DIFFUSIBLE ANTIGENS IN STAPHYLOCOCCAL CULTURES.

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BACTERIAL growth in fluid media produces a mixture of many substances, and to demonstrate the toxicity of resulting filtrates or supernatants their actions on the cells or tissues of animals must be investigated. Toxic effects may be due to one or to several substances. The first step in their analysis is to find suitable indicator effects which are capable of quantitative measurement. This has inherent difficulties, as a single product may have several indicator effects; or conversely, a single effect may be due to several different toxins in the same culture filtrate. Quantitative assessment of the effect would then measure only the constituent present in highest titre. Since diffusible bacterial toxins are antigenic, the components of a toxic mixture may be distinguished by quantitative neutralization combined with the use of indicator effects. The analysis is based on the assumption that when a single toxin has two or more indicator effects, the quantitative relationship of the effects should remain constant when measured by antisera prepared against filtrates from different strains of the same species. Should this relationship not hold, the effect must be due to different toxins and multiplicity of toxins is inferred. Though the theoretical basis of this approach is sound and simple, the practical difficulties encountered are considerable. The greater the number of toxins producing multiple indicator effects the more complex the analysis becomes. For this reason the toxins of many organisms have not so far been successfully analysed.

The most complete analysis of bacterial toxins has been achieved with the clostridia. There appears to be a set number of toxins characterizing each species. Not all of these toxins are produced by every member; some may be shared by all virulent members, while others occur in combinations defining individual types. Several types of *Cl. welchii* can be differentiated in this way by their toxin patterns. Furthermore, the various types so defined show well-marked differences in their host range and the types of lesions caused—differences which are not surprising, since toxin production is the key to virulence in these organisms. With staphylococci no such classification is as yet possible. Burnet (1929) found that three activities of staphylococcal filtrates—haemolysis of rabbit cells *in vitro*, necrotic skin lesions in rabbits and lethal effect on intravenous injection—were manifestations of a single antigenic substance. With the description of fresh indicator effects the concept of multiple toxin production by staphylococci has become accepted, and the toxin investigated by Burnet is known as the α haemolysin. It is now known that antigenically different haemolysins (α and β) are formed, but the evidence for the separate existence of α_2, δ and

γ is not conclusive. In addition coagulase, leucocidin, fibrinolysin, enterotoxin, spreading factor, lipase, protease and proinvasin are said to complete the offensive arsenal of staphylococci. These individual effects have been much investigated, but little is known of the pattern in which they occur in human and animal strains. Analogy with the clostridia suggests that toxin patterns may partly determine the nature of the lesion caused and so explain why some strains produce relatively unimportant skin lesions while others are invasive.

Instead of investigating the individual toxic effects of different strains, we set out to demonstrate the antigenically different diffusible substances formed. With this approach we have at present confined ourselves to the investigation of the number of antigens formed by a series of staphylococci of human and animal origin, tested by reference sera prepared against the Wood 46 strain. We hope later to be able to identify the observed antigenic reactions with specific indicator effects. Since the diffusible antigens are not all necessarily toxins, the number of antigens demonstrable may exceed the number of true toxins formed. Moreover, by the technique employed only those antigens will be revealed for which the antiserum used contains sufficient antibody, and other toxins may be present but undetected. In spite of these limitations evidence will be presented that in fact a good correlation exists between the production of diffusible antigens and other criteria of pathogenicity. A plate method described previously (Elek, 1948) offered a means of enumerating the diffusible antigens of staphylococci, and in a later paper (Elek, 1949) various methods were discussed by which individual antigen-antibody lines could be identified with some indicator effects. A modification of the method is now used, and the results are correlated with coagulase production, degree of pigmentation, gelatin liquefaction, mannitol fermentation, penicillin sensitivity and strain origin.

METHODS

Plate technique for the demonstration of diffusible antigens.

