ANTIGENIC PROPERTIES OF *STAPHYLOCOCCUS AUREUS*

PER OEDING

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| I. Introduction | 374 |
| I. Antigenic Structure | 374 |
| A. What Can Be Learned from Attempts at Serologic Type Differentiation | 374 |
| B. Carbohydrate A and Other Carbohydrate Substances | 375 |
| C. Verwey's Protein Antigen | 377 |
| D. Nucleoproteins, Heterophile Antigens | 378 |
| E. Hemagglutination | 380 |
| F. Agar Precipitation | 381 |
| G. Distribution of Antigens and Chemical Structures in the Cell | 382 |
| H. Animal Strains | 384 |
| I. "Normal" Antibodies | 385 |
| III. Serologic Typing | 385 |
| A. Methods and Techniques | 385 |
| 1. Cowan's Method | 386 |
| 2. Oeding's Method | 386 |
| 3. Technical Problems | 387 |
| B. Epidemiologic Value | 388 |
| 1. Reproducibility | 388 |
| 2. Field Examinations | 389 |
| 3. Correlation to Disease | 390 |
| 4. Correlation to Antibiotic Sensitivity | 390 |
| 5. Correlation to Phage Types and Groups | 391 |
| IV. Conclusion | 391 |
| V. References | 392 |

I. INTRODUCTION

The antigenic properties of *Staphylococcus aureus* have been considered difficult to explore. Methods which were used with success on related bacteria, failed with staphylococci. There has been a general feeling that the antigenic structure of this organism is so complex that a systematic classification can hardly be achieved. Consequently most work has been done on the cultural and biochemical behavior of the organism, whereas there has been little interest in its serologic and immunologic properties.

Staphylococci are extremely widespread and *S. aureus* is one of the organisms most frequently found in human infections. At present staphylococcal hospital infections are a world-wide problem of major importance. Knowledge of the antigens of *S. aureus* and their activities is not merely of interest for epidemiologic typing, but may also give possibilities for successful research in the field of immunology and infection.

Extensive reviews have recently been written on *S. aureus* in general (20) and on its pathogenicity (5). As there is today an increasing interest in the antigenic properties of *S. aureus*, a review may be of help to those working or planning to work in this field. This review has been limited to the bacterial antigens of *S. aureus* and serologic typing, whereas toxins, pathogenicity, and immunity in man are not covered.

II. ANTIGENIC STRUCTURE

A. WHAT CAN BE LEARNED FROM ATTEMPTS AT SEROLOGIC TYPE DIFFERENTIATION

A serologic distinction between pathogenic and saprophytic staphylococci was first made by Kolle and Otto (53). They found that strains from purulent lesions were all agglutinated in a serum against one type, whereas strains from other sources were not agglutinated. Several authors in the following years confirmed that pathogenic and saprophytic staphylococci could be separated by agglutination. However, it soon became apparent that some strains of pathogenic
staphylococci agglutinated only weakly or not at all in sera against pathogenic staphylococci used for group-differentiation, whereas other strains agglutinated weakly in heterologous group serum also.

Even from these early investigations important conclusions can be drawn on the antigenic structure of S. aureus:

1. S. aureus and S. albus\(^1\) strains have mainly different antigens. At least one common agglutinin seems to be present in some strains. This has later been confirmed by Oeding (66) and Pillet and Orta (88).

2. S. aureus strains have at least one common agglutinin. By agglutinin absorption it was shown (Hine (35); Julianelle (44); and later workers) that S. aureus has a very complex antigenic structure with a number of more or less shared antigens.

In most earlier studies, immunization and agglutination were performed with boiled bacteria. This was done partly because heating resulted in fewer cross-reactions, thus facilitating serologic classification. Andersen (2) and Oeding (66) tried to get a more complete picture of the antigens of S. aureus by including heat-labile antigens. Oeding (66), by immunizing with formalin-killed vaccines and making cross-absorptions and agglutinations with live bacteria, worked out an antigenic scheme for S. aureus. Ten antigens were recorded and given letters from a to k. At least one heat-stable antigen (d) is shared by all S. aureus strains.

