A STUDY OF THE SEROLOGICAL TYPE DIFFERENTIATION OF *STAPHYLOCOCCUS PYOGENES**

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CONTENTS

INTRODUCTION

Studies on the epidemiology of staphylococcal infections have in the past been hampered by the inability to differentiate specific types among infecting strains of the genus *Staphylococcus*; moreover, the ubiquitous nature of staphylococci makes it more than usually difficult to prevent or control outbreaks of infection due to these organisms. In infant nurseries, maternity homes and operating theatres, as well as in outbreaks of food-poisoning due to staphylococcal enterotoxin, the origin of infection and paths of spread can be traced only if the types of organism causing infection can be shown to be identical with those isolated from other sources in the patient's environment.

It is now generally accepted that the criterion of potential pathogenicity for staphylococci is the ability to coagulate human or rabbit plasma under standard conditions (Cruickshank, 1937). The great majority of strains of *Staph. aureus* are coagulase-positive and the great majority of strains of *Staph. albus* and, so far as is known, most strains of *Staph. citreus* are coagulase-negative. However, coagulase-negative strains of golden staphylococci and coagulase-positive strains of white and lemon-yellow staphylococci do occur. Staphylococci which are coagulase-positive and therefore considered, regardless of pigment production, to be potentially pathogenic whether isolated from infected sources or from healthy nose, throat, skin, fomites, dust, etc., will, as suggested by Hine (1922) and Cowan (1938), be referred to as *Staph. pyogenes*.

In recent years two investigations (Cowan, 1939; Christie & Keogh, 1940) have been reported describing procedures for typing staphylococci by serological methods. Both studies showed the difficulties experienced because of the considerable antigenic overlap between strains, and neither technique has been widely adopted in routine epidemiological investigations. The object of the present work was, therefore, to explore the possibility of preparing specific sera which would give clear-cut results by means of a fairly simple technique, and at the same time to study the antigenic structure of the organisms by serological methods. The application of serological typing in epidemiological investigations of staphylococcal infections such as pemphigus neonatorum (Hobbs, 1944; Allison & Hobbs, 1947) mastitis in women (Hobbs, 1944) and sycosis barbae (Hobbs, Carruthers & Gough, 1947) was studied at the same time.

The bacteriophage typing of staphylococci introduced by Fisk (1942) and amplified by Wilson & Atkinson (1945), promises to be a useful method of differentiation, and some preliminary observations on a comparison of the serological and bacteriophage methods of typing are included.

* The name *Staphylococcus pyogenes* denotes coagulase-positive strains of *Staph. aureus*, this property being at present recognized as the *in vitro* test of actual or potential pathogenicity; such strains may be pigmented or non-pigmented.

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REVIEW OF PREVIOUS WORK

Much of the early work on staphylococci combined biochemical and cultural examinations with immunological studies. Julianelle (1922) divided twenty-five strains into three serological groups by means of agglutination reactions. He found that staphylococci fixed complement but complement fixation tests showed no difference between strains.

Julianelle & Wieghard (1934, 1935) and Wieghard & Julianelle (1935) distinguished pathogenic type A from non-pathogenic type B strains of staphylococci by precipitation of purified carbohydrates with staphylococcal antisera. Virulence was claimed to be associated with the type A polysaccharide. They also described a protein substance which was antigenic but shared in common by all staphylococci. Lancefield (1925) showed by serological methods that the protein of staphylococci possessed antigens which were common to both haemolytic and non-haemolytic streptococci and to pneumoccoci.

Hine (1922), using agglutination tests at 55° C. with absorbed sera, demonstrated two groups: I, *Staph. pyogenes*, and II, *Staph. epidermidis*; the first group divisible into three serological types and the second group into two serological types. He found that agglutinability varied from strain to strain and that suspensions in saline lost their agglutinability after some months.

Yonemura (1936) divided 324 strains of *Staph. pyogenes*, isolated from a variety of infections, into nine specific types by means of serum absorption and agglutination tests. Few details are given of the methods used.

Hegemann (1937) distinguished between pathogenic and saprophytic staphylococci by precipitation methods. He found that complement fixation was unsatisfactory and claimed to have improved the specificity of agglutination reactions by treating his suspensions with antiformin.

Thompson & Khorazo (1937) divided 286 strains of staphylococci into four groups on the basis of precipitation, haemotoxin and pigment formation, fermentation of mannitol and production of coagulase.

Cowan (1938) showed by precipitation tests that the majority of *Staph. pyogenes* fell into one serological group corresponding to Julianelle's type A and that most of the saprophytic staphylococci belonged to group B, Julianelle's type B. Two further groups were described and a fifth contained all that remained ungrouped. Preliminary investigations by slide-agglutination reactions revealed three types and the possibility of sub-types. Cowan suggested that the division made by the precipitin reaction should be defined as a series of groups leaving the way open for a further subdivision into types by agglutination methods in much the same way as the haemolytic streptococci are classified. Later Cowan (1939) evolved a simple and fairly rapid slide-agglutination technique for the serological typing of staphylococci, and described the three types I, II and III placing all strains not fitting clearly into one or other of these types into an 'atypical' group which was subsequently divided into five tentative subtypes (Cowan, personal communication).

Christie & Keogh (1940) with nine sera, including three prepared from Cowan's original types, and using Cowan's slide-agglutination technique, classified pathogenic staphylococci into ten types based on major and minor agglutinogens. Although they used absorbed sera they still obtained cross-reactions, but relied on the major reactions for identification.

Verwey (1940) prepared a type-specific protein fraction independent of the type-specific carbohydrate of Julianelle & Wieghard. If a more practicable and less complicated method of extraction were found this technique might prove useful for type differentiation.

Durfee (1942) studied the relationship between the agglutination reactions and the production of polysaccharide, α-haemolysin and leucocidin by various strains of staphylococci. She found that most of the strains belonged to Cowan's types I and III and produced Julianelle 'A' polysaccharide. Strains which produced leucocidin were mainly Cowan's type II and produced less 'A' polysaccharide than other strains. A fourth type was added to Cowan's three.

Andersen (1943) described a pemphigus outbreak in Sønderborg. She was able to identify the infecting strain of coagulase-positive staphylococcus by means of slide-agglutination reactions and divided all the staphylococci isolated from cases and carriers into three serological types using formolized organisms for the preparation and absorption of sera and trypsin-digested staphylococcal antigens as suspensions for agglutination.

During recent years the discovery of bacteriophages active against staphylococci has prepared the way for a second method of type differentiation, and much promising work has developed along these lines.

Burnet & Lush (1935) found that bacteriophages which lysed *Staph. aureus* were divisible into 'strong' and 'weak' and that they could be correlated with three serologically distinct types.

Fisk (1942) found 44 % of bacteriophage-carrying *Staph. pyogenes* amongst forty-three strains, and isolated twenty-four different phages. No phages were found amongst non-pathogenic staphylococci. In a further paper Fisk (1942) tested the susceptibility of ninety-five strains of *Staph. pyogenes* to twenty-seven different phages and was able to

distribute forty-four of the ninety-five cultures among thirty-seven different groups.

Wilson & Atkinson (1945) extended the bacteriophage method of typing of *Staph. pyogenes*, and prepared lytic filtrates from eighteen strains by means of which twenty-one staphylococcal types and subtypes were recognized. They were able to type 60% of 460 strains, while a further 22·6% were acted on by phage but did not fit into any of the twenty-one types. This method of typing was applied successfully to problems in the field, particularly those connected with the causal organism of staphylococcal enterotoxin food poisoning.