The base medium consisted of Difco heart-infusion broth with 0·2 per cent of potassium dihydrogen phosphate (KH_2PO_4) and 0·03 per cent magnesium sulphate ($MgSO_4.7H_2O$). The pH was adjusted to 7·4, and 1·5 per cent New Zealand agar powder was added. After filtration through paper pulp the medium was sterilized by autoclaving at 15lb. per square inch for 15 minutes. Sterile horse blood was centrifuged and the red cells washed three times with normal saline. The red cells were then resuspended to their original volume in saline. A 10 per cent solution of white saponin in distilled water was prepared and sterilized in the autoclave at 10 lb. per square inch for 30 minutes ; one volume of this saponin solution was added to twenty volumes of the reconstituted horse red cells, and the mixture incubated for 15 to 30 minutes to complete lysis. The laked cells were stored in the refrigerator and were found to be satisfactory for several weeks. Flat-bottomed petri dishes of three-and-a-half inch diameter were used. Into each of these 0·2 ml. of the laked red cells and 12 ml. of the base medium were poured. Even mixing was ensured by gentle rotation of the plate. Finally before the agar had set a filterstrip soaked in staphylococcal antitoxin was embedded below the surface in the middle of the plate. The filterstrips measured 60 mm. by 15 mm., and had been sterilized in the hot-air

oven. The antitoxin used was Refined Staphylococcal Antitoxin Globulins (Burroughs Wellcome & Co.,) of a strength of 1250 units per ml. The plates thus prepared were dried for 2 to 3 hours and were used the same day. Not more than four strains were inoculated on each plate in the form of streaks at right angles to the filterstrip. The plates were placed in air-tight jars which were then filled with a mixture of 30 per cent carbon dioxide and 70 per cent air through a reducing valve from a steel cylinder, and incubated at 37° C. for four days ; final readings were taken after a further 48 hours at room temperature.

Coagulase production.

Human citrated plasma from the blood bank was diluted 1 in 10 with sterile nutrient broth immediately before use. A Wassermann tube containing 0·5 ml. of this mixture was inoculated with an entire colony from an overnight blood agar culture. The tubes were placed in a water-bath at 37° C., and readings were made 3 hours later. The negative tubes were then left at room temperature for the final reading after 24 hours. Two control tubes were included with each group, one containing the diluted plasma uninoculated to detect spontaneous clotting, and the other inoculated with a known-coagulase positive strain. Those strains which appeared negative by this technique were checked by using two drops of an overnight broth culture in place of the solid growth.

Pigment production.

Milk agar plates were poured from an autoclaved mixture of one part whole cow's milk and four parts of ordinary nutrient agar. Streak inoculations were made and the plates were incubated in air at 37° C. for 48 hours, and then left at room temperature for a further three days before being read. The following four types of pigmentation were differentiated : albus, off-white, aureus and citreus. The aureus strains were graded arbitrarily + to +++ according to depth of colour.

Mannitol fermentation.

Bijou bottles containing slopes of ordinary nutrient agar incorporating 1 per cent mannitol and Andrade's indicator were inoculated over the surface. They were incubated in air at 37° C. and read up to one week ; the development of a red colour was recorded as positive.

Gelatin liquefaction.

Nutrient broth was made up with 12 per cent Gurr's gelatin and sloped in tubes. Strains were streaked over the surface and left at room temperature for three weeks, readings being taken weekly. Slight pitting around the growth was accepted as positive. Strains found to be negative after this time were incubated at 37° C. in air for a further 48 hours and then placed in the refrigerator to cool ; failure to resolidify was regarded as a positive result. Considerable incubation was required for many of the strains before liquefaction occurred. Incubation at room temperature for three weeks was found to be more sensitive

than incubation for 48 hours at 37° C., as with the former method a small degree of surface-pitting could be demonstrated, while this was missed with the latter procedure.

Penicillin sensitivity.

Strains were streaked over horse blood agar plates, and a filterstrip soaked in penicillin solution (500 units per ml.) was placed at right angles to the centre of the streaks. The Oxford staphylococcus was always included as a control. Sensitivity or resistance was recorded after overnight incubation at 37° C. in air.

MATERIAL.

