO MOUSE PATHOGENICITY

PAGES

INTRODUCTION

Injection of micro-organisms pathogenic for one animal species into anther animal naturally resistant may or may not cause infection. Should infection result, the chances are that the natural and experimental infections will resemble one another partially. Even where the natural and experimental diseases are of a pattern essentially the same, as in human and guinea-pig tuberculosis, such considerable differences exist that caution in interpretation of the findings derived from the experiments is necessary. Nevertheless there is often no alternative to the use of laboratory animals in studies of the mechanism of pathogenicity.

Our understanding of the process whereby organisms become established in a host, multiply, and produce lesions is far from complete. In the case of some pathogens, such as the toxin producers, C.diphtheriae, Cl.tetani, the over-riding factor i.e. the toxin has been found. Proof of the role of these substances is derived from the fact that it is often possible to interrupt the course of disease with the appropriate antiserum. With regard to Staph.aureus there is abundant information regarding substances which, by their activity both in vivo and in vitro play a role in the disease. So far, however, none of the known staphylococcal products

has been shown to be predominant. The two most important would appear to be coagulase which, it has been suggested, is essential for the staphylococcus to become established in the host (Smith, Hale and Smith, 1947), and the alpha toxin, which is produced by almost all pathogenic strains and exerts a severe toxic effect on many animals.

As a natural pathogen Staph.aureus affects only a few species: bovines, horses, human beings, and ovines. In addition to the usual difficulty of interpreting results of experimental infection the staphylococcus presents the complication that the natural disease differs from species to species. In human beings it may be a trivial superficial sepsis or less commonly a severe septicaemia; horses suffer from botriomycosis, cows from mastitis, and young lambs from septicaemia. In the search for an experimental infection it must clearly be kept in mind that staphylococcal infections take many forms and it is by no means clear which type of infection one should expect in the experimental animal. Admittedly both serological tests and phage typing, may in the future, satisfactorily separate strains responsible for characteristic infections in different animals. On the other hand, anticipating the results recorded in this section, variants of one and the same strain differ not only in degree of pathogenicity but also in the type of infection and the

character of the lesions. There is a good reason, however, to hope that with more results available the puzzle of staphylococcal infection, both natural and experimental, will resolve into a pattern. The differences between the various types of infection may well depend not so much on the organisms themselves as on the hosts.

Experimental studies in the natural hosts have, of course, been limited for ethical or economical reasons so that for the most part the in vivo investigations of staphylococcal infections have been confined to laboratory animals. The choice of the mouse in the present work, rather than of the more susceptible rabbit, was governed by the consideration of the ease with which large numbers of animals could be obtained; the difficulty of obtaining serological information from the smaller animal was accepted. Objection to the use of mice on the grounds that very large numbers of organisms are required to establish infection is a criticism which may levelled at experimental staphylococcal infection in any host including man. Thus Elek (1956) found that as many as 7×10^6 organisms were required to cause a purulent skin lesion in man - a naturally susceptible host. On the other hand two facts suggested that mice would be suitable; in the first place, coagulase, which is thought to contribute to the pathogenic effect by its clotting action, does clot

mouse plasma (Gorrill, 1951; Selbie and Simon, 1952), and secondly mice are susceptible to the lethal action of alpha toxin (Parish and Clarke, 1932; Dolman, 1952).

The mouse has been used by a number of workers in the last fifteen years, and a considerable knowledge has accumulated regarding the course of the disease and type of lesion produced. Unfortunately it is often difficult to compare the results which have been obtained for a number of reasons. First of all the route of inoculation used by different workers was not the same; organisms were introduced into the peritoneum, intramuscularly, intravenously, or intradermally. Secondly, the nature of the inoculum differed: in some instances, whole broth cultures were used, in others the organisms were washed and suspended either in saline or broth. The criterion of pathogenicity also varied considerably; the presence of organisms in the heart blood, the formation of localised abscesses, the extent of the lesions and death of the animal, all were used. Notwithstanding the variety of procedures, criteria and interpretations, several workers have come to the conclusion that mouse pathogenicity of a strain is linked with the amount of alpha lysin produced in vitro (Christie, North and Parkin, 1946; Selbie and Simon, 1952; Howard, 1954).

The present investigation is an attempt to correlate

mouse pathogenicity with alpha, beta and gamma lysins and with coagulase by comparing the *in vivo* effect of variants differing in their *in vitro* production of these substances.

MATERIALS AND METHODS

Mice. Two strains of albino mice were used, W-Swiss and Porton, weighing between 20 and 24 g.

Staphylococci. The organisms used were all old laboratory strains and included:

Strain F Originally isolated from a lamb dying of septicaemia by Dr. Angus Foggie, and subsequently passaged many times through mice by Dr. Paul Browning.

Strain 5 Human in origin, it was isolated in this laboratory about 10 years ago from a breast abscess.

Strain 491 A human strain highly pathogenic for mice, supplied by Dr. E.S. Duthie.

Strain Newman This strain, also from Dr. Duthie is noted for its high coagulase production.

Strain G Originally isolated post-mortem from a fatal case of staphylococcal septicaemia in a child. It was supplied by Dr. Browning.

Wood 46 This is the well known alpha lysin producing strain. It was suppled by Dr. A.E. Frances formerly of Wellcome Laboratories, Beckenham. This strain is labelled CN 56 in the BW collection.

Of these strains, two, 5 and F, each produced four variants, namely, 5R, 5RS, 5AS, 5ABS, FABS, FBS, FRS and FR; the remaining four strains each provided one variant. After selecting and plating single colonies from at least six successive 24 hour old cultures it was found that cultures of each variant contained a homogeneous population of individuals alike in morphology, haemolysin and coagulase production; the variants remained stable when cultured on solid media, but not in fluid media (see page 84).