The introduction of an antigenic scheme was considered by Oeding to be a necessary development which might stimulate further research on staphylococcal serology. Other workers have followed this line (8, 25, 57, 105, 110), although the results are usually not directly comparable because different strains and designations of antigens have been used. Grün (25), however, using Oeding's technique and strains, confirmed the results obtained by the latter. Grün (28)

\(^1\) Editor's note: Since in many cases one can not ascertain which of the present designations, Staphylococcus aureus or Staphylococcus epidermidis (Breed, R. S., Murray, E. G. D., and Smith, N. R. 1957 Bergey's manual of determinative bacteriology, 7th ed. The Williams & Wilkins Co., Baltimore), fits strains of staphylococci designated as Staphylococcus albus and Staphylococcus citreus in the literature, the older specific epithets are retained in this review.

also described a new antigen which he designated as \(l\), present chiefly in animal strains but also in human ones. Haukenes and Oeding (33) recently discovered by agglutinin absorptions and by agar precipitation that the \(e\) antigen consists of two distinct antigens and that there is also a previously unrecognized antigen present in the strains examined. The new antigens were given the letters \(m\) and \(n\).

Strains are characterized by their antigenic patterns, and groups and types by the similarity of antigenic patterns and not by antigens characteristic of each group or type. Thus in staphylococci one can hardly speak of type-specific antigens. Major and minor antigens may be more adequate terms.

When suspensions of staphylococci are heated, a significant reduction in the agglutinating ability of the antigens in factor sera is first observed at 100 C, and then a reactivation at 120 C (68). Naturally this may be of great consequence in the determination of heat-stable antigens in staphylococci. The observation was at first difficult to explain, but when the significance of blocking antigens became apparent, it seemed to be connected with this.

It was demonstrated (3, 68) that one antigen might block agglutination of other antigens presumably situated deeper in the cell. Inagglutinability due to blocking antigens was shown to be of great consequence in the determination of antigens of a strain (Oeding (72)). Although any antigen, and possibly also inert material, may block agglutination, the \(\tau\) antigen is usually the cause. As a rule the blocking effect is insignificant in cultures 3 to 5 hr old. It increases with the duration of incubation and may be complete in cultures 18 to 24 hr old. Furthermore, blocking increases when incubation takes place at temperatures lower than 37 C and decreases at higher temperatures. The discovery of blocking antigens also gave an indication of the distribution of the antigens in the cell (68).

It has been pointed out that extensive quantitative variation of the staphylococcal antigens from one strain to another makes classification difficult (36, 66). The ability of the antigens to mediate agglutination and to produce antibodies also varies from one strain to another. In one strain an antigen may be a good agglutinogen but poorly antigenic (66, 105). We have also observed that although a weak antigen may not
be recognized by agglutination, it can absorb the homologous antibody completely.

According to the sensitivity to heating and to the action of proteolytic enzymes (3, 68, 89), an impression of the nature of the antigens was obtained. The resistant antigens were supposed to have a carbohydrate structure, whereas the labile ones would be proteins or possess determinate groups of protein nature. The majority of the antigens recorded by Oeding (68) were found to be heat-stable and possibly carbohydrates, for example the d antigen shared by all Staphylococci. Pillet et al. (89) considered the agglutinogens to be chiefly of protein nature, a view which is now shared in this laboratory in the light of recent observations. Oeding’s e antigen was definitely heat-labile and was supposed to be a protein. This is also the case with the newly described antigens m and n (33). However, certain antigens (a, b, c) seem to have one heat-labile and another heat-stable component (Oeding (68)). This was also demonstrated by Grün (29) in his new l antigen. Recent investigations indicate that this peculiar observation may be explained by the presence in the factor sera of more than one antibody (33).