Three hundred and fifty-nine strains were examined, Of these, 200 were selected from routine human material, and were regarded as pathogens because they came from lesions typically caused by staphylococci. They were mostly isolated in pure primary culture and were all coagulase-positive. Nine strains were from carbuncles, 24 from boils, 21 from breast abscesses, 16 from conjunctivitis in the newborn, 20 from whitlows, 54 from miscellaneous abscesses, 6 from otitis, 6 from osteomyelitis and 10 from post-operative infections. The remaining 34 strains were from a variety of conditions. The second group consisted of 100 unselected strains from the skin and nose of hospital in-patients with no skin or nose lesions. Ninety consecutive patients were swabbed, using a dry swab for both nostrils, and a swab moistened with broth rubbed over an area about the size of a square inch of the upper abdomen. Each swab was inoculated on to a nutrient agar plate and a 7·5 per cent sodium chloride-nutrient agar plate. Of the 100 strains 26 were found to be coagulase-positive. Twenty-one of the coagulase-positive strains were isolated from the nose (23·3 per cent of the patients) and only 5 (5·5 per cent) from the skin. All the strains obtained from lesions and from carriers were tested either within a few days of isolation (without subculturing), or they were freeze-dried forthwith and tested immediately after reconstitution. A further 59 strains were from animal lesions. These were not freshly isolated strains, but were all coagulase-positive.

RESULTS.

The serum used throughout these experiments was one which had been prepared against the Wood 46 strain, and when this organism was tested by the technique described, it was found to produce no fewer than ten lines. A line drawing of the pattern obtained, with the Wood 46 uppermost and another strain below, is shown in Fig. 1, while a photograph of the typical appearances given by two different strains is shown in Fig. 2. Staphylococci other than the Wood 46 generally produced fewer lines. The maximum number of lines formed was constant for each strain, though their position relative to each other was somewhat variable. Most of the lines appeared within 24 to 48 hours, but some appeared only after several days. The early lines increased in density and length with incubation and were easily seen; the later lines were much finer, and occasionally they were difficult to see. Sometimes a narrow zone of turbidity was formed which later differentiated into fine lines.

Coagulase production.

The most striking experimental finding was the complete agreement between coagulase activity and line production. All the coagulase-positive strains from human sources flocculated with the Wood 46 antitoxin, whereas none of the coagulase-negative strains did so (Table I). In the animal pathogens there is also a close correlation between coagulase production and the ability to produce lines, but the agreement is not absolute. Coagulase-positive skin strains produce fewer lines than the proved pathogens, the difference being highly significant statistically. Moreover, there is a highly significant difference between the average number of lines produced by the human and animal pathogens.

TABLE I.—*Comparison of Line Production by Strains Obtained from Various Sources.*

Group.	Number of strains examined.	Number of strains producing lines.	Mean number of lines.
Coagulase-positive human pathogens	200	200	6·37
Coagulase-positive animal pathogens	59	55	4·69
Coagulase-positive skin and nose strains	26	26	5·19
Coagulase-negative skin and nose strains	74	none	*nil*

Type of lesion.

When the human pathogens are grouped according to the lesions from which they were isolated there is seen to be no statistically significant difference in the number of lines produced by the different groups (Table II).

TABLE II.—*Comparison of Line Production in Strains from Different Lesions.*

Lesion.	Number of strains.	Mean number of lines.
Breast abscesses	21	6·33
Boils	24	6·12
Carbuncles	9	6·00
Various abscesses	54	6·61
Osteomyelitis	6	6·00
Conjunctivitis of infants	16	6·69
Otitis	6	6·66
Postoperative wound sepsis	10	6·30
Whitlows	20	6·25
Unclassified lesions	34	6·24

Penicillin resistance.

Strains showing resistance to penicillin do not produce fewer lines than those which are sensitive. Of 183 human pathogens examined 131 (71·5 per cent) were sensitive, the average number of lines produced being 6·34. The 52 penicillin resistant strains averaged 6·60 lines per strain. This difference is not statistically significant.

Pigment production.

The incidence of various degrees of pigmentation in the groups investigated is shown in Table III. There is a striking difference in the distribution of the various grades of pigmentation between the coagulase-positive and coagulase-negative groups. This is shown graphically in Fig. 3. In the coagulase-positive