Preparation of organisms for injection. Organisms from 24 hour old nutrient-agar cultures were washed three times in, and suspended in meat extract broth. The number of organisms in suspension was estimated by comparison with standard Brown's opacity tubes (Burroughs Wellcome). Serial doubling dilutions were made in broth and 0.5 ml. volumes used as inoculum.

Degree of pathogenicity. This was measured by estimating the smallest intravenous dose of organisms to kill 50 per cent of the animals with 10 days (LD_{50}). The 50 per cent end-point was calculated according to procedure of Reed and Muench (1938). Preliminary estimations of the LD_{50} were made on W-Swiss mice, using at least 50 animals for each variant. Very similar results were obtained using Porton mice and since larger numbers of these were available this strain was used

in the final experiments to assess the LD_{50}. In each experiment 4 or 5 decreasing doses were tested using 10 animals for each dose. The time limit of 10 days was chosen because interest was mainly in acute disease and, as reported by Gorrill (1951), most deaths occur within this period when staphylococci are injected intravenously.

Post-mortem examinations. Each animal which died on or before the 10th day was examined post-morteum. Lungs, heart, kidneys, liver and spleen were all examined macroscopically for lesions.

Histological examination was carried out on the organs of 55 animals.

Bacteriological examination. Numerous cultures were made from the heart, spleen and kidneys of the animals. Staphylococci which were found in these cultures were examined for haemolysin and coagulase production.

Coagulase production. This was tested by the method of Fisk (1940); 0.1 ml. of an 18-hour meat-extract broth culture was added to approximately 0.5 ml. of human plasma diluted 1 in 10 in saline containing 20 per cent broth. Readings were made after 3 hours' incubation at 37°C and after a further 15 hours at room temperature. A modification of this method, in which mouse plasma is substituted for human plasma, was also used (Gorrill, 1951). Mouse plasma

was obtained by the method of Himmelweit quoted by Gorrill (1951).

Slide test for clumping of staphylococci by plasma. In this test both human (Cadness-Graves, Williams, Harper and Miles, 1943) and mouse plasma were used (Gorrill, 1951; Selbie and Simon, 1952). Staphylococci from 24-hour agar cultures on solid media were suspended in a drop of saline on a slide. To this suspension was added a loopful of undiluted plasma. The slide was then rocked, and if any clumping occurred within 1 minute a positive result was recorded.

Haemolysin production. Burnet's method (1930) was used to prepare alpha, beta, and gamma lysins which were estimated as described in Section III. Delta lysin was estimated only qualitatively on horse blood agar plates.

Antitoxin. Refined staphylococcal antiserum (BW) Batch Number RA 362 A was used in the mouse protection experiments and in the demonstration of diffusible antigens in agar (Howard, 1954).

Multiplication of staphylococci in vivo. The technique used was almost exactly the same as that used by Smith and Dubos (1956). Of 10 mice injected with a known number of organisms intravenously, half were killed within 10 minutes, and the remainder 20 hours after inoculation. The animals were killed with ether and the heart, lungs, spleen, kidneys and liver removed a septically. The organs were

put in to 100 ml. of saline and broken down in an MSE tissue homogeniser for about 45 seconds. One standard loopful (delivering about 0.005 ml.) of the homogenate was then plated on a nutrient agar plate and the colonies counted after 24 hours' incubation at 37°C. The number of organisms from one animal was estimated by multiplying the number of colonies by the dilution factor which was 2×10^4.

RESULTS

Table 13 shows the variants arranged in order of pathogenicity as determined by the LD_{50} which is shown in column 1; production of alpha, beta, gamma, and delta lysins are also shown; coagulase production refers to tests using mouse instead of human plasma; mouse plasma has also been used to show clumping of staphylococci in the slide test (Gorrill, 1951; Selbie and Simon, 1952). Each of these *in vitro* characters will be considered separately in relation to the lethal effect of staphylococci in mice.

Table 12

Variant	Degree of pathogen-icity LD_{50} (millions) (orgs given) (intravenly)	Alpha lysin titre	Beta lysin titre	Gamma lysin titre	Delta lysin titre	Coagulase type of clot Mouse plasma	Slide test Mouse plasma
FABS	0.3	4000	8000	256	+	large	+
491	1.5	2000	0	64	+	large	+
FBS	16	128	128	32	0	large	+
FR	16	8	4000	1000	0	large	+
Newman	29	500	0	500	+	small	+
5ABS	76	16000	8000	256	+	small	0
5AS	85	16000	0	500	+	small	0
G	107	4000	0	128	+	small	+
5R	600	64	0	500	0	large	+
FRS	>1200	0	32	0	0	small	+
5RS	>1200	0	16	0	0	small	+
Wood 46	>1200	8000	0	500	+	large	0

Table 12 shows variants arranged in order of pathogenicity expressed as LD_{50}, also their alpha, beta, gamma and delta lysin, coagulase production and slide clumping test.

The LD_{50} of each strain was calculated by the Reed-Muench procedure. Formal statistical analysis of these values was deemed unnecessary since the only differences which have been considered significant are those in which the LD_{50} differs by more than 30 times.

COAGULASE

Human plasma was clotted by all 12 variants tested by Fisk's method; 10 of the variants gave good fibrin clots within 3 hours at 37°C; the remaining two, 5RS and FRS, gave only weak clots after a further 15 hours' incubation at 20°C. With mouse plasma the only variants which gave really good clots within 3 hours were 491 and FR, the rest were positive only after incubating at 20°C overnight, and of these, 6 caused only a very small granular deposit of fibrin, just recognisable as a clot (see table 12).

The work of Wilson Smith and his colleagues (1947) and of Boake (1956) suggests that efficient clotting is essential to the establishment of infection in mice as well as in guinea-pigs. Although the highly pathogenic strain 491 clots mouse plasma rapidly with a good fibrin clot, there is no difference between FABS and Wood 46 in either the quality of the clot or the speed with which it is produced, yet one variant is highly lethal and the other is not.