B. Carbohydrate A and Other Carbohydrate Substances

Julianelle and Wieghard (46, 47) demonstrated that Staphylococcus aureus and Staphylococcus albus each had a carbohydrate antigen shared by all strains in the two groups, respectively. The precipitinogen present in Staphylococcus aureus was named carbohydrate A, that present in Staphylococcus albus, carbohydrate B. There was no precipitation in sera against pneumococci, Klebsiella, or typhoid bacilli.

Staphylococci were extracted with hot 0.0625 N hydrochloric acid, and all material precipitable with 40 per cent NaOH and subsequently with 50 per cent trichloroacetic acid was removed. The Molisch test was strongly positive, whereas the biuret test was variable. The total nitrogen was about 4 per cent. The high percentage of phosphorus (about 6 per cent) suggested that the substances might be phosphoric acid derivatives. The two substances differed clearly in their optical rotation and in the sugars after hydrolysis. The reducing sugars were not identified (113).

The purified substances did not engender antibodies when injected into rabbits, but precipitated in dilutions up to 1 to 8 million in homologous immune serum. The carbohydrates had little ability to form antibodies even when whole organisms were injected. Cutaneous reactions of the immediate “weal and erythema type” were observed with high dilutions of carbohydrate A, but not with carbohydrate B in patients with staphylococcal infections. Circulating precipitins were present only in patients with chronic and severe staphylococcal infections (45, 46, 48).

The existence of the two group-specific carbohydrate precipitinogens of Julianelle and Wieghard has been confirmed by a number of authors (12, 107, 109). It was, however, observed that these substances were not present in all strains of each group. Staphylococcus albus strains lacking carbohydrate B were separated by Thompson and Khorazo (107) into one clearly defined and two less clearly defined groups, and Staphylococcus aureus strains lacking carbohydrate A were placed in another group by Cowan (12). In both instances very crude extracts were used and it is not known whether substances specific for the new groups exist.

The technique used by Julianelle and Wieghard for extraction and purification is rather drastic. This fact, and the not insignificant nitrogen content indicate that carbohydrate A was not produced in its pure form. Serologically active carbohydrates have been produced with significantly lower nitrogen values (73) or practically nitrogen free (38, 108), but it is not known whether these substances contain the same active principle.

Various methods for extraction and fractionation of carbohydrate materials from Staphylococcus aureus have been tried. The procedure described by Verwey (109) seems to be comparatively gentle and was found to give satisfactory separation of carbohydrate, protein, and nucleoprotein. The extract obtained after grinding the staphylococci is precipitated with 0.1 N hydrochloric acid, first at pH 5.2 and then at pH 3.5. These two fractions contain the bulk of the nucleoprotein. After a protein fraction has been removed by precipitation with 50 per cent trichloroacetic acid, a carbohydrate is precipitated from the supernatant with alcohol. According to Verwey this substance, like carbohydrate A, had a faintly positive biuret reaction, contained nitrogen and phosphorus but not pentoses, and was group specific. Similar substances were isolated by Inoue (40) and Kahn et al. (49). The carbohy-
drate isolated by Inoue was negative on the biuret test, contained 3.5 per cent nitrogen and 1.6 per cent phosphorus, and had a reducing power after hydrolysis of 28.5 per cent. Pentoses were present, whereas glucosamine was not demonstrated. Kahn et al. considered the carbohydrate to be the most significant staphylococcal substance for cutaneous reactions in hypersensitive individuals.