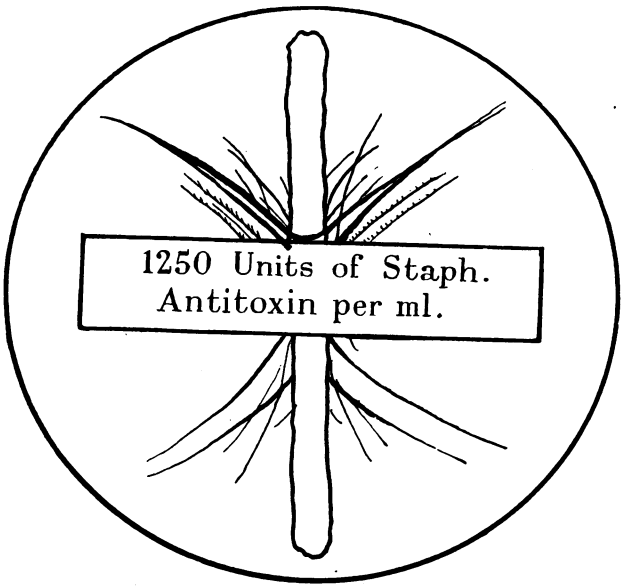

FIG. 1.—A line drawing of the patterns obtained with two different strains of staphylococci; the uppermost strain is the Wood 46.

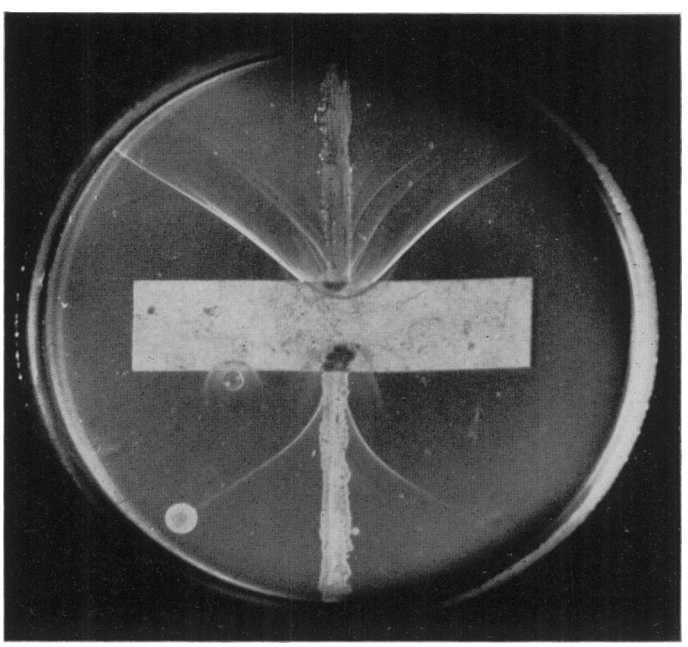

FIG. 2.—A photograph showing the patterns obtained with two different strains of staphylococci.

TABLE III.—*The Relationship between the Degree of Pigmentation and Line Production.*

Group.	Number of strains.	Albus.		Off-white.		Aureus +.		Aureus ++.		Aureus +++.	
		Number of strains.	Mean number of lines.	Number of strains.	Mean number of lines.	Number of strains.	Mean number of lines.	Number of strains.	Mean number of lines.	Number of strains.	Mean number of lines.
Coagulase-positive	281	21	4·10	22	5·45	69	5·81	97	6·29	72	6·15
Coagulase-negative	72	60	*nil*	3	*nil*	6	*nil*	3	*nil*	*nil*	*nil*