I. THE APPLICATION OF METHODS ALREADY DESCRIBED, TO THE SEROLOGICAL TYPING OF *STAPH. PYOGENES*

(1) *The method of Cowan*

As a preliminary, trials were made using the technique of Cowan (1939), and sera were prepared according to his recommendations. Briefly, the method depends upon the slide-agglutination reactions obtained with sera made from Cowan's three strains of *Staph. pyogenes*, types I, II and III, and absorbed sera obtained by the absorption of type I serum with type II and type III strains separately, and of type III serum with type I and type II strains separately. According to the results obtained with these three unabsorbed and four absorbed sera, Cowan divided strains into three main types, I, II and III, placing all those strains which failed to fit clearly into these three types into an 'atypical' group (Cowan, 1939). Arising from this 'atypical' group he suggested five subtypes (personal communication) I*a*, and I*b*, III*a*, III*b* and III*c*, and Gillespie (personal communication) suggested a sixth subtype III*d*. Although satisfied with his three clear-cut types, I, II and III, Cowan has expressed some reservation over the validity of his subtypes.

Strains for typing were inoculated into 10 ml. quantities of a suitable broth in centrifuge tubes, the inoculum consisting of 0·4–0·6 ml. of an overnight broth culture. After 4–5 hr. at 37° C. in an incubator or 3–4 hr. in a 37° C. water-bath, the cultures were killed by rapidly bringing to boiling-point, cooled at once and centrifuged. The deposit was suspended in 0·5–1·0 ml. of broth supernatant or saline to give a suspension of suitable opacity. Individual batches of nutrient broth often varied, not only in their ability to encourage growth but also with regard to some factor relating to the antigenicity of the organisms. Some batches of broth gave granular suspensions, others gave gelatinous suspensions, some encouraged group reactions while others inhibited the slide agglutination of certain strains. Accordingly digest or extract broths were chosen which produced rapid growth, smooth suspensions and clear-cut slide-agglutination results.

Results. Table 1 shows the results obtained by the typing of 1552 strains isolated from a variety of sources including pemphigus neonatorum, staphylococcal conjunctivitis in infants, mastitis, sycosis barbae, staphylococcal food poisoning and upper respiratory tracts normal and abnormal. Among typed strains type I*b*, which occurred most frequently, was commonly found as a cause of pemphigus neonatorum occurring endemically over a period of 2 years in the maternity block of a local hospital (Allison & Hobbs, 1947). Type I was the next most frequent type encountered from a variety of sources including pemphigus lesions, boils, sycosis barbae and the upper respiratory tract. Type III*b* was most commonly isolated from breast milk or abscess pus from cases of mastitis, and type III*c* was found frequently to be the cause of staphylococcal food poisoning. It will be seen that 13% of the strains were not agglutinated by any serum, and that a further 13%, although readily agglutinable, could not be identified as belonging to any one of Cowan's types or suggested subtypes; the latter have been called 'indefinite' types. The inagglutinable and 'indefinite' strains thus comprised 26% of the total number of strains examined.

Table 1. *The serological type distribution of* 1552 *strains of* Staphylococcus pyogenes *isolated from pemphigus neonatorum, staphylococcal conjunctivitis, mastitis, sycosis barbae, food poisoning and human upper respiratory tracts*

Serological type (Cowan)	No.	%
I	268	17·3
I*a*	127	8·2
I*b*	387	24·9
II	84	5·4
III	7	0·4
III*a*	18	1·2
III*b*	88	5·7
III*c*	146	9·4
III*d*	20	1·3
Agglutinable but not typed ('Indefinite')	206	13·3
Inagglutinable	201	12·9
Total	1552	100·0

One reason for the high proportion of untypable strains was the granularity of some of the suspensions prepared for typing. It was observed that suspensions for slide agglutination prepared from living cultures grown in Hartley broth containing 0·5% glucose for 18–20 hr. at 37° C. were often smooth, while 4–5 hr. suspensions from Hartley broth without glucose were granular. Unfortunately some strains grown in glucose broth became inagglutinable although remaining perfectly smooth.

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Table 2 gives the comparative results obtained for the typing of 335 strains of *Staph. pyogenes* using these two media. It will be seen that twenty-eight out of thirty-one strains giving granular suspensions in young Hartley broth cultures yielded smooth suspensions in overnight glucose broth culture and of these sixteen were typed, the remaining twelve being inagglutinable. Approximately the same numbers of 'indefinite' strains, that is strains which could not be fitted into any particular type, were found from suspensions of overnight glucose broth cultures as from suspensions of young Hartley broth cultures. Some strains which were inagglutinable on first isolation were readily identified after they had been kept for a few weeks in agar stabs at room temperature.

Table 2. *Comparison between suspensions from (a) heat-killed 4–5 hr. Hartley broth and (b) living 18 hr. 0·5% glucose broth cultures for typing* Staphylococcus pyogenes *by slide agglutination*

	Hartley broth suspensions		0·5% glucose broth suspensions	
	No.	%	No.	%
Typed	211	62·9	227	67·7
Granular	31	9·3	3	0·9
Inagglutinable	79	23·6	91	27·2
Agglutinable but not typed ('Indefinite')	14	4·2	14	4·2
Total	335	100·0	335	100·0

The cross-reactions obtained amongst many of the subtypes presented the greatest difficulty, while minor variations amongst different colonies of presumably the same type fished from one primary culture were also troublesome. With experience many minor variations could be ignored but there still remained a confusing series of gradations between the subtypes, particularly those of type III, which made it almost impossible to fit some strains into a recognized type. Furthermore, any two sets of absorbed sera prepared from the same types and absorbed by the same heterologous strains rarely seemed identical. Batches of absorbed sera prepared from the same strains and absorbed by the same heterologous strains by two laboratories have given very different results, as, in fact, has the interpretation of results by two investigators using the same batches of sera.

Two further sera were added to those prepared from Cowan's three types. One was prepared from a strain regularly isolated from pus or milk in a series of cases of breast abscess occurring in one nursing home and which on first isolation was nearly always inagglutinable; weak slide-agglutination reactions after subculture suggested that the strain was probably type III*b*. The other strain appeared to be endemic in the maternity block of a local hospital and was responsible for many cases of pemphigus neonatorum. The agglutination reactions of this organism generally resembled those of type I*b*, but it was often granular and frequently showed weak slide-agglutination reactions. The introduction of these two sera used unabsorbed in a dilution of 1 : 5 reduced the proportion of inagglutinable strains. Of 101 strains which would otherwise have been untypable, seventy-eight reacted with one or other of these sera leaving a total of twenty-three which could not be classified. The two additional sera made little difference to the proportion of 'indefinite' strains.

(2) *The method of Christie & Keogh* (1940)

Christie & Keogh described nine types of *Staph. pyogenes*, three of which were Cowan's main types I, II and III. Their method of differentiation appeared to depend partly on unabsorbed and partly on absorbed sera. Type I was used for absorbing most of the sera; antiserum for type I was absorbed with Cowan's type III strain and antisera for types II and 7 with strain MS48 (Christie & Keogh's type 4–5). Identification was carried out by means of the slide-agglutination technique and depended on a pattern of reactions. The strong reactions were considered to be due to major and the weak reactions to minor agglutinogens. Table 3 shows the slide-agglutination titres of the sera, absorbed in the manner recommended by Christie & Keogh, against the homologous and heterologous strains. The method showed much the same disadvantages as were found in Cowan's method. The antigenic overlap was still troublesome with some sera, and the results obtained with absorbed sera were not always consistent. There were indications, however, that by means of absorption some sera could be made specific and that where this was not possible a pattern of reactions made identification possible.

It seemed probable that (1) there might be further types which could be recognized in the same way by preparation of more antisera, and (2) improved methods of absorption might lead to greater specificity.