Since the qualitative test for coagulase did not differentiate between pathogenic and non-pathogenic variants it was decided to examine the relationship of the amount of coagulase produced to the degree of pathogenicity. Coagulase was estimated roughly by measuring the clotting times of

the supernatants of 3 day broth cultures on mouse plasma in the presence of merthiolate (Duthie, 1954b; Lominski, Morrison and Smith, 1955). Variants FR and 5R each had clotting times of only 15 minutes but their pathogenicity differed greatly while the most pathogenic variant FABS, had a clotting time of between 12 and 18 hours. The degree of pathogenicity is therefore not related to the amount of coagulase formed.

Duthie (1954a) has demonstrated that the factor which causes clumping of staphylococci in the slide test is distinct from coagulase. The last column in table 12 shows that a positive reaction may be obtained from non-pathogenic variants FRS and 5RS, and a negative result from pathogenic variants 5AS and 5ABS. There is no correlation between slide tests on mouse plasma and mouse pathogenicity. (All the variants were slide test positive using human plasma).

ALPHA LYSIN

From table 12 it can be seen that the amount of alpha lysin produced by a variant *in vitro* indicates neither that a strain is pathogenic nor, if it is pathogenic, the severity of the infection which it causes.

Production of alpha lysin in vivo. It could be that, although a variant produces alpha lysin *in vitro*, it does not necessarily do so *in vivo* and *vice versa*. Rogers (1956) has suggested

that the production of some toxins *in vivo* may be blocked by macro-ions such as chondroitin sulphate, nucleoproteins and hyaluronic acid. In the present work the results of two groups of experiments appear to show that strong alpha producers *in vitro* also produce alpha toxin *in vivo*; weak alpha toxin producers *in vitro* do not give high yields *in vivo*.

Experiment 1. Immunity to alpha toxin following infection

Seventeen apparently healthy mice which had survived an LD_{50} dose of variants producing *in vitro* either high or low yields of alpha toxin were challenged intraperitoneally, 6-8 weeks later with a small amount of alpha toxin (125 MHD) prepared from variant 5AS.

Among these, all of 11 mice previously infected with a high or medium alpha lysin producing variant withstood the challenge, whereas 6 of 6 previously infected with variants producing little alpha lysin died (see table 13).

Table 13

Variant	Titre of alpha lysin produced *in vitro*	Effect of intraperitoneal injection of alpha toxin on mice previously infected
	Reciprocal	Deaths/group infected
Newman	500	0/6
5AS	1600	0/2
5ABS	1600	0/3
5R	64	5/5
FBS	128	1/1

Table 13. Active immunity to alpha toxin in mice previously infected with variants producing higher yields of this toxin *in vitro* is inferred from the results recorded in this table. The amount of toxin injected into each mouse was equivalent to 125 minimum haemolytic doses measured against rabbit RBC.

The results of these experiments may reasonably be interpreted as indicating that animals which withstood the intraperitoneal injection of toxin were immunised actively against alpha lysin produced by the infecting organisms *in vivo*.

Experiment 2. Passive immunisation against alpha toxin produced in vivo

Mice were injected intraperitoneally with about 400 units of refined staphylococcal antiserum 18 hours before being challenged with organisms intravenously. The results of these experiments are summarised in table 14.

Table 14

Variant	Production of alpha lysin *in vitro* (reciprocal)	Dose of organisms	Deaths/group of mice injected	
			Protected	Unprotected
5ABS	16000	LD_{50}x1	6/10	10/11
5AS	16000	LD_{50}x1	4/10	10/10
FABS	4000	LD_{50}x4	3/5	6/6

Table 14, shows the protective effect of staphylococcal antitoxin against 3 variants producing high yields of alpha toxin *in vitro*.

Since the serum affords some degree of protection against pathogenic variants producing alpha lysin but not against FBS, a poor alpha lysin producer, the results again suggest that alpha lysin is produced by some variants *in vivo* and plays a part in the disease caused by them. The results correspond with the findings of Burnet (1929), who found that protection of rabbits by antitoxin was only partial, from which he concluded that it was unlikely that acute killing of rabbits was wholly due to the exotoxin, i.e. alpha toxin. Similar results were obtained by Downie (1936).

However tempting it may appear to attribute pathogenicity to alpha lysin production a glance at table 12 shows that in mice at least no such relationship exists. Strain Wood 46 noted for its high yields of alpha lysin, is non-pathogenic, and variants 5AS and 5ABS produce more than 491 and are less pathogenic. On the other hand FBS, producing little alpha lysin, and FR, only traces, are both moderately pathogenic.

BETA-LYSIN

Referring to table 12 again it may be seen that beta lysin does not play an important part in pathogenicity. Variant FR produces much more beta lysin than FBS yet the lethal effect is the same in each case. Variants 5AS and 5ABS provide even better evidence of the insignificance of beta lysin in relation to fatal infections in mice. These

two variants compared morphologically, biochemically, as well as for coagulase and haemolysins show as the only difference that 5ABS produces high yields of beta lysin and 5AS none; despite the high yield of beta lysin of one of them, both have virtually the same LD_{50}.

In view of these results and the fact that beta lysin has been found non-toxic for mice (Bryce and Rountree, 1936; Smith and Price, 1938b), it appears unlikely that beta lysin plays any major part in staphylococcal infections of mice. None of this evidence, however, excludes the possibility that beta lysin acts as an auxiliary pathogenic factor.

GAMMA LYSIN

As with coagulase and alpha and beta lysins there is abundant evidence that neither qualitatively nor quantitatively does gamma lysin play an overriding part in determining mouse pathogenicity of the variants. However, since evidence has been obtained that gamma lysin is toxic not only for rabbits (Smith and Price, 1938b) but also to some extent for mice it is possible that gamma lysin, as well as alpha lysin, although not as an overriding factor, contributes to pathogenicity.