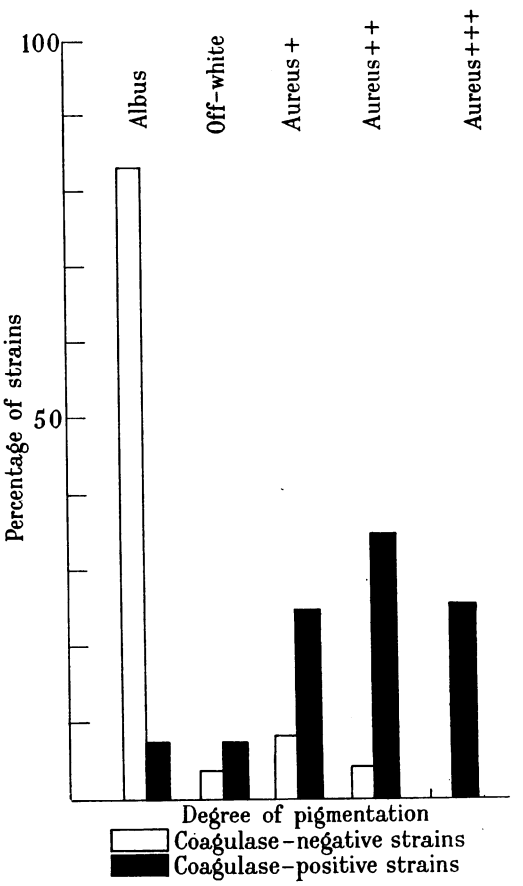

FIG. 3.—Graph illustrating the distribution of the various degrees of pigmentation among 281 coagulase-positive strains and 72 coagulase-negative strains.

group the number of lines produced can be correlated with the degree of pigmentation. There is a highly significant increase in the number of lines as colour deepens from albus to aureus +++. This can be shown by plotting the five categories of pigmentation against the mean number of lines produced. The

best fitting straight line—representing the regression of number of lines on grade of colour—passes through 4·81 for albus and 6·48 for aureus +++ (Fig. 4).

Mannitol fermentation.

In a group of 246 pathogenic strains most were mannitol-positive (Table IV). Among the animal pathogens no significant difference in line production was found between the positive and negative strains. The group of carrier strains was too small for analysis, but there appears to be a significant difference in line production within the group of human pathogens.

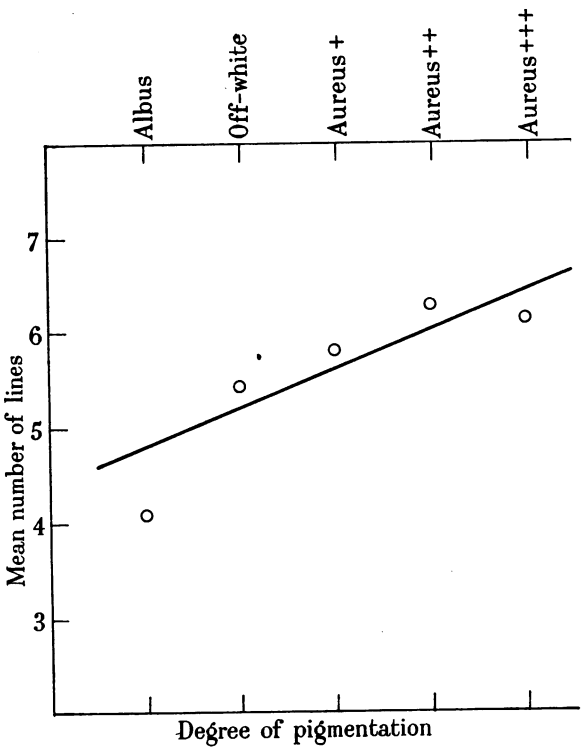

FIG. 4.—Graph showing the increase in the mean number of lines with increasing degree of pigmentation.

TABLE IV.—*Relation of Line Production to Mannitol Fermentation.*

Strains.	Total numbers examined.	Mannitol fermentation.		Mean number of lines.	
		+	−	Mannitol +.	Mannitol −.
Human pathogens	188	182	6	6·41	4·50
Animal pathogens	58	46	12	4·60	4·66
Coagulase-positive carrier strains	15	13	2	6·08	3·00

Gelatin liquefaction.

Of 195 coagulase-positive strains only 123 were gelatin liquefiers (63 per cent). There is a reduction in the mean number of lines produced by gelatin-negative strains in both the human and animal groups of pathogens (Table V).

TABLE V.—*Relation of Gelatin Liquefaction to Lines.*

Strains.	Total number examined.	Gelatin		Average number of lines.	
		+	−	Gelatin +.	Gelatin −.
Human pathogens	165	99	66	7·33	6·69
Animal pathogens	27	22	5	5·63	3·00
Coagulase-positive carrier strains	3	2	1	7·50	9·00

DISCUSSION.