II. EXPERIMENTS ON THE SEROLOGICAL REACTIONS OF STAPHYLOCOCCI

(1) *Preparation of sera*

Sera were prepared from Cowan's types I, II and III, from Christie & Keogh's types 4, 5, 6, 7, 8 and 9, from twelve strains of *Staph. pyogenes* freshly isolated from staphylococcal lesions which could not be typed satisfactorily by Cowan's technique, and from one strain of *Staph. albus*. The first eleven sera were prepared according to the technique described by Cowan (1939). Broth cultures, incubated 18–20 hr., were killed by rapidly bringing to boiling-point,

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cooled, centrifuged and resuspended in a similar volume of saline. Nine inoculations were given intravenously to rabbits on three consecutive days of three successive weeks, 0·5 ml. the first week and 1·0 ml. the second and third weeks. If the slide-agglutination titre of the serum taken 5–6 days after the last dose was unsatisfactory a further three daily inoculations of 1·0 ml. each were given the following week. The remaining sera were prepared as follows: 75 ml. quantities of broth in centrifuge tubes were inoculated with about 0·4 ml. of an 18-hr. broth culture and incubated for 18–20 hr. at 37° C. After centrifugation 18 ml. of 75% (v/v) of ethyl alcohol was added to the deposit and the alcohol suspension was stored in the cold room for 1 hr. The centrifuged deposits from 300 ml. broth were drained, resuspended in 15 ml. saline and tested for sterility. Suspensions prepared in this way contained approximately 20,000–25,000 million organisms per ml. Inoculations graded from 0·1 to 1·0 ml. were given on three successive days of three successive weeks. By both methods the homologous titres of sera ranged from 1: 500 to 1: 8000 approximately by slide agglutination. Tube-agglutination titres in the water-bath at 52° C., or by the technique used for the Vi agglutination of *Salmonella typhi* were sometimes higher, occasionally ten times the slide-agglutination titre.

Owing to the shortage of rabbits no attempt was made to passage strains of *Staph. pyogenes* prior to the preparation of vaccines as recommended by Cowan. Sera from all normal rabbits to be used for immunization were tested for natural antibodies to staphylococci. In a few instances low titres were obtained but never higher than 1: 8 by slide agglutination.

(2) *Agglutination. Techniques and results with unabsorbed sera*

Techniques. Three methods were used to estimate the titres of all newly prepared sera for the homologous strain of *Staphylococcus pyogenes*, and also for many heterologous strains. For the preparation of staphylococcal suspensions to be used as antigens, the age of the culture, the effect of using live suspensions or those killed by heat, phenol, formol or alcohol and growth in various media were investigated.

Slide agglutination. A preliminary slide-agglutination titration was used because it was simple, quick and yielded more clear-cut results than tube agglutination in which the end-point was often difficult to define. Dilutions of serum in drops of saline were made on a waxed slide using a Pasteur pipette. A loopful of each dilution was transferred to a microscope slide divided into sections by grease pencil, to each a loopful of suspension was added and the slide was rocked gently for 3–5 min. or until the end titre was definite. Suspensions in saline or broth of the spun deposit from 4–5 hr. cultures of heat-killed organisms grown in meat digest broth produced the best and most regular results.

Table 3. *Slide-agglutination titres of staphylococcal sera prepared and absorbed by the technique recommended by Christie & Keogh* (1940)

	Absorbed sera								
Suspension	CI/III	CII/MS48	CIII/I	CK4/I	CK5/I	CK6/I	CK7/MS48	CK8/I	CK9/I
CI	**1/40**	0	0	0	1/20	0	0	0	0
CII	0	**1/320**	0	0	0	0	0	0	0
CIII	0	0	**1/80**	0	0	1/40	0	0	0
CK4	0	0	0	**0**	0	0	0	0	0
CK5	0	0	0	0	**1/80**	0	0	0	0
CK6	0	0	0	0	0	**1/20**	0	0	0
CK7	0	0	0	0	0	0	**1/1280**	0	0
CK8	0	0	1/10	0	0	Tr.	0	**1/20**	0
CK9	0	0	0	0	1/20	0	0	0	**1/160**

0 = No agglutination. Tr. = Trace agglutination.

Water-bath agglutination. After numerous trials in which the method of preparation of the suspension was varied as well as the temperature and incubation period required for the test, it was decided to use the following technique.

Cultures incubated for 4 or 5 hr. in (*a*) meat digest broth, or (*b*) CCY fluid media (Gladstone & Fildes, 1940) to which was added an extra 5·0 % of yeast extract, were killed by rapidly bringing to the boil. The suspensions were either standardized to contain approximately 1000×10^6 organisms per ml. and used in equal volumes with the diluted serum or concentrated from 10 to 0·25 ml. by centrifugation when one drop was added to each serum dilution.

The tubes were incubated in a water-bath at 52° C. and read after 4 and 24 hr. The agglutination particles were very fine and the end-point often difficult to determine.

Vi technique for agglutination. The technique was similar to that described by Felix for the determination of Vi antibody to *Salmonella typhi*. Wide-bore,

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3 in. × ½ in. round-bottomed tubes were used and one drop of concentrated suspension, prepared as described above, was added to each serum dilution. The tubes were incubated for 2 hr. at 37° C. and left overnight at room temperature. The test was read by examination of the amount and nature of deposit rather than by the clearing of the supernatant. End-points were usually more clear-cut than in the Dreyer tube method.

Finally, the standard technique adopted for the investigation of all freshly prepared sera was as follows: two to three centrifuge tubes, each containing 10 ml. of meat digest broth, were inoculated with approximately 0·4 ml. of an 18 hr. broth culture of each organism to be tested. The cultures were incubated for 4–5 hr. at 37° C., killed by bringing rapidly to boiling-point, cooled and centrifuged. To the centrifuged deposits from 10 ml. of culture sufficient saline was added for the appropriate test, approximately 0·25 ml. for a concentrated suspension, 5·0 ml. for the water-bath technique and about 0·4 ml. for slide-agglutination titrations.

Agglutination reactions with unabsorbed sera. To study the cross-reactions between strains agglutination tests with unabsorbed sera, using the three methods described above, were carried out with all the available sera against homologous and heterologous organisms. There was extensive cross-agglutination so that differentiation between strains was impossible. The only conclusion reached was that the homologous titres were usually, but not always, amongst the highest. Table 4 shows a set of fourteen reactions using unabsorbed sera and the slide-agglutination technique. The heterologous cross-reactions were not so marked as with tube agglutination and a slightly higher degree of specificity was shown—particularly with types II, 7 and 9. It was obvious from these results, however, that without careful absorption none of these sera except those prepared from II and 7, would be sufficiently specific for type differentiation.

(3) *Absorption*

The object was to obtain a set of specific sera incorporating sera prepared from the strains described by Cowan and by Christie & Keogh, as well as additional type-specific sera from freshly isolated strains from staphylococcal lesions, not identifiable with the sera already prepared.

Most absorptions were carried out with the equivalent of one 16–18 hr. agar plate (4 in. diameter) culture of *Staphylococcus pyogenes* to 0·5 ml. of serum diluted 1: 5. The growth was washed off with saline, killed by boiling and centrifuged. To the deposited culture from one agar plate were added 0·4 ml. saline and 0·1 ml. serum; the deposit was emulsified in the diluted serum, incubated at 37° C. for 1 hr. with occasional shaking and centrifuged. Merthiolate 1: 10,000 was added as preservative to the absorbed serum or the serum dilution was made in saline containing 1: 10,000 mercuric iodide. The absorbed serum was titrated against the absorbing strain and the homologous organism on a slide. If the serum still agglutinated the absorbing strain a second absorption was carried out with the deposited growth from a second plate of culture, and treated as already described. If the absorbed serum failed to agglutinate its homologous strain the absorption was repeated with a fresh serum dilution using either a smaller dose of the original absorbing strain or a different strain of staphylococcus. Finally, each absorbed serum was titrated against its homologous organism and also against the heterologous strains by means of slide agglutination. A quantitative and therefore more standard method of absorption has been used recently and work is continuing along these lines.

At first one particular type of *Staph. pyogenes* was used to absorb all sera. Christie & Keogh's type 8 strain was the first choice because the unabsorbed serum prepared from it agglutinated to high titre all the strains tested except Cowan's type II and the strain was, therefore, thought to possess much 'group' antigen. Table 5 shows the results of absorption with CK8. The specificity of many of the sera was greatly improved although they showed much reduced titres for the homologous strain, but others still showed considerable cross-reactions; types I and '10667' sera and four other sera not shown in the table were particularly unsatisfactory. Serum CK4 absorbed with strain CK8 failed to react with any of the strains.