Toxicity of gamma lysin for mice. Preparations from 3 variants FR, AR, and 5R were tested for toxicity by injecting intravenously 0.5 to 0.75 ml. of each into mice; the gamma

haemolytic titre of these preparations was between 1 in 500 and 1 in 1000; alpha lysin was at most 1 in 64, and only FR contained beta lysin (non-toxic for mice). Following the injection death occurred in less than 30 minutes, the animals apparently dying of asphyxia. Although Smith and Price (1938b) reported that gamma lysin is non-toxic for mice it should be noted that in the present experiments the amount of gamma lysin was about 2 to 4 times greater. A few experiments were carried out which indicate that staphylococcal antisera containing gamma antibody (and also alpha antibody) passively protect mice against gamma toxin. So far the protective effect of a monovalent gamma antiserum has not been tried.

DELTA LYSIN

The production of this lysin has only been measured qualitatively, but since it is produced both by the non-pathogenic strain Wood 46 and the most pathogenic variant FABS, and since two strains of moderate pathogenicity, i.e. FR and FBS, do not produce it, it cannot be an essential factor of fatal staphylococcal infections in mice. Delta lysin may, however, contribute to mouse pathogenicity by acting as a leucocidin (Gladstone and Van Heyningen, 1957), capable of rupturing the polymorphs of mice (as well as of other animals).

LEUCOCIDIN OF THE PANTON-VALENTINE (PV) TYPE

Since PV leucocidin is known to have no action on mouse leucocytes *in vitro*, it might be surmised that this substance is irrelevant to mouse infections. Nevertheless there is evidence that it is produced to high titres by variants causing severe human infections (Panton and Valentine, 1932; Valentine, 1936; Valentine and Butler, 1939) and it seemed interesting to determine its production by some of the variants used in the present work.

Estimations of PV leucocidin were kindly carried out by Dr. Gladstone who used an antigen-antibody combining technique with an antitoxic serum of fixed arbitrary unitage. As may be seen from the table 15 it was not possible to relate the amount of PV leucocidin to mouse pathogenicity.

Table 15

Variant	Degree of Pathogenicity LD_{50} (millions)	Panton-Valentine Leucocidin (L+/ml.)
FABS	0.5	0.2
491	2	0.1
FBS	18	0.1
Newman	37	0.8
5ABS	75	0.1
5AS	75	0.1
G	150	0.1
5R	600	0.1

Table 15. Variants arranged in order of pathogenicity showing LD50 and P-V leucocidin production expressed as the largest volume not neutralised by 1 arbitrary unit in 1 ml. of serum (Gladstone and Van Heyningen, 1957).

ANTIGENIC PATTERN

Judged by the LD_{50} there is no single pattern of haemolysin and coagulase production associated with a high degree of pathogenicity for mice, nor does it appear that any of the substances is an overriding factor. Howard (1954) suggested that strains producing the largest number of antigen-antibody flocculation lines (double diffusion technique in agar) are most pathogenic. Some of the variants in the present work were examined in this way; they were stroked out on nutrient agar at right angles to strips of filter paper soaked in staphylococcal antitoxin RA 362A (BW). The plates were examined after 48 hours at 37°C in a mixture of air and 30 per cent CO2, and after a further 5 or 6 days at 22°C in air. From table 16 it may be seen that there is no relationship between the number of lines and the severity of the disease.

Table 16

Variants	Degree of Pathogenicity LD_{50} (millions)	Number of flocculation lines	
		After 48 hours	After 6 days
FABS	0.5	4	7
491	2	4	7
FR	18	1	5
FBS	18	1	3
5AS	75	4	6
5ABS	75	4	7
N2	37	4	7
G	150	4	4
5R	600	1	3
Wood 46	1200	5	8

Table 16. Variants arranged in order of pathogenicity, showing the LD_{50} and the number of antigen-antibody flocculation lines produced by each of them.

As was to be expected the variant which gave rise to the largest number of flocculation lines was Wood 46 since it was a gainst products of this organism that the immune serum was prepared. This strain however, is non-pathogenic for mice. The number of diffusible antigens as determined by this serum gives no indication of pathogenicity.

MULTIPLICATION OF STAPHYLOCOCCI IN VIVO

Several workers have recently emphasised that an essential character of a pathogenic organism is its ability to grow in a host. Smith and Dubos (1956) have shown that in the kidneys of mice pathogenic staphylococci multiply more rapidly than avirulent coagulase negative staphylococci. Ekstedt (1956) attempting to relate pathogenicity to multiplication in vitro has shown that coagulase negative strains do not grow in human serum. It was therefore thought interesting to compare the rate of multiplication in mice of a highly and a poorly pathogenic variant. The technique used was substantially that of Smith and Dubos, with the difference that in the present work counts of bacteria in each organ separately were not made, but a total count of bacteria present in the heart, lungs, kidneys, spleen and liver taken together.

The invivo multiplication of the variants FABS and 5R is shown in table 17.

Table 17

Variant	No. of organisms injected (Viable count)	Number of organisms isolated from the organs of mice	
		After 10 min.	After 20 hrs.
FABS	2.10^5	4	140
		4	3
		5	130
		9	50
		11	55
5R	1.10^5	1	14
		5	0
		0	2
		3	1
		5	-

Table 17 showing the multiplication in vivo of FABS, a highly pathogenic variant and 5R, which is poorly pathogenic.

+ Each figure is the number of colonies growing from the organs of one mouse. The total number of organisms from each animal may by calculated by multiplying this figure by 2.10^4, the dilution factor.

Despite the small number of animals used for this experiment the result obtained strongly suggests that the highly pathogenic variant does multiply more rapidly *in vivo*. Before any general conclusion of this nature could be drawn many more strains will have to be examined and the number of mice considerably increased.