Fellowes and Routh (22) and Oeding (71), examining various extraction methods, got the most satisfactory results with the hot formamide method. The carbohydrate obtained by Fellowes and Routh in the supernatant, after precipitation with 2.5 volumes of alcohol, was obviously rather impure. It had a positive biuret reaction, contained 6.5 to 8 per cent nitrogen, and induced antibody formation when injected into rabbits. The amount of reducing sugars after hydrolysis was about 20 per cent and pentoses and glucosamine were demonstrated. Oeding (71) isolated two carbohydrates by alcohol fractionation of the formamide extract. The fraction which precipitated with 2 volumes of alcohol was apparently thrown away by Fellowes and Routh. It precipitated together with the protein when acid was added and was nondialyzable, whereas the carbohydrate precipitated by 4 volumes of alcohol was partly dialyzable. These substances did not contain free protein but there was still some nitrogen and both contained pentoses. The 4-volume substance may be identical with carbohydrate A.

Sasaki (personal communication) also obtained two carbohydrate substances from a formamide extract of one S. aureus strain. In both substances glucose, galactose, xylose, and glucosamine were identified by paper chromatography.

Hoffstadt and Clark (38) and Maggi (60) isolated different carbohydrates from smooth and rough variants of S. aureus. The rough-variant carbohydrate of Hoffstadt and Clark was apparently antigenically inert.

Jensen (42, 43) extracted from S. aureus by means of phosphate buffer or heating to 100 C a substance which was present in the majority of S. aureus but not in S. albus strains and which was toxic to guinea pig ileum. The substance was labile on autoclaving and storage. The biuret test was negative and the substance was considered to be a carbohydrate. Few chemical data are given. It seems that this product must be a mixture (see below).

In this laboratory, staphylococci are now frozen, crushed in a bacteria press, and extracted with phosphate buffer. A crude fraction containing the bulk of the carbohydrate is obtained by acid or alcohol precipitation, and the material is purified by ion exchange chromatography. The group-specific substance thus obtained is probably identical with the active material in carbohydrate A. It is active in very high dilution in the ring test, where a sharp disc is formed, and does not induce antibodies in rabbits or sensitize erythrocytes to agglutination. A characteristic band is formed by agar precipitation. The biuret reaction is negative, the Molisch test very weakly positive. The substance has a reducing activity after hydrolysis of 30 per cent. Glucosamine and probably related structures constitute an important part of the substance, whereas other sugars have not been demonstrated by two-dimensional chromatography (Haukenes, unpublished data).

The investigations referred to above show that there are more antigens of carbohydrate nature than the group A substance present in strains of S. aureus. Comparison between the carbohydrates isolated by different authors is extremely difficult. Precipitation will differ according to the methods used for extraction and purification, and with the concentration and purity of the substance. Bindings may be released with one method whereas new bindings may be formed with another method. Furthermore, serologic investigations have shown that the numbers and quantities of carbohydrate antigens other than the d antigen vary from strain to strain. It is likely that the substances described have been mixtures of serologically different carbohydrates, possibly containing other material also. The true nature of carbohydrate A is still unknown.

C. Verwey's Protein Antigen

Verwey (109) demonstrated a small amount of a protein fraction in S. aureus which was group specific and serologically active in high dilution. After nucleoproteins had been removed by precipitation with 0.1 N hydrochloric acid at pH 5.2 and pH 3.5, this protein was brought down by addition of 50 per cent trichloroacetic acid to a final concentration of 11 to 14 per cent.
TABLE 1