Since this method of absorption gave promising results, but still did not yield monospecific sera a second set of absorbed sera was prepared using Cowan's type I strain for absorption. Type I was chosen because so many types appeared to possess antigens in common with the 'group' constituent. Table 6 shows the slide-agglutination titres for sixteen strains absorbed with type 1. The results resemble those shown in Table 5. Types containing a large amount of the I antigen were agglutinated feebly or not at all. Many of the specific titres were very low and there was still an antigenic overlap between certain strains.

A third strain '7163' was tested for its ability to absorb out all group agglutinins. This strain, resembling Cowan's subtype 1*b*, was responsible for many cases of pemphigus neonatorum in Cardiff. Strains of this type were usually readily agglutinable, very often granular and occasionally specific for '7163' serum only.

The results of absorption with strain '7163' are shown in Table 7. The specific titres were moderately good and somewhat better than those obtained

Table 4. *Slide agglutination of* Staphylococcus pyogenes (*one strain of* Staphylococcus albus *included*). *Unabsorbed sera*

	Sera													
Suspensions	CI	CII	CIII	CK4	CK5	CK6	CK7	CK8	CK9	10,667	7163	1636	2411	*S. Albus*
CI	**1/1024**	0	1/64	1/8	1/256	1/8	1/16	1/512	1/128	**1/64**	1/64	1/8192	1/32	0
CII	0	**1/1024**	0	0	0	0	0	0	0	0	1/32	0	Tr.	0
CIII	1/1024	0	**1/1024**	1/32	1/64	1/128	1/16	1/128	1/128	1/4096	1/128	1/2048	1/2048	1/1024
CK4	1/8192	0	1/256	**1/512**	1/8192	1/2048	1/2048	1/4096	1/1024	1/4096	1/8192	1/8192	1/4096	1/64
CK5	1/512	0	1/64	1/64	**1/2048**	1/128	1/64	1/256	1/256	1/256	1/256	1/2048	1/1024	1/16
CK6	1/1024	0	1/64	1/64	1/2048	**1/1024**	1/128	1/256	1/256	1/512	1/512	1/512	1/4096	0
CK7	0	0	0	0	0	0	**1/16,384**	1/64	Tr.	0	0	1/8	1/1024	0
CK8	1/4096	1/32	1/64	1/1024	1/512	1/512	1/64	**1/4096**	1/512	1/256	1/1024	1/16,384	1/8192	1/8
CK9	Tr.	1/64	1/64	1/64	1/1024	1/128	1/128	1/2048	**1/32,768**	1/2048	1/1024	1/512	1/512	1/128
10,667	1/16	0	0	0	1/32	1/16	1/16	0	1/512	**1/256**	1/32	1/256	1/64	1/512
7163	1/4096	1/32	1/32	1/1024	1/512	1/16	1/32	1/128	1/16	1/32	**1/4096**	1/256	1/32	0
1636	1/2560	1/20	1/160	1/320	1/320	1/320	1/80	1/1280	1/1280	1/320	1/320	**1/4096**	1/128	0
2411	1/4096	1/512	1/512	1/512	1/128	1/128	1/256	1/64	1/64	1/2048	1/1024	1/256	**1/8192**	1/16
S. Albus	1/8	0	1/512	1/16	1/16	1/16	Tr.	1/16	1/16	1/32	1/8	0	0	**1/512**

0 = No agglutination. Tr. = Trace agglutination.

Table 5. *Slide-agglutination titrations of sera absorbed with* Staphylococcus pyogenes, *type CK* 8

	Sera												
Suspensions	CI	CII	CIII	CK4	CK5	CK6	CK7	CK8	CK9	10,667	7163	1636	2411
CI	**1/1280**	0	0	0	1/40	0	0	0	0	0	0	0	0
CII	1/320	**1/40**	0	0	1/40	0	0	0	0	0	0	0	1/20
CIII	1/640	0	**1/80**	0	0	1/40	0	0	0	1/2560	0	0	1/20
CK4	1/640	0	0	**0**	0	0	0	0	0	1/640	0	Tr.	0
CK5	1/640	0	0	0	**1/160**	0	0	0	0	1/10	0	0	1/20
CK6	0	0	0	0	0	**1/80**	0	0	0	1/320	0	0	0
CK7	0	0	0	0	0	0	**1/80**	0	0	0	0	0	0
CK8	0	0	0	0	0	0	0	**0**	0	0	0	0	0
CK9	1/640	0	0	0	1/160	0	0	0	**1/160**	1/320	0	0	0
10,667	1/640	0	0	0	0	0	0	0	1/20	**1/1280**	1/40	0	0
7163	0	0	0	0	0	0	0	0	0	0	**0**	0	0
1636	0	0	1/40	0	0	0	0	0	0	0	0	**1/160**	0
2411	0	0	0	0	0	0	0	0	0	0	0	0	**1/80**

0 = No agglutination. Tr. = Trace agglutination.

for the previous two absorptions; furthermore, although antigenic overlap existed as before there appeared to be a higher degree of specificity for many of the absorbed sera. As for sera absorbed with CK 8 all specific agglutinins were removed from the sera of CK 4, CK 8, '7163' and in addition from '1636'. That these strains are not identical may be inferred from Tables 5–8.

A study of Tables 5–7 shows that the types of *Staph. pyogenes* appear to fall into five main groups, the types within each group being very similar but not identical as shown by mirror absorption tests. The first group may be exemplified by types I and CK 5 which behave similarly towards sera prepared against each other apart from differences in titre, but vary in their reactions to other heterologous sera, CK 5 being agglutinated by the absorbed sera of '10667' and '2411', while type I is not. The second group includes type II, one of the most specific of the types so far isolated; akin to it was another type '10154' (not shown in the tables) which appeared to be transitional between groups I and II as it agglutinated readily with sera prepared against both types, while another strain CK–MS 48 behaved similarly. Type CK 9 and '2411' were transitional between CK 5 and the fourth group containing types III, CK 6 and '10667'. The fifth group contains types CK 4, CK 8 and '1636' and probably '7163'; all show a close relationship, and it may be seen in Table 7 that type '7163' appears to absorb all agglutinins from CK 4, CK 8 and '1636'. Mirror absorption tests indicated that these strains were not identical (Table 8).

In general, most newly isolated strains could be merged into one or other of these five groups, although the antigenic structure of such strains did not necessarily coincide with that of the strains already studied. CK 7 was rarely found; its antiserum could be made type-specific, so that this strain falls into a separate group, the sixth. There was a small seventh group of miscellaneous strains which were either agglutinated weakly or not at all by the other sera; they were of rare occurrence. One strain '7211', appeared to correspond with strain '235' described by Durfee (1942), and regarded by her as supplementary to Cowan's three types.

These results led to two possible hypotheses to explain the antigenic make-up of *Staph. pyogenes*. The first being that each of the five main groups possessed an antigen for each group and common to members of types within the group. On this assumption the first group would be dominated by the type I antigen, the second by type II, the fourth by type III, the fifth by type 8 or '7163', and the third by a balance between antigens I and III. The second hypothesis would disregard this grouping and consider each strain as capable of recognition on a pattern of reactions dominated by one or more major antigens. The first hypothesis would explain Cowan's scheme and the second that of Christie & Keogh. The discussion deals more fully with these suggestions.