LESIONS CAUSED BY VARIANTS WITH DIFFERENT IN VITRO PROPERTIES

No macroscopic lesions were seen in animals dying within 24 hours of inoculation, but in nearly all animals which died between the 2nd and 10th days macroscopic lesions were found. The organs affected were the heart or kidneys, or both; lesions were not found in the lungs, spleen, or liver, but occasionally abscesses were found scattered over the abdominal and thoracic walls.

So far, it has been shown that variants differ in pathogenicity as determined by the LD_{50}, but from the type of lesion found post mortem, it is apparent that qualitative pathogenic differences also exist. The variants fall into three groups according to the type of lesion produced.

Group 1 (FABS, FBS, G, 491, 5AS and 5ABS). Animals dying of infection caused by these variants showed pale infarcts of various sizes up to 4 mm. in diameter, lesions affecting a third of the ventricle wall or the whole pole of a kidney. Not infrequently abscesses were formed but there was little

pus formation in lesions found in animals dying within 5 days.

Group 2 (5R and FR). Although differing considerably in LD_{50}, these two variants gave rise to a lesions which were macroscopically identical and consisted of tiny white patches less than 0.5 in diameter, scattered throughout the myocardium and kidneys.

Group 3 (Newman). This variant caused well defined purulent lesions of between 1 and 2 mm. in diameter in the heart and kidneys.

Histology. The organs of 35 animals were examined. In group 1 the lesions consisted of masses of gram-positive cocci surrounded by large areas of necrosis of the myocardium or renal parenchyma. In group 2 the small lesions were abscesses with numerous polymorphs and gram-positive cocci. No histological lesions were found in lungs, liver, spleen, pancreas or suprarenals. The organs from animals in group 3 were not examined histologically and information that the lesions were frankly purulent is based on smears.

Comparison of the type of lesion with the in vitro properties shows that in group 1 the colonial morphology of the variants is smooth, and although they differ in the amounts of haemolysins produced, the alpha titre is higher than the gamma in each of them; the main feature of the lesions is

necrosis, presumably the result of toxic action. In group 2, the colonial morphology is rough; coagulase and gamma lysin production is high; alpha lysin production is low. A possible explanation for the numerous small abscesses is that the rough organisms readily establish themselves in the tissues on account of their high coagulase production, but the lesions remain small because toxin production is not high.

Post mortem cultures. Many of the organs of animals dying of infection were cultured. The organisms isolated from the heart or kidneys had invariably properties identical with those injected. No instance of variation in vivo was encountered.

CHANGES IN PATHOGENICITY ASSOCIATED WITH S-R VARIATION

Colonial variation from smooth to rough would at first sight appear to be associated with a decrease of pathogenicity (see table 12). However, in strain F the variant FR is known to be derived from variant FBS (see section III); there is no difference between the two in degree of pathogenicity as measured by the LD_{50}, though the disease caused by these two variants differs qualitatively. The smooth variant causes diffuse sometimes purulent lesions which may be large; the rough variant numerous tiny lesions. With strain 5 there is no strict information regarding the origin of variant 5R

(see section III). It may have been derived from a variant not now in existence and of unknown pathogenicity; conclusions are impossible.

The reverse colonial variation from rough to smooth is represented by 5R - 5RS and FR - FRS. In each case the smooth variant is less active in vitro producing little coagulase and practically no haemolysin; in vivo is non-pathogenic.

PATHOGENICITY FOR RABBITS

A few preliminary experiments were carried out in an attempt to assess roughly the comparative pathogenicity of five of the variants for rabbits. Doses of 300×10^6 and 30×10^6 were injected intravenously into chinchilla rabbits of approximately 3 kg. Two animals were used for each dose.

At the larger dose, vvariants FABS, FBS, and FR were killed 2 of 2 in 48 hours, 5AS 2 of 2 in 6 days; none of the animals receiving the smaller dose died within 28 days. Variant 5R failed to kill at either dose. From these few experiments it would appear that there is some degree of parallelism between pathogenicity for mice and rabbits.

DISCUSSION

Tager and Hales (1947) were unable to relate the quantitative production of coagulase and alpha toxin to the nature and severity of human staphylococcal infections. Panton and Valentine (Panton and Valentine, 1932; Valentine, 1936) found that all strains causing severe lesions in man, such as osteomyelitis, gave high yields of PV leucocidin, whereas strains from superficial lesions did not. Madison (1935) found that the proportion of strains producing fibrinolysin was higher in deep than in superficial infections. Again, most workers find that about 95 per cent of all human pathogenic strain produce alpha haemolysin, but in osteomyelitis the incidence of such strains is lower (Lack and Wailling, 1954).

A priori it is difficult to believe that quantitative production of potent toxins and aggressins should have no influence on the nature of infection. Indeed in the present work the demonstration of different types of lesions in the mouse caused by variants with different in vitro properties suggests that the nature of the infection may to a large extent reflect the properties of the infecting organism. By more detailed investigation it may prove possible in the future to assess the role of individual staphylococcal products.

On the other hand the present investigation shows that

neither coagulase nor any of the haemolysins is an overriding factor in determining mouse pathogenicity, and it seems unlikely that any of the other staphyloccal products not examined here, such as fibrinolysin or hyaluronidase, would play such a dominant role. Many workers have reached a similar negative conclusion with regard to human infections. Also the present work does not suggest that strains with the widest antigenic pattern are particularly pathogenic for the mouse, but these experiments were limited to one antiserum and only a few strains.

It may be suggested that the failure of a coagulase positive variant, producing abundant toxin (Wood 46) to cause infection could be explained by its inability to multiply in vivo (Christie North and Parkin, 1946). The most likely explanation of such a wide difference in pathogenicity between variants seemingly identical in in vitro properties is to assume the existence of a toxin as yet unrecognised.