It must be emphasized that when any strain is tested against a reference serum the number of flocculation lines demonstrable cannot exceed that of the constituent antibodies. In this work the number of the lines was limited by the diffusible antigens formed by the Wood 46 strain. Though this is generally regarded as a representative example of toxicogenic staphylococci, it is now known not to produce certain toxins, notably the enterotoxin and the β-haemolysin. It is not suggested that all the lines produced can be identified with toxins, but with suitable sera the recognition of the α- and β-haemolysins is relatively easy. In this connection it may be noted that analogous work with diphtheria bacilli (Elek, 1948; Ouchterlony, 1948) has shown that apart from the diphtheria toxin, which is believed to be a single entity, at least two other diffusible antigens having no lethal or dermotoxic action can be demonstrated after prolonged incubation. Similar work with strains of *Cl. welchii*, however, showed a correlation between the number of lines produced and the number of toxins known to be associated with various types (Elek, 1949). In the present work, since the antiserum was prepared against a representative toxicogenic strain, the majority of its antibodies are likely to be antitoxins; consequently the formation of flocculation lines is most probably an index of toxicogenicity.

While there is complete agreement between coagulase activity and line production amongst human pathogens, there is a slight discrepancy in the animal group. In this connection it should be stressed that the animal strains were not freshly isolated, and loss of toxicogenicity might have occurred. There are three possible explanations to account for the difference between the average number of lines produced by the human and animal pathogens. Firstly as a result of repeated subculturing not only did four of the strains lose completely the ability to produce toxin, but there might have been a reduction in the number of toxins in the remainder. Secondly, the animal strains may produce toxins different from those of the human strains, and not neutralized by the antitoxin used. This would result in an apparent reduction of the number of lines. Thirdly, it is possible that animal strains normally produce fewer diffusible antigens. It is known, for example, that animal strains of staphylococci generally fail to produce fibrinolysin. The fact that skin strains produce fewer lines than those obtained from lesions suggests that they produce fewer toxins. If this were so, coagulase production would slightly overestimate the potential pathogens amongst skin strains. It must be remembered that coagulase testing is based on the

observation that all strains from lesions are positive ; from this it has been inferred that all coagulase-positive strains are potentially pathogenic. As yet there is no simple means of assessing the pathogenicity of coagulase-positive strains obtained from sources other than lesions. It may well be that routine coagulase testing of carrier strains—although detecting all the pathogens—may in fact include some that are less likely to produce human lesions.

The absolute agreement between line production and coagulase formation in human strains and the similar agreement for animal strains are of great interest, for the association between coagulase production and pathogenicity is now well established. The fact that virulence of staphylococci is linked with coagulation of plasma and the ability to produce toxin must be more than coincidence. Indeed the question of virulence as applied to staphylococci requires clarification. MacLeod and Pappenheimer (1948) defined pathogenicity as the capacity of micro-organisms to produce disease, and virulence as a measure of pathogenicity. Further, they state that diseases due to bacteria fall into three groups : in the first toxin production is the major factor, invasion being absent ; in the second group the organism is invasive without producing toxin ; and in the third both toxin production and invasion contribute to virulence. Blair (1948) classes staphylococci in the third group. The pathogenicity of any strain in his view represents the total effect of both factors working together at times one or the other being predominant. In some cases exotoxin is undoubtedly responsible for the manifestations of disease, but more often invasion of the tissues is the main feature. He concludes that " a small number of pathogenic staphylococci produce either no exotoxin or only an insignificant amount ; infections due to these non-toxicogenic organisms are referable to their invasive capacity." Wilson and Miles (1946) on the other hand suggest that it is better to retain the term " virulent " in its correct sense of poisonous, without any implication as to how the poisonous effect is produced. In their view " no mechanical theory of the pathogenic action of bacteria is compatible with our knowledge of the way in which the tissues deal with inert particles which had gained access to them. The basis of all harmful effects of bacterial infection is quite certainly chemical . . . " The two views are not necessarily contradictory ; the latter attempts to explain in chemical terms the mode of invasiveness. It places on the broadest possible basis our concept of toxins. Nevertheless the experimental findings sharply contradict Blair's statement that non-toxicogenic strains may produce disease. Amongst 200 strains examined from lesions not one was found to lack toxin production, in spite of the artificial limitations set by the use of a single reference serum.

The failure to find any difference in the number of lines between strains obtained from different human lesions bears out the view that as regards toxin patterns there is no difference between strains causing relatively mild surface lesions and those causing more serious infections. How far quantitative differences in toxin production come into play cannot, of course, be assessed by the method employed.