To investigate the identity of all these strains and to prove or disprove their position as individual types more than seventy absorptions were carried out, apart from those shown in Tables 5–7. Where two strains showed such resemblance that they appeared almost identical their sera were mirror-absorbed. It is impossible to show the results of all these absorptions, but a few have been selected and are given in Table 8. These results indicate that there exist among strains of *Staph. pyogenes* types which occur in closely related pairs or small groups. The relationship of the paired organisms appears to be such that one possesses all the antigenic structure of the other plus a specific antigen. For example, a serum prepared against type III absorbed with CK 6 still retains enough specific substance to agglutinate type III, but serum CK 6 absorbed with type III fails to react with CK 6 showing that it has been completely absorbed. Similarly, serum CK 8 absorbed with type CK 4 still retains its specificity for CK 8, but serum CK 4 is completely absorbed by type CK 8. It seemed from the results shown in Table 8 that to obtain sera specific for some of these strains, each serum should be absorbed with a strain closely related antigenically to the homologous organism. Instead, therefore, of using one particular strain to absorb all sera, a carefully selected absorbing strain would be required for each serum. The results obtained with such a set of selectively absorbed sera are given in Table 9. It shows that a good degree of specificity can be reached by careful choice of the absorbing strain, but that in some instances the designation of a type name to the strain will depend on a pattern of agglutination reactions over a range of sera rather than a specific reaction with one serum alone. Strain '11529' proved very useful for absorbing '7163' serum; by Cowan's typing it showed almost identical reactions with '7163', yet when used as an absorbing agent it consistently provided an almost specific serum for '7163'.

It seems probable that among *Staph. pyogenes* there are large numbers of strains varying antigenically only slightly one from another. Whether these differences are of such importance as to warrant a separate type designation for each variation is a matter for discussion. These results suggest that there exists a small number of well-differentiated types around which occur a number of strains showing minor antigenic variations. A comparison carried out between the bacteriophage and serological methods of typing, and discussed in a later section, seems to support this view, but this should not detract from the suggestion that those strains showing a very mixed antigenic structure may be recognized as types by a pattern of reactions with a series of absorbed sera.

Table 6. *Slide-agglutination titrations of sera absorbed with* Staphylococcus pyogenes, *type CI*

	Sera												
Suspensions	CI	CII	CIII	CK 4	CK 5	CK 6	CK 7	CK 8	CK 9	10,667	7163	1636	2411
CI	**0**	0	0	0	0	0	0	0	0	0	0	0	0
CII	0	**1/20**	0	0	0	0	0	0	0	0	0	0	0
CIII	0	0	**1/80**	0	0	1/40	0	0	0	1/80	0	1/80	0
CK 4	0	0	0	**0**	0	0	0	0	0	1/40	0	Tr.	0
CK 5	0	0	0	0	**1/80**	0	0	0	0	0	0	0	1/40
CK 6	0	0	0	0	0	**1/20**	0	0	0	1/40	0	0	0
CK 7	0	0	0	0	0	0	**1/160**	0	0	0	0	0	Tr.
CK 8	0	0	1/10	0	0	Tr.	0	**1/20**	0	0	0	1/160	Tr.
CK 9	0	0	0	0	1/20	0	0	0	**1/160**	1/20	0	0	0
10,667	0	0	±	0	0	0	0	0	1/80	**1/2560**	0	1/10	0
7163	0	0	0	0	0	0	0	0	0	0	**1/2560**	1/10	0
1636	0	0	Tr.	0	0	0	0	Tr.	0	1/320	0	**1/160**	0
2411	0	0	0	0	0	0	0	0	0	0	0	0	**1/80**

± = Weak agglutination, titre not ascertained. Tr. = Trace agglutination. 0 = No agglutination.

Table 7. *Slide-agglutination titrations of sera absorbed with* Staphylococcus pyogenes *type* 7163

	Sera												
Suspensions	CI	CII	CIII	CK 4	CK 5	CK 6	CK 7	CK 8	CK 9	10,667	7163	1636	2411
CI	**1/40**	0	0	0	0	0	0	0	0	0	0	0	0
CII	0	**1/1280**	0	0	0	0	0	0	0	0	0	0	0
CIII	1/20	0	**1/1280**	0	0	1/160	0	0	0	0	0	0	+ + + +
CK 4	0	0	0	**0**	0	0	0	0	0	0	0	0	+
CK 5	1/10	0	0	0	**1/320**	0	0	0	0	0	0	0	+ + + +
CK 6	0	0	0	0	0	**1/80**	0	0	0	0	0	0	0
CK 7	0	0	0	0	0	0	**1/640**	0	0	0	0	0	0
CK 8	1/20	0	1/10	0	0	0	0	**0**	0	0	0	0	+
CK 9	0	0	0	0	1/40	0	0	0	**1/1280**	1/20	0	0	0
10,667	0	0	1/20	0	0	0	0	0	1/20	**1/1280**	0	0	0
7163	0	0	0	0	0	0	0	0	0	0	**0**	0	0
1636	0	0	0	0	0	0	0	0	0	0	0	**0**	0
2411	0	0	0	0	0	0	0	0	0	0	0	0	+ + + +

+ + + + = Rapid, well-marked agglutination (titre not ascertained).
+ = Slow agglutination (titre not ascertained).
0 = No agglutination.

Table 8. *Slide-agglutination reactions of 'mirror' absorptions*

Suspensions	Sera											
	III / CK 6	CK 6 / III	III / 10,667	10,667 / III	CK 5 / CK 9	CK 9 / CK 5	CK 9 / 10,667	10,667 / CK 9	CK 8 / CK 4	CK 4 / CK 8	CK 8 / 1636	1636 / CK 8
CI	0	0	0	0	0	0	0	0	0	0	0	0
CII	0	0	0	0	0	0	0	0	0	0	0	0
CIII	**1/1280**	0	**1/320**	0	0	Tr.	0	0	+	0	0	Tr.
CK 4	0	0	0	Tr.	0	0	0	0	0	**0**	0	Tr.
CK 5	0	0	0	0	**1/20**	0	0	0	0	0	0	0
CK 6	0	**0**	0	0	0	0	0	0	Tr.	0	0	0
CK 7	0	0	0	0	0	Tr.	0	0	0	0	0	0
CK 8	0	0	0	0	0	0	0	0	+ + + +	0	**0**	0
CK 9	0	0	0	0	0	**1/2560**	**1/40**	0	0	0	0	0
10,667	0	0	0	**1/160**	0	1/160	0	**1/160**	0	0	0	0
7163	0	0	0	1/40	0	0	0	0	+ + +	0	0	+
1636	0	0	0	0	0	0	0	0	0	0	0	+ +
2411	0	0	0	0	0	0	0	0	0	0	0	0

+ + + + = Rapid, well-marked agglutination (titre not ascertained).
+ = Slow agglutination (titre not ascertained).
Tr. = Trace and very weak agglutination.

Table 9. *Slide-agglutination reactions of the final set of absorbed sera*

Suspensions	Sera												
	I / CK 8	II / 7163	III / 7163	CK 4 / MS 48	CK 5 / 7163	CK 6 / 7163	CK 7 / 7163	CK 8 / CK 4	CK 9 / 10.667	10,667 / 1636	7163 / 11,529	1636 / 10,667	2411 / 1636
CI	+ + +	0	0	0	0	0	0	0	0	0	0	0	0
CII	0	+ + +	0	0	0	0	0	0	0	0	0	0	0
CIII	0	0	+ + +	+	0	+ +	0	0	0	0	0	0	0
CK 4	0	0	0	+ +	0	0	0	0	0	0	0	0	0
CK 5	0	0	0	0	+ +	0	0	0	0	0	0	0	0
CK 6	0	0	0	0	0	+	0	0	0	0	0	0	0
CK 7	0	0	0	0	0	0	+ + +	0	0	0	0	0	0
CK 8	0	0	0	+ +	0	0	0	+ +	0	0	+ +	+	0
CK 9	0	0	0	0	0	0	0	0	+ + +	0	0	0	0
10,667	0	0	+ +	0	0	0	0	0	0	+ + +	0	0	0
7163	0	0	0	0	0	0	0	0	0	0	+ + +	0	0
1636	0	0	+	0	0	0	0	+	0	0	+	+ + +	0
2411	0	0	0	0	0	0	0	0	0	0	0	+ +	+ + +

+ to + + + = Degrees of agglutination. 0 = No agglutination.