There is little evidence as to the possible nature of such a substance. Forssman (1935-1938) was able to protect rabbits by vaccinating them with formolised organisms, and Farrell and Kitching (1940) protected mice by passive immunisation with horse sera also prepared against formolised organisms. Protective action of sera prepared by Farrell and Kitching could not be related to anti-alpha haemolysin or antileucocidin titres. On the other hand a number of

workers have failed to protect animals with vaccines of a similar nature (Kitching and Farrell, 1936; Downie, 1937; Flaum, 1938). For the present, therefore, further investigations are required to assess the importance of the protective action of cellular antigens.

Previous workers (Christie, North and Parkin, 1946; Selbie and Simon, 1952; Howard, 1954; Anderson, 1956) have investigated strains whose pathogenicity for susceptible animals could to some extent be inferred from the source from which they were isolated; those from lesions being pathogenic, and those from healthy carriers either pathogenic or non-pathogenic. In the present work it is not possible to relate directly mouse pathogenicity to pathogenicity for susceptible animals because the variants were chosen either for their <u>in vitro</u> properties or for their known pathogenicity for mice. Human and experimental infections in mice are similar in that none of the known toxins plays a determining role.

SUMMARY

1. Twelve variants of Staph.aureus representing a wide range in the quantitative production of coagulase, alpha, beta and gamma toxins, and leucocidin of the Panton-Valentine type, have been tested for mouse pathogenicity. None of these substances is an overriding factor in causing mouse pathogenicity.

2. The degree of pathogenicity is not determined by the amount of coagulase, alpha, beta or gamma lysin or PV leucocidin produced in vitro by a variant.

3. Evidence is presented that pathogenic variants giving high yields of alpha toxin in vitro also produce this toxin in vivo.

4. There is no correlation between the number of diffusible antigens (as determined against a serum prepared against the products of strain Wood 46) and mouse pathogenicity.

5. Of two variants tested the more pathogenic multiplied more rapidly in vivo.

6. Smooth colony variants, producing more alpha than gamma lysin caused necrotic lesions; rough colony variants, giving high yields of gamma lysin and coagulase with only traces of alpha lysin, caused numerous tiny abscesses infiltrated with

polymorphonuclear leucocytes.

7. A smooth variant and the rough variant derived from it were equally pathogenic for mice, as measured by the LD_{50}.

8. Results of preliminary experiments suggest a degree of parallelism in pathogenicity of strains for mice and rabbits.

DISCUSSION

The purpose of this section is to sum up the more important findings of the thesis and examine what light they shed on the mechanism of pathogenicity of Staph.aureus.

Experiments are described in which intravenous injection of coagulase into rabbits resulted in a fall in blood fibrinogen and in the deposition of intravascular fibrin. From previous work (Smith, Hale and Smith, 1947; Lominski and Roberts, 1946; Lominski, 1949) there was only indirect evidence suggesting that coagulase by its clotting action helps to establish infection. Against this Menkin and Walston, (1955), Fisher, (1956) and Lack and Wailling, (1954) failed to demonstrate clotting in vivo. The present experiments show clearly that, if coagulase does act as an aggressin the hypothesis of Wilson Smith (that it does so by its clotting action) is valid.

An association was shown to exist between colonial morphology and coagulase production rough variants giving higher yields than smooth variants of the corresponding strain. Rough variants should prove a useful source of coagulase; indeed they have already been used by Barber and Wildy (1958) in studies relating the antigenic type of coagulase to the phage type of the organism producing it.

Comparison of the haemolytic activities of colonial variants revealed that although either type (smooth or rough) produced both alpha and gamma toxins, in smooth variants the yield of both toxins tends to be high (with alpha in excess) whereas in rough variants gamma is usually the major toxin with only traces of alpha present. Rough variants may be used for the production of gamma toxin relatively free of alpha toxin.

In the course of the work the frequent production of gamma toxin by alpha toxin-producing variants (Smith and Price, 1938b Marks, 1951) was confirmed. It was found that even the classical alpha toxin-producing strain Wood 46, yields some gamma toxin. From this it may be inferred that existing descriptions of the properties of alpha toxin are probably based on examinations of material containing an unknown amount of gamma toxin.

Gamma toxin, produced by rough variants, was examined in vitro: a novel finding was that its lytic action for sheep RBC at 37°C is inhibited by beta lysin. The inhibition is reminiscent of the inhibition of alpha by beta lysin already described by Christie and Graydon (1941). Alpha and gamma toxin are in many ways similar: both are best demonstrated by their lytic action on rabbits RBC and dermonecrotic lethal for rabbits. The fact that both are

inhibited by beta lysin suggests that possibly the mechanism by which they exert their lytic action is also the same.

It is well known that strains of Staph.aureus readily throw off variants (Biggar, Boland and O'Meara, 1937; Burnet, 1930; Rountree, 1936; Elek and Levy, 1954; Smith, Morrison and Lominski, 1952). In relating the pathogenic effect of a staphylococcus to its quantitative production of toxins the use of a culture which is stable and homogeneous is essential. Rogers (1953) found that cultures of Staph.aureus are composed of several homogeneous populations each with a different level of hyaluronidase production. Similarly, experiments described in Section III show that within strains there may exist a number of populations differing in the quantative production of gamma and alpha toxin. This finding has conceivably a bearing on experiments related to animal pathogenicity. In previous work (Selbie and Simon, 1952; Howard 1954; Anderson 1956) mouse pathogenicity has been compared with toxin production, but since no evidence is given as to the homogeneity of cultures in respect of the toxins under consideration the information may well be misleading. In the present experiments whenever pathogenicity was related to toxin production the cultures were, as far as possible, ascertained to be homogeneous.