Some properties of carbohydrates and proteins derived from Staphylococcus aureus

<table>
<thead>
<tr>
<th>Reference</th>
<th>Molisch Test</th>
<th>Biuret Test</th>
<th>N</th>
<th>P</th>
<th>Reducing Sugar</th>
<th>Glucosamine</th>
<th>Gel-Diffusion</th>
<th>Nature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Julianelle and Wieghard (46, 47)</td>
<td>Strong</td>
<td>Variable</td>
<td>4.1</td>
<td>6.3</td>
<td>26</td>
<td></td>
<td></td>
<td>Carbohydrate (A), group specific Carbohydrate</td>
</tr>
<tr>
<td>Hoffstadt and Clark (38)</td>
<td>Strong</td>
<td>Negative</td>
<td>0.1-0.3</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td>Carbohydrate, group specific Carbohydrate</td>
</tr>
<tr>
<td>Inoue (40)</td>
<td>Strong</td>
<td>Weak</td>
<td>3.5</td>
<td>1.6</td>
<td>28.5</td>
<td>0</td>
<td></td>
<td>Carbohydrate, group specific Carbohydrate</td>
</tr>
<tr>
<td>Verwey (109)</td>
<td>Positive</td>
<td>Weak</td>
<td>6.5-8.2</td>
<td>1.0-1.2</td>
<td>18.3-21.6</td>
<td>6.7-9.9</td>
<td>Characteristic band</td>
<td>Carbohydrate, group specific</td>
</tr>
<tr>
<td>Fellowes and Routh (22)</td>
<td>Weak</td>
<td>Negative</td>
<td>2.5</td>
<td>6</td>
<td>30</td>
<td>19</td>
<td></td>
<td>Carbohydrate, group specific</td>
</tr>
<tr>
<td>Haukenes (unpublished data)</td>
<td>Weak</td>
<td>Strong</td>
<td>14</td>
<td>&lt;0.1</td>
<td></td>
<td></td>
<td></td>
<td>Protein, group specific Protein</td>
</tr>
<tr>
<td>Verwey (109)</td>
<td>Positive</td>
<td>Positive</td>
<td>11</td>
<td>1.5</td>
<td>8.4</td>
<td>Positive</td>
<td></td>
<td>Protein, group specific Protein</td>
</tr>
<tr>
<td>Inoue (40)</td>
<td>Strong</td>
<td>Positive</td>
<td>12.1</td>
<td>1.3</td>
<td></td>
<td></td>
<td></td>
<td>Protein, group specific Protein</td>
</tr>
<tr>
<td>Dworetzky et al. (18)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The substance was strongly positive to the biuret test and weakly positive to the Molisch test. It contained about 14 per cent nitrogen and less than 0.1 per cent phosphorus, and was destroyed by trypsin. Pentoses were not present.

Similar substances have been isolated by Inoue (40) and Kahn et al. (49), although they gave slightly lower values for protein and higher values for phosphorus. The material described by Inoue contained glucosamine. Its serologic specificity was not described. The protein material of Kahn et al. was reported to be serologically highly specific and to give skin reactions both in normal and hypersensitive individuals. Sasaki (personal communication) examined several protein fractions from yellow and white staphylococci by paper chromatography, and found them to be almost homogeneous.

Very little chemical work has therefore been done on the protein antigens of S. aureus. The protein of Verwey seems to be a clear-cut entity but little is known of its chemical structure or immunologic significance. Although it is said to be group specific, we do not know to what extent it is present in strains of S. aureus. By serologic investigations several antigens of protein nature have been found in S. aureus. These proteins are, however, not group antigens but more or less “specific.” Verwey’s protein may be a mixture from a serologic point of view, or other protein antigens may have been lost, probably into the bulk of nucleoproteins. Furthermore, the possibility of isolating protein antigens will depend upon the strains used.

D. Nucleoproteins, Heterophile Antigens

Serologically active nucleoprotein materials have been isolated from staphylococci by a number of authors. The nucleoproteins were extracted with 0.01 N NaOH and precipitated with dilute acetic acid by Lancefield (55), Boor and Miller (6), and Julianelle and Wieghard (48). Verwey (109) obtained the bulk of the nucleoproteins by precipitation with 0.1 N hydrochloric acid at pH 5.2 and then at pH 3.5. This procedure was adopted by Kahn et al. (49) and Dworetzky et al. (18). The latter authors demonstrated 12.5 per cent nitrogen and 3.7 per cent phosphorus in the nucleoprotein fraction of one strain of S. aureus, and they as well as Boor and Miller found indications of a carbohydrate radical.