A few absorptions were carried out using mixed absorbing cultures, but they were not very successful as absorption with two or more strains made the sera too weak for slide-agglutination purposes. Tube agglutination including both water-bath and Vi techniques was used for titrating a few of the absorbed sera. The method was moderately successful, although end-points were difficult to read because of the very fine agglutination. Slide agglutination was preferred because of its simplicity, speed and sharp end-points.

(4) *Precipitation*

Lancefield's technique for the acid extraction of streptococci for grouping purposes, Lancefield (1928, 1933), was used for the preparation of extracts from eleven serologically distinct types of *Staph. pyogenes*, as well as from a coagulase-negative strain of *Staph. aureus*, four strains of *Staph. albus*, a diphtheroid, and a yeast. All extracts were tested against unabsorbed antisera prepared from the eleven types of *Staph. pyogenes*. The precipitin reactions with the extracts prepared from the strains of *Staph. pyogenes* failed to show serological differentiation. Most strains of *Staph. albus* gave weak reactions similar to those obtained from coagulase-negative *Staph. aureus*. One strain of *Staph. albus*, however, failed to give any precipitation. Extracts from the diphtheroid and yeast showed no reactions. Apart from revealing a difference between the coagulase-positive and negative strains, there appeared to be little value in the precipitin test carried out by this particular technique and using unabsorbed sera.

(5) *Preliminary comparison between serological and bacteriophage methods of typing*

Subcultures of the strains of *Staph. pyogenes* isolated during epidemics of pemphigus neonatorum and breast abscess were sent to Oxford for typing by bacteriophage, while serological typing of the strains was carried out at Cardiff. Similarly, strains from Oxford were sent to Cardiff for serological examination. Although the first comparisons of these two methods of identification were undertaken during the early days of both serological and bacteriophage investigations, useful information was obtained on the degree of correlation between the two. Many of the phage filtrates were of low titre and had not been classified into any order except their precedence in isolation and much of the serological typing was carried out using Cowan's method only, with unabsorbed and absorbed sera prepared from Cowan's three types.

A review of the results obtained for 382 strains typed by both methods showed that the serological types could be divided into five groups; this grouping was governed by the phage typing results which indicated little or no difference between serological types I and I*a*, I/II and II, III*a* and III*b*, III*c* and III*d*. Several different phage filtrates may be active against strains belonging to one serological type but there was a general trend for (1) types I and I*a* to be lysed by phages 29, 44 or 44*a*, or to remain unidentified, indicating either that no phage had as yet been found to lyse those strains or that they were phage resistant; (2) type I*b* to be lysed by phages 3, 3*a* and 51; (3) type II to be lysed by phages 29, 51 and 44 or 44*a*; (4) types III*a* and III*b* to be lysed by one or both of phages 44 and 44*a*; and (5) types III*c* and III*d* to be lysed by phages 6, 47, 42 and 4.

In this series of 382 strains, 51 (13·3 %) were untypable by phage but could be identified serologically. Most of the 76 (19·7 %) strains which were untypable by serological methods were lysed by phages 44 or 44*a*. An antiserum subsequently made to one of these strains isolated from a breast abscess, one of a series of mastitis cases occurring in a maternity home, showed that the strain reacting with phages 44 or 44*a* belonged to one serological type.

Tables 10 and 11 show the results of examination by both methods of 256 more recently isolated strains of *Staph. pyogenes*. These strains were isolated from outbreaks of infection including pemphigus, breast abscess, boils and staphylococcal food poisoning and were from widely scattered areas. The nomenclature of the serological types has been changed. Roman figures are still given to Cowan's original three types, followed by arabic figures representing Christie & Keogh's types 4–9, while sera prepared from freshly isolated strains have been given the laboratory number of the relevant strain. There have been alterations also in the bacteriophage nomenclature as a number of new bacteriophages have been added to the series and some have been discarded; the bacteriophage numbers in the tables represent the actual phage filtrates active against the strains and not phage types.

Table 10 shows the frequency distribution of the phage patterns corresponding to the various serological types, while Table 11 shows the frequency distribution of individual phages in relation to each serological type.

Table 10 is designed to illustrate the variety of phage reactions which may occur for any one serological type. The second and fifth columns show the total number of strains examined by both methods of typing, and include not only the infecting types from outbreaks of staphylococcal infection or food poisoning but also other strains isolated from carriers and contacts during the investigation of these outbreaks. A small number of strains isolated from nose, throat, skin and wounds during surveys of staphylococcal carriage not related to outbreaks of infection are also included. The third and last columns show the number of outbreaks covered. The figures indicate

Table 10. *The relationship of serological types to bacteriophage pattern reactions*

Serological			Bacteriophage		
Type	Total no. of strains examined	No. of outbreaks covered	Filtrate or filtrate pattern	No. of strains examined	No. of outbreaks covered
CI	55	14	**52**, 29/52, 29/44/52, 52/52A, 42C/42D/52, 47/52/52A	13	9
			47C, 42B/42C/47C, 42B/47C	13	3
			29, 29/42C	3	2
			31	3	2
			52A	1	1
			42C	1	1
			Not typable	21	8
CII	31	14	**3C**, 3B/3C, 3A/3B/3C, 3A/3B/3C/51	17	11
			3B	2	1
			51	1	1
			Not typable	11	7
CIII	35	14	**47**, 6/47	23	11
			47A	2	2
			7	2	1
			Not typable	8	4
CK4	31	7	47B	18	2
			29	5	1
			31, 31/52	2	2
			44/52	1	1
			Not typable	5	4
CK5	38	17	**29**, 29/31, 29/29A/31, 29/29A/31/52, 29/31/52, 29/31/47C/52, 29/42B/47C/52	10	9
			42B, 42B/47C/52	4	4
			29A/31	3	1
			52A, 42C/52A	2	1
			31/52, 42C/44/52	2	1
			Not typable	17	9
CK8	14	4	3A	5	2
			42C	2	—
			31	2	—
			Not typable	5	3
CK9	4	1	42E	4	1
7163	30	11	**3A**, 3A/3B/3C	16	7
			Not typable	14	6
10,667	10	2	**31**, 31/44	4	2
			44	1	—
			42D	2	1
			Not typable	3	1
1636	8	3	31/44	4	1
			3A	2	1
			42C	1	1
			Not typable	1	1

Note: Clarendon type signifies the phage reaction common to the series.

that in general the different types came from many outbreaks as well as from individuals within the same outbreak and that strains were derived from various parts of the country and not confined to one outbreak or one locality.

When two or more strains of *Staph. pyogenes* were isolated from different sources in one individual they were counted as one if they belonged to the same serological type. Multiple strains isolated from the same individual, but differing from each other serologically, were counted as separate individual types.

Considering each serological type and comparing the phages which react on that type the following points may be observed: many (21 or 40·4 %) of the serological type I strains were not identified by phage, while the remainder fell mostly into the 47*c*, 52 and 29 phage pattern. The diversity of the filtrates acting on type I strains indicates that these reactions

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may be of a 'group' nature and that no specific phage has as yet been found for type I strain. Type II strains were lysed chiefly by either or both of phages 3B and 3C and occasionally by phage 3A; the reaction to phage 3A is difficult to understand because strain '7163', which is lysed almost exclusively by phage 3A, is quite dissimilar serologically to type II. Phages 3B and 3C had been prepared since the preliminary comparative work described above. Most type III (similar to Cowan's III*c*) strains were lysed by phages 6 and 47, but sometimes by phage 47 alone. The results for type 4 may be a little misleading as the strains which were lysed by phage 47B came from two outbreaks only. Six further strains identified as type 4, but which showed a slightly different pattern of serological reactions, were lysed by one or more of phages 29, 31, 44 and 52, a reaction pattern often shown by type I strains. Phages 29 and 31 were also prominent in lysing type 5 strains and either alone or in combination with other filtrates reacted on fourteen out of thirty-eight strains of type 5, seventeen of the remaining twenty-four strains being untypable by phage. Type 5 strains were found to resemble type I strains serologically and this may account for the similarity in phage reactions. Omitting type 8, for the moment, it will be seen that the four type 9 strains were lysed by the phage 42E. Type '7163' was lysed very constantly by phage 3A; the reason why one strain was lysed not only by phage 3A but also by phages 3B and 3C is not easy to understand because, as pointed out above, there appeared to be little or no resemblance serologically between types '7163' and II. Type '10667' which appears to be a clear-cut serological type, was not reacted on specifically by any phage unless it be 42D, but it also proved susceptible to phages 31 and 44 either alone or in combination.