Experiments in mice have revealed great differences in

pathogenicity between strains. Table 12 of Section IV shows the variants arranged in decreasing order of pathogenicity, as measured by LD_{50}, ranging from 0.3 million to more than 1200 million. This arrangement has proved useful in assessing the relative importance of alpha, gamma and beta toxins, coagulase and the Panton-Valentine leucocidin. But it should be pointed out that the quantitative production of none of the substances under consideration, either singly or jointly, could fully account for the differences in degree of pathogenicity. It is possible to have two organisms with the same pattern of toxin production differing in pathogenicity and also two organism of the same pathogenicity but differing in pattern.

This finding, at least so far as alpha toxin is concerned, is not in agreement with the findings of previous workers. Thus it has been stated that for full mouse pathogenicity a strain must produce alpha toxin (Christie, North and Parkin, 1946). Other workers (Selbie and Simon, 1952; Howard, 1954) also refer to the association between high alpha toxin production a nd virulence for mice. It emerges clearly from the present experiments that the amount of alpha toxin produced <u>in vitro</u> gives no indication of the degree of

pathogenicity, at least for mice. Rough strains yielding only traces of alpha toxin are pathogenic, and although the most virulent strains produce alpha toxin they produce less than strain Wood 46 which does not kill 50 per cent of a batch of mice even when injected in doses of 1200 million.

Similarly, it has been shown that the degree of pathogenicity of strains is not dependent on their quantitative production of beta toxin, gamma toxin, coagulase or the Panton-Valentine leucocidin.

Although evidence has been presented that none of the known toxins plays an over-riding part in staphylococcal infection there is no evidence to the effect that they do notcontribute to pathogenicity. On the contrary, alpha toxin is lethal for mice (Dolman, 1952) and it appears that both in experimental (Burnet, 1929; Downie, 1936) and in natural infections (Blair, 1939) alpha toxin may play a part. The results of a few preliminary experiments (page 109) are in agreement with this view; alpha antitoxin gave some degree of protection to mice infected with strong alpha toxin-producing variants. It appears that alpha toxin production is often associated with pathogenicity, but the

present experiments indicate that the pathogenicity of a strain need not exclusively be determined by its capacity to give high yields of this toxin *in vitro*.

Again, with regard to coagulase, Soake (1956) has shown that immunisation against coagulase protects against a coagulase-positive strain, but not against one which is coagulase-negative. It is possible that the importance of toxins and aggressins may vary from strain to strain. It may be supposed that some strains may readily become established in the host but do not produce sufficient toxin to cause severe lesions, and that others although strong toxin producers *in vitro* lack the factors which would allow it to invade.

Finally it should be borne in mind that *smooth* and *rough* variants produce histologically different lesions and it is therefore possible that the mechanism by which they cause death is also different. From the pattern of toxins and coagulase it may be possible, within limits, to forecast the type of disease induced rather than its severity. As far as the killing capacity of a strain is concerned it seems, safe to speculate that there remains unrecognised a factor, or factors, which determine the

pathogenic action of staphylococci in mice, possibly their pathogenic action in general. The demonstration and identification of these missing factors, along with a more intimate understanding of the *in vivo* action of the known toxins and aggressins may well provide the key to staphylococcal pathogenicity.

SUMMARY

1. An association between colonial morphology and coagulase production *in vitro* is demonstrated; *rough* variants give high yields and *smooth* variants give low yields of coagulase.

2. The roughness of *rough* variants can be accentuated by the addition of blood or certain complex naphtalene compounds to solid media.

3. In their biochemical properties *rough* variants attack the same sugars as the *smooth* variants but generally are much less active in the liquefaction of gelatin.

4. Of 10 *rough* variants, 9 belonged to the same or a similar phage type as the *smooth* variant of the same strain.

5. It has been demonstrated that coagulase may exert its clotting action *in vivo*. 2 mgm. doses of a purified coagulase injected intravenously into rabbits causes death with massive intravascular thrombosis; 0.125 mgm. of the same coagulase was followed by a fall in blood fibrinogen of more than 60 per cent.

6. The quantitative production of haemolysins, with special reference to alpha and gamma lysins, by *smooth* and *rough* variants has been examined. Both types of variant produce alpha and gamma lysin but in *smooth* alpha tends

to predominate, whereas in rough variants gamma is more often the major lysin.

7. Smooth - Rough variation is associated with a loss of alpha lysin. Rough variants give rise to smooth colonial variants characterised by an almost complete lack of haemolytic activity.

8. Gamma lysin resembles alpha lysin in that its lytic action on sheep REC at 37°C is inhibited by beta lysin.

9. Twelve variants of Staph.aureus representing a wide range in the quantitative production of coagulase, alpha, beta and gamma toxins, and leucocidin of the Panton-Valentine type, have been tested for pathogenicity. None of these substances is an over-riding factor in mouse pathogenicity.

10. A smooth variant and the rough derived from it were equally pathogenic for mice as measured by the LD_{50}. However, lesions produced by the two variants differed qualilatively.

11. The relevance of this work to an understanding of the mechanism of pathogenicity of Staph.aureus is discussed briefly.

REFERENCES

ANDERSON, K. (1956) J.Clin.Path., 9, 257.

BARBER, M. (1947) J.Path.Bact., 59, 275.

BARBER, M. and WILDY, P. (1958) J.gen.Microbiol., 18, 92.

BIGGER, J.W., BOLAND, C.R. and O'MEARA, R.A.Q. (1927) J.Path.Bact., 30, 261.

BLAIR, J.E. (1959) Bact.Rev., 3, 97.

BOAKE, W.C. (1956) J.Immunol., 76, 89.

BRYCE, L.M. and ROUNTREE, P.M. (1936) J.Path.Bact., 43, 173.

BRYSON, V. (1956) Ann.N.Y.Acad.Sci., 65, Art.3.

BURNET, F.M. (1929) J.Path.Bact., 32, 717.

BURNET, F.M. (1930) J.Path.Bact., 33, 1.

BURNET, F.M. (1931) J.Path.Bact., 34, 759.