Lancefield (55) reported cross-precipitation between nucleoproteins from staphylococci,
TABLE 2
Cross-reactivity of "nucleoproteins" derived from Staphylococcus aureus

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococci</td>
<td>100%</td>
</tr>
<tr>
<td>Pneumococci</td>
<td>90%</td>
</tr>
<tr>
<td>Meningococci</td>
<td>80%</td>
</tr>
</tbody>
</table>

Staphylococci have been found to absorb antibodies to blood groups A and B from human sera (39). This reaction was thought to be due to polysaccharides. The sharing of a common antigen in these species is very unlikely, since they do not cross-agglutinate (20).

Cross-reactivity between staphylococci and Listeria monocytogenes has been demonstrated by Seeliger and Sulzbacher (100). Of the staphylococcal strains examined, 30 to 50 per cent gave agglutination and complement fixation in live and in autoclaved conditions with antisera against live L. monocytogenes types 1, 2, and 3. Soluble carbohydrate materials gave only a unilateral cross-reaction between L. monocytogenes type 3 serum and staphylococcal extracts. The latter result was confirmed using an agar precipitation technique. Three separate bands were found to be common to the above mentioned system. Parts of the antigenic complexes other than the extractable carbohydrate material must therefore be responsible for the extended cross-reactions observed with whole bacteria. The nature of the cross-reacting substance is unknown (100).

In the course of purification of staphylococcal extracts by column chromatography, Haucken (unpublished data) isolated one contaminating strain of Alcaligenes and one of Bacillus subtilis, both of which gave bands with staphylococcal rabbit antiserum by agar precipitation.

Mitchell and Moyle (62, 63) discovered that lipid-free extracts of staphylococci contained phosphorus, amounting to more than 30 per cent of that which could be accounted for by the nucleic acid content. The excess phosphate was separated from the other phosphorus compounds after fractional extraction of staphylococci in dilute acid or alkali. Excess phosphate was demonstrated in a number of gram-positive organisms, suggesting that a glycerophosphate compound similar to that of S. aureus may be of general occurrence in gram-positive bacteria and yeasts.

Recently McCarty (58) recognized in streptococci a cross-precipitating substance which could be extracted at pH 2 at 100 C, at pH 9, by trypsin treatment, or with hot formaldehyde. The sodium salt of the purified antigen was essentially nitrogen free and on the border line of dialyzability through cellophane. Paper chromatography of acid hydrolyzates showed no carbohydrate or...
TABLE 3
Cross-reactivity observed between Staphylococcus aureus and:

1. Human blood groups A and B. (Illehnann-Christ and Nagel (30)).
2. Listeria monocytogenes. (Seeliger and Sulzbacher (100)).
3. Alcaligenes, Bacillus subtilis. (Haukenes, unpublished data.)
4. Staphylococcus albus, streptococci, pneumococci, B. subtilis. Hemagglutination, non-species-specific substance. (Rants et al. (92, 93)).
5. Glycerophosphate extracted from streptococci. Reacts also with S. albus and Bacillus species. (McCarty (58)).

Amino acids. A single organic phosphate was demonstrated, its mobility being identical with that of glycerophosphate. Synthetic polyglycerophosphates inhibited the precipitin reaction. The substance was estimated to constitute about 1 per cent of the dry weight of the streptococcal cells.

This antigen was found in many different groups of streptococci, including nonhemolytic species, in S. aureus and S. albus, and in aerobic sporulating bacilli including B. subtilis. It was not present in pneumococci, clostridia, corynebacteria, yeasts, Micrococcus lysodeikticus, or Staphylococcus citreus.

There is not complete accordace between the distribution of the excess phosphate of Mitchell and Moyle and the phosphate antigen of McCarty. The explanation is probably that the antigenic substance constitutes only a part of the excess phosphate. The phosphate antigen does not precipitate with acid and can therefore hardly be present in the nucleoproteins of Lanefield and others.