Types 8 and '1636' are serologically very similar, but not identical (see Table 8). It may be seen that their phage reactions are similar and that they are both lysed by phages 3A, 42C and 31. There is a serological relationship between these two types and '7163', but the strains were found not to be identical.

Of these 259 strains, 85 or 32·8% were untypable by phage, whereas all gave positive agglutination reactions with the battery of absorbed sera in use.

Table 11. *The relationship of serological types to individual bacteriophage reactions*

Serological type	No. exd.	Bacteriophage filtrate										
CI	55	Phage filtrate	52	47C	29	42C	52A	31	42B	47	44	42D	N.T.
		No. examined	13	13	8	4	3	3	2	1	1	1	21
CII	31	Phage filtrate	3C	3B	3A	51	N.T.						
		No. examined	17	11	2	2	11						
CIII	35	Phage filtrate	47	6	47A	7	N.T.						
		No. examined	23	16	2	2	8						
CK4	31	Phage filtrate	47B	29	31	52	44	N.T.					
		No. examined	18	5	2	2	1	5					
CK5	38	Phage filtrate	31	29	29A	52	42B	47C	42C	44	N.T.		
		No. examined	11	9	4	7	6	3	1	1	17		
CK8	14	Phage filtrate	3A	42C	31	N.T.							
		No. examined	5	2	2	5							
CK9	4	Phage filtrate	42E										
		No. examined	4										
7163	30	Phage filtrate	3A	3B	3C	N.T.							
		No. examined	16	1	1	14							
10,667	10	Phage filtrate	44	31	42D	N.T.							
		No. examined	3	3	2	3							
1636	8	Phage filtrate	31	44	3A	42C	N.T.						
		No. examined	4	4	2	1	1						

N.T. = Not tested.

The agglutination reactions were not always monospecific, and as in phage typing, identification was frequently based on the identical pattern reactions with several absorbed sera.

It sometimes happened that within the same outbreak, the infecting strain isolated from different sources was phage typed in some instances and proved completely phage-resistant in others; there was no evidence that the serological reactions of a strain were affected by the development of resistance to phage, and all the strains could be identified serologically.

With both methods of typing it was found that those strains giving a pattern of reactions sometimes varied slightly in the patterns which they formed; this was particularly noticeable when strains from

the one individual were isolated and examined on repeated occasions.

These results indicate that certain serological types are lysed by certain phage filtrates or groups of filtrates, and from these observations two hypotheses arise. First, that there exist amongst staphylococci antigenic types or complexes specifically susceptible to the action of certain phage filtrates. The correlation is not complete but it is implied. The antigens are heat stable and in this respect resemble the somatic antigens of Salmonella organisms, and the type specific 'M' antigens of *Str. pyogenes*. Secondly, instead of a large number of phage filtrates, each of which is capable of lysing a different type of staphylococcus, there may be groups (or subtypes) of phage filtrates the individual members of which react on one type of staphylococcus. It has been suggested (Smith, 1948) that staphylococci may be divided by phage methods into 'genetic types' that differ from each other according to the following groupings:

'Genetic type'	Phage filtrates						
1	3A	3B	3C	51	—	—	—
2	6	7	42B	47	47C	—	—
3	29	29A	31	42C	44	52	52A
4	42D	—	—	—	—	—	—
5	42E	—	—	—	—	—	—
6	47A	—	—	—	—	—	—
7	44A	—	—	—	—	—	—
8	47B	—	—	—	—	—	—

These type differences are not related to acquired phage resistance.

Strains that are susceptible to any of the phages forming one of the groups are considered as one 'genetic type'; the term 'genetic type' is used to distinguish them from phage types.

While there were objections to this method of classification the agreement between these suggested groupings and those indicated in Table 11 is fairly high. There are a few notable discrepancies, for example, the placing of phage 3A in group 1 with phages 3B and 3C because phage 3A usually lyses a serological type, '7163', unrelated serologically to the types associated with phages 3B and 3C. Again phage 42B is placed in group 2 when it appears to be more closely related to serological type I, although it is often associated with the 6/47 complex. These discrepancies show that the correlation is not yet complete, although there are strong indications that the action of phage filtrates on the organisms are dependent on certain antigenic components on or in the cell. These observations tend to support the suggestion that there may exist among both serological and bacteriophage types a small number of specific types around which are grouped varying numbers of subtypes.

It is possible that all the cocci of a particular strain may not be equally endowed with subsidiary or group antigens and this may apply also to different strains of the same type.

To summarize, it appears that there is a relationship between the results obtained by serological and bacteriophage typing. A number of discrepancies are evident but it is hoped that further work may reveal an explanation for them and show the connexion between the action of bacteriophage-containing filtrates and the antigenic structure of the bacterial cell.

III. SEROLOGICAL TYPING APPLIED TO PROBLEMS IN THE FIELD

Serological typing of *Staph. pyogenes* was applied to the epidemiological investigation of three types of staphylococcal infection. First, outbreaks of staphylococcal infection in small nursing homes consisting of cases of pemphigus neonatorum and breast abscess; two such outbreaks have been reported in the *Monthly Bulletin of the Emergency Public Health Laboratory Service* (Hobbs, 1944). The first deals with an outbreak of pemphigus neonatorum in which the infecting strain of *Staph. pyogenes* was identified as type I*b*; a heavy nasal carrier of the same type was found among the nursing staff. The nurse was due to be transferred and there was no further trouble after she had left. The second outbreak deals with a nursing home in which a series of cases of breast abscess occurred from each of which the same type of *Staph. pyogenes* was isolated, type III*b*. Unfortunately the source was not discovered. The infecting strain was frequently found to be serologically untypable on first isolation, but by means of the bacteriophage method of typing it was possible to identify all the strains as belonging to one type which apparently corresponded to the serological type III*b*. This was confirmed after preparation of an antiserum against this strain.

Serological typing was also applied to the problem of pemphigus neonatorum and staphylococcal conjunctivitis ('sticky eye') in the maternity block of a large hospital (Allison & Hobbs, 1947). There were 111 cases of pemphigus and twenty-one cases of 'sticky eye'. The pemphigus occurred in a series of sharp outbreaks, one of which involved twenty-one cases. In the periods between outbreaks cases continued to occur at irregular intervals. Bacteriological examination of lesions from infants and of swabs from the nose and throat of staff were made in two outbreaks caused by different serological types of *Staph. pyogenes*, and a third which appeared to be commencing as the investigation terminated. The infecting strain, present in nearly 100% of lesions, was present also in the noses of a high percentage of the nursing staff and was in fact the predominant

organism. In the first two outbreaks the swabbing was carried out shortly after the maximum number of cases had occurred but the last set of swabs was taken at the commencement of infection among the infants. It seemed, therefore, that a high carrier rate of an invasive strain of *Staph. pyogenes* among the nursing staff provided a high concentration of that strain in the nursery environment and under conditions of overcrowding predisposed the infant population to infection by that strain. A comparison was made also between the types of *Staph. pyogenes* found in nose, eyes and lesion of infected infants and attempts were made to trace the paths of spread of infection.

A third type of staphylococcal infection to which serological typing of *Staph. pyogenes* was successfully applied was sycosis barbae (Hobbs *et al.* 1947). Examination of strains of *Staph. pyogenes* isolated from the lesion and nose of individual patients showed that the serological type causing the infection was also present in large numbers in the patient's nose. Provided the organism was penicillin-sensitive treatment of the lesion and the nose with penicillin cream resulted in at least temporary cure, both of the lesion and of the nasal carrier state. When, however, the nose was not treated, or the organism persisted in the nose in spite of treatment, relapses occurred regularly. Occasionally there was recurrence of infection due to a different serological type of *Staph. pyogenes* and again the fresh type was found in the patient's nose.