CADNESS-GRAVES, B., WILLIAMS, R.E.O., HARPER, G.J. and MILES, A.A. (1943) Lancet, i, 736.

CHRISTIE, R. and GRAYDON, J.J. (1941) Aust.J.exp.Biol.Med. Sci., 19, 9.

CHRISTIE, R., NORTH, E.A. and PARKIN, B.J. (1946) Aust.J.exp. Biol.Med.Sci., 24, 73.

DOLMAN, C.E. (1932) Can.Pub.Hlth.J., 23, 125.

DOWNIE, A.W. (1936) J.Path.Bact., 44, 573.

DUTHIE, E.S. (1954a) J.gen.Microbiol., 10, 427.

(1954b) J.gen.Microbiol., 10, 437.

EKSTEDT, R.D. (1956) Ann.N.Y.Acad.Sci., 65, Art. 3.

J.Bacteriol., 72, 157.

ELEK, S.D. (1956) Ann.N.Y.Acad.Sci., 65, Art. 3.

ELEK, S.D. and LEVY, E. (1950) J.Path.Bact., 62, 541.

(1954) J.Path.Bact., 68, 31.

FISHER, A.M. (1936) Bull.Johns Hopkins Hosp., 59, 393.

FISK, A. (1940) Brit.J.exp.Path., 21, 311.

FLAUM, A. (1938) Acta.Path. et microbiol.Scand., Suppl. 35, 1.

FORSSMAN, J. (1935-38) Acta.Path. et microbiol.Scand., 12-15.

GILLESPIE, W.A. and SIMPSON, P.M. (1948) Brit.Med.J., ii, 902.

GLADSTONE, G.P. and van HEYNINGEN, W.E. (1957) Brit.J.exp.Path., 38, 123.

GLENNY, A.T. and STEVENS, M.F. (1935) J.Path.Bact., 40, 201.

GORRILL, R.H. (1951) Brit.J.exp.Path., 32, 151.

HADLEY, P. (1937) J.Infect.Dis., 60, 129.

HOFFSTADT, R.E. and YOUMANS, G.P. (1932) J.Infect.Dis., 51, 217.

HOWARD, J.G. (1954) J.Path.Bact., 68, 177.

KITCHING, J.S. and FARRELL, L.N. (1936) Am.J.Hyg., 24, 268.

LACK, C. and WAILLING, D.G. (1954) J.Path.Bact., 68, 431.

LAMMANA, C. and MALLETTE, M.F. (1953) Basic Bacteriology, Baltimore: Williams and Wilkins.

LOMINSKI, I. and LENDRUM, A.C. (1942) J.Path.Bact., 54, 421.

LOMINSKI, I. MORRISON, R.B. and SMITH, D.D. (1955) J.gen. Microbiol., 13, 446.

LOMINSKI, I. and ROBERTS, G.B.S. (1946) J.Path.Bact., 58, 187.

MADISON, R.R. (1935-36) Proc.Soc.exp.Biol.Med., 33, 209.

MARKS, J. (1951) J.Hyg.Camb., 49, 52.

(1952) J.Path.Bact., 64, 175.

MARKS, J. and VAUGHAN, A.C.T. (1950) J.Path.Bact., 62, 597.

MENKIN, V. and WALSTON, H.D. (1934-35) Proc.Soc.exp.Biol.Med., 32, 1259.

MORGAN, F.G. and GRAYDON, J.J. (1936) J.Path.Bact., 43, 385.

NAIDU, P.M.N. (1934) Zbl.Bakt., 1 Abt.Orig., 132, 47.

PANTON, P.N. and VALENTINE, F.C.O. (1932) Lancet, 506.

PARISH, H.J. and CLARKE, W.H.M. (1932) J.Path.Bact., 35, 251.

REED, L.J. and MUENCH, H. (1938) Ann.J.Hyg., 27, 493.

ROGERS, H.J. (1953) J.Path.Bact., 66, 545.

(1956) Ann.N.Y.Acad.Sci., 65, Art. 3, 132.

ROUNTREE, P.M. (1936) Aust.J.exp.Biol.Med.Sci., 14, 93.

SELBIE, F.R. and SIMON, R.D. (1952) Brit.J.exp.Path., 33, 316.

SEXTON, W.A. (1953) Chemical Constitution and Biological Activity 2nd Edition, London: Spon Ltd. page 301.

SMITH, D.D. (1956) Nature, 178, 1060.

SMITH, D.D. and JOHNSTONE, J.M. (1956) Nature, 178, 982.

SMITH, D.D. MORRISON, R.B. and LOMINSKI, I. (1952) J.Path.Bact., 64, 567.

SMITH, J.M. and DUBOS, J.R. (1956) J.Exp.Med., 103, 87.

SMITH, M.L. and PRICE, S.A. (1938a) J.Path.Bact., 47, 361.

(1938b) J.Path.Bact., 47, 379.

SMITH, W. HALE, J.H. and SMITH, M.M. (1947) Brit.J.exp. Path., 28, 57.

SCHWABACHER, H. CUNLIFFE, A.C. WILLIAMS, R.E.O. and HARPER, G.J. (1945) Brit.J.exp.Path., 26, 134.

TAGER, M. and HALES, H.B. (1947) Yale J.Biol.Med., 20, 41.

TOPLEY, W.W.C. and WILSON, G.S. (1955) The Principles of Bacteriology and Immunology, 4th edition, London: Arnold and Co. (Revised by WILSON, G.S. and MILES, A.A.).

WILLIAMS, R.E.O. and HARPER, G.J. (1946) Brit.J.exp.Path., 27, 72.; (1947) J.Path.Bact.,

WISE, R.I. (1956) Ann.N.Y.Acad.Sci., 65, Art. 3, 169.

VALENTINE, F.C.O. (1936) Lancet, 526.

VALENTINE, F.C.O. and BUTLER, E.C.B. (1939) Lancet, 973.