As it is common experience that penicillin-resistant strains are as virulent as sensitive ones (Spink, Hall and Ferris, 1945 ; North and Christie, 1946), it is not surprising to find that they fail to show a reduction in the number of lines. It is interesting to note, however, that the ratio of sensitive to insensitive strains varied according to the lesion. Taking conjunctivitis of infants and postoperative

sepsis as a group representing hospital infections (26 strains), the ratio of sensitive to resistant strains was 0·4 to 1, whereas in the remainder of the infections the ratio was 3·5 to 1. It seems therefore that lesions which apparently originated in hospital are associated with a higher incidence of penicillin-resistant strains than those arising outside (Barber and Rozwadowska-Dowzenko, 1948; Martyn, 1949; Forbes, 1949; Barber, Hayhoe and Whitehead, 1949; Rountree and Thomson, 1949, and Cairns and Summers, 1950).

Although pigment production is no longer used as a criterion of pathogenicity, it is interesting to note that the greater the degree of pigmentation the more lines are produced. This appears to be in good agreement with the old observation that the deeply pigmented strains are more likely to be pathogenic. Lack of mannitol fermentation and of gelatinase production in human pathogens appear to be associated with the production of fewer lines.

SUMMARY.

An experimental technique based on the double diffusion gradient principle designed to reveal different diffusible antigens in staphylococcal plate cultures is described. Flocculation is observed in the form of lines which permit an assessment of the number of antigens produced by different strains. A single representative antitoxin prepared against the Wood 46 strain showed that antigens were produced by all coagulase-positive strains but none by coagulase-negative staphylococci in a group of 359 strains examined. This absolute correlation is brought forward as evidence that the production of diffusible antigens is a *sine qua non* of virulence. Although it is not claimed that each flocculation line produced represents a toxin, it is certain that every diffusible toxin will yield a line in the presence of sufficient antibody. The number of lines is therefore likely to be a rough measure of the toxin pattern.

The mean number of lines produced by animal pathogens was significantly lower than that produced by the human pathogens. Coagulase-positive skin strains also produced fewer lines than strains from lesions. Possible explanations for these differences are discussed.

Lesion of origin and penicillin-resistance have no correlation with line production. Increasing degrees of pigmentation, mannitol fermentation and gelatinase production are associated with a greater number of lines.

There is no evidence that invasive staphylococci may fail to produce exotoxin.

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REFERENCES.

BARBER, M., HAYHOE, F. G. J., AND WHITEHEAD, J. E. M.—(1949) *Lancet*, ii, 1120.

Idem AND ROZWADOWSKA-DOWZENKO, M.—(1948) *Ibid.*, i, 641.

BLAIR, J. E.—(1948) In 'Bacterial and Mycotic Infections of Man.' Edited by R. J. Dubos; p. 331. Philadelphia (J. B. Lippincott Co.).

BURNET, F. M.—(1929) *J. Path. Bact.*, **32**, 717.
CAIRNS, H. J. F., AND SUMMERS, G. A. C.—(1950) *Lancet*, i, 446.
ELEK, S. D.—(1948) *Brit. med. J.*, i, 493.—(1949) *Brit. J. exp. Path.*, **30**, 484.
FORBES, G. B.—(1949) *Brit. med. J.*, ii, 569.
MACLEOD, C. M., AND PAPPENHEIMER, A. M.—(1948) In 'Bacterial and Mycotic Infections of Man.' Edited by R. J. Dubos; pp. 68 and 69. Philadelphia (J. B. Lippincott Co.).
MARTYN, G.—(1949) *Brit. med. J.*, i, 710.
NORTH, E. A., AND CHRISTIE, R.—(1946) *Med. J. Aust.*, i, 176.
OUCHTERLONY, Ö.—(1948) *Acta path. microbiol. scand.*, **25**, 186.
ROUNTREE, P. M., AND THOMSON, E. F.—(1949) *Lancet*, i, 501.
SPINK, W. W., HALL, W. H., AND FERRIS, U.—(1945) *J. Amer. med. Ass.*, **128**, 555.
WILSON, G. S., AND MILES, A. A.—(1946) 'Topley and Wilson's Principles of Bacteriology and Immunity,' pp. 1005 and 1007. London (Edward Arnold & Co.).