A few outbreaks of food-poisoning due to staphylococcal enterotoxin were investigated and serological typing was employed to identify the nasal or hand carrier of the infecting strain among food-handling personnel.

DISCUSSION

Serological work on the type differentiation of staphylococci has progressed by slow stages from the work of Julianelle (1922) and Hine (1922), who demonstrated the possibility of division by agglutination into a limited number of groups or types, to that of Cowan (1939) and Christie & Keogh (1940), which extended the number of specific types and simplified the technique so that it could be applied less laboriously to epidemiological investigations. Cowan's method limited the number of distinct types to three and suggested a further division into five sub-types related to these, while Christie & Keogh, relying principally on the reaction of major agglutinins, defined nine types, including Cowan's original three.

The work described above suggests a further increase in the number of supposedly specific types, recognizable by selective absorption of sera. Consideration of the collected findings, however, suggests that it is still a matter for discussion whether there exists a large number of types capable of recognition by specific absorbed sera or whether in reality there are only a small number of specific types around which are grouped many strains showing minor variations from the main type. A classification for *Staph. pyogenes*, based on the labelling of primary antigens or on the recognition of types by a pattern of reactions, would be advantageous in allowing the inclusion of new types along with those already formulated regardless of whether they possessed combinations of antigens already known or new primary antigens. Similar hypotheses have been suggested for the Flexner group of organisms.

The serology so far suggests, therefore, either that types such as Cowan's I, II, and III, type 7 of Christie & Keogh, and the more recently isolated strain '7163' represent specific types, while many other strains are connected with these types through a common antigen, or that there exist very many primary antigens in strains which are identifiable at present by a pattern of major and minor agglutination reactions only. It is possible that an analogous system of antigens may exist in the staphylococci as has already been found in the morphologically similar group of streptococci which are divisible into large groups by the possession of somatic polysaccharides and into types by specific proteins, the 'M' substances and more generally distributed 'T' substances, the constitution of which have not been fully worked out. It is hoped that the staphylococci will be studied further along these lines. The recent work of Andersen (personal communication) suggests that the staphylococcal antigens are both protein and polysaccharide in nature. She has prepared in rabbits antisera from suspensions of organisms treated in different ways. Antigens prepared by similar methods and used for absorption and slide-agglutination tests could be divided into three groups depending on their stability to trypsin and heat. By means of two of these antigenic groups she has been able to differentiate five 'types' of staphylococci. Her results suggest the presence in staphylococci of group-specific and type-specific antigens which may correspond to the 'T' and 'M' antigens of group A streptococci (Lancefield, 1940).

One of the objects of this investigation has been to co-ordinate the existing methods of serological typing and to explore the possibility of increasing the number of types. The original nomenclatures have, therefore, been maintained throughout, namely Cowan's roman numerals I, II and III, Christie & Keogh's arabic 4, 5, 6, 7, 8 and 9, while further types added during the investigation are referred to by their laboratory numbers. The question of re-numbering the types was considered, but it seemed unwise to do so at this stage. Further work on the serology of staphylococci along the lines of Andersen's investigations might clarify the antigenic

structure and thus provide a firmer basis for reclassification. Meanwhile, it is suggested that for convenience strains '10667', '7163', '1636', and '2411' be regarded as representative of provisional types 10, 11, 12 and 13.

So far, therefore, it has been established that certain strains of *Staph. pyogenes* belong to recognizable serological types. These types, whether isolated from local outbreaks of staphylococcal infection or from outbreaks occurring in other parts of the country, have been consistently identified by the use of absorbed sera. There are other strains which can be identified only by a pattern of agglutination reactions using a battery of absorbed sera. It is suggested that provided these results are reproducible they may be regarded as valid types and not merely as variations of one another. The evidence is such that the identification and correlation of types may be carried out not only within a single outbreak of staphylococcal infection but from outbreaks in different localities.

When the same strains are tested by both bacteriophage and serological methods of typing there is a fairly close correlation between results obtained by the two methods in the case of clear-cut specific types and strains from a single outbreak, but there is not always agreement on the identification of ill-defined types. Bacteriophage differentiation has produced a greater division of strains than is available by serological methods, but these divisions may not be legitimate. It is frequently found that the identity of strains from different sources in an outbreak depends on a pattern of reactions in much the same way as has been described for agglutination reactions. Further work on comparisons between the two methods may lead to a better understanding of the fundamental nature of bacteriophage differentiation. As Dubos (1945) points out 'enzymic decomposition, agglutination, precipitation, inhibition of growth, lysis, etc., are only the secondary manifestations of a primary reaction, the specificity of which depends upon the union between the cellular receptors on the one hand, and the biological reagent, be it enzyme, antibody or bacteriophage, on the other'.

SUMMARY AND CONCLUSIONS

1. Of 1552 strains of *Staph. pyogenes* tested by the slide-agglutination technique, using the absorbed and unabsorbed sera suggested by Cowan, 74% of strains were identified while 26% remained untyped. Of untyped strains, half were inagglutinable while the remainder gave 'indefinite' results and remained unclassified. The introduction of two new sera, used diluted but unabsorbed, considerably reduced the number of untyped organisms but made little difference to the proportion of 'indefinite' strains. The method was simple and quick, but the variety of cross-reactions often prevented a clear-cut differentiation of strains and the number of types identifiable was limited.

2. Of three methods of agglutination, titrations on glass slides were preferred because by this technique end-points were clearer than those obtained in a water-bath or by the method for determination of Vi antibody.

3. Agglutination of seventeen strains of *Staph. pyogenes* with homologous and heterologous sera showed that the use of unabsorbed sera was of limited value for typing because of the marked cross-reactions.

4. Christie & Keogh's division of *Staph. pyogenes* into nine types was promising but their absorbing methods allowed much antigenic overlap.

5. Experiments with absorbed sera showed that no single strain of *Staph. pyogenes* could be used successfully to absorb all sera. There appeared to be groups or pairs of types in which serologically the individual members resembled each other very closely and yet were not identical. In these instances the absorption of a serum by a heterologous strain nearly related to the homologous strain proved of most value in the preparation of specific sera.

6. A set of seventeen absorbed sera was prepared from Cowan's three types of *Staph. pyogenes*, from six of Christie & Keogh's type strains and from eight additional strains freshly isolated from staphylococcal lesions. With some absorbed sera there was specific agglutination with the homologous strain and no heterologous reactions, other sera reacted with several strains and could not be made specific.

7. Seven specific serological types were recognized while a further eight types showed less specific reactions and were identifiable by a pattern of reactions. A number of strains differed slightly one from another in their agglutination reactions, and it was not possible to decide whether these were additional types or variants within types.

8. In a small series of experiments the precipitin reaction, using unabsorbed sera, seemed of little value in the differentiation of strains of *Staph. pyogenes*.

9. Of 259 coagulase-positive strains examined during 1946 and 1947, all were identified by agglutination reactions, although they varied in their specificity; 67·5% of these strains were identified by staphylococcal bacteriophage reactions.

10. A preliminary comparison of serological and bacteriophage types showed a fairly regular but not complete correlation between the results obtained by the two methods. A number of serological types were regularly lysed by the same bacteriophage filtrates; a few were consistently untypable by phage indicating that filtrates had not as yet been found for them or that the strains were phage resistant.

Others which were not clear-cut in their reactions

and which were recognized by patterns of reactions with both sera and phage filtrates did not always show a close correlation in the typing results.

11. The application of both serological and bacteriophage typing to the investigation of outbreaks of staphylococcal infection has proved to be of value in tracing sources and paths of spread of infection. In several instances the use of both methods of typing has led to conclusions which would not have been warranted if one method alone had been used.

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