Three observations have been described which are difficult to explain but may be due to heterophile antigens. Dudgeon and Bamforth (15) found precipitins in no less than 87 per cent of the sera from patients with serum sickness against a 1-month-old broth culture filtrate of S. aureus. No precipitation was seen with extracts of E. coli or Salmonella typhosa. There may be a connection with the serologic similarity demonstrated between staphylococci and L. monocytogenes (100), glandular fever (23), and blood groups (39).

Penner and Voldrich (81) found that S. aureus agglutinated in high titers in all tuberculous pleural effusions examined, and less frequently in nontuberculous effusions. There was no agglutination of S. albus or S. citreus, or of other bacteria. The agglutinating substance was not destroyed by heat, whereas the factor present in the effusions was inactivated at 60 C. Shrigley (101) observed that approximately 46 per cent of S. aureus strains and possibly strains of S. albus, but not of S. citreus or other organisms tested, were agglutinated in normal or influenza virus-infected chorioallantoic fluid. The fluids were rendered inactive after heating to 56 C. The active substance was nondialyzable and considered to be a protein.

There is a possibility that these two observations can be explained by the same mechanism. The tuberculous effusions and the chorioallantoic fluid are similar in so far as both agglutinate S. aureus exclusively and both are highly susceptible to heat. Although the agglutination may be hard to explain in terms of antigen-antibody reactions, some kind of heterophile antigen may be involved. Heterophile antigens in S. aureus, demonstrated by indirect hemagglutination, will be treated in the following section, after which a concluding remark will follow.

E. Hemagglutination

Keogh et al. (50) found that staphylococci among other bacteria were able to sensitize erythrocytes to agglutination in the presence of antiserum. Later authors confirmed that culture filtrates (64, 93) and extracts of S. aureus have erythrocyte sensitizing properties. Active material was present in the insoluble fraction after extraction with 90 per cent phenol (50, 73, 94), and in the soluble fraction after extraction with 88 per cent phenol (93). Furthermore active material could be extracted with glycine (34), hot formamide (73, 95), hot hydrochloric acid (93), by simple heating to 100 C, or by means of proteolytic enzymes (78).

Little has been done to isolate the active principle. Most investigations on the properties of the sensitizing substance have been made with simple culture filtrates or very crude extracts. It is generally assumed that the sensitizing substance of staphylococci, as of other bacteria, is a polysaccharide, although Sachse (95) considers it to be a nucleoprotein. The sensitizing property has been shown to resist autoclaving.
(59, 73) and formamide extraction at 150 C (73, 95). On the other hand, the sensitizing substance with heterophile properties described by Rantz et al. (92) was shown to be stable at 100 C at acid pH, but labile at neutrality and alkaline pH. This was confirmed by Neter and Gorzynski (64). At room temperature the sensitizing property was destroyed both by acid and alkali (73). The sensitizing substance is not destroyed by trypsin (59, 73, 92) and is nondialyzable (78, 92).

Oeding (73), working with more purified materials, found them almost nitrogen-free. The sensitizing property was gradually lost on storage in an ice chest (73, 94). According to Oeding (73) this indicates a labile grouping in the active polysaccharide, necessary for its sensitizing activity but without significance to precipitation, which was unimpaired.

Antibodies to the sensitizing antigen were easily produced in rabbits when crude material was injected. Purified material, which is not antigenic, becomes so when attached to erythrocytes (73).

The reports of various authors are quite conflicting regarding the properties of the sensitizing substance. This is not surprising as different staphylococcal strains and even different bacterial species have been compared. Furthermore, the sensitizing substance may have different properties in crude preparations than in purified extracts. The investigations hitherto reported can hardly tell us whether more than one sensitizing antigen is present in S. aureus and it can be discussed whether it is justified to describe the properties, as has been done here, as if we were dealing with only one substance.

However, the sensitizing agents described by the majority of authors show obvious similarities in that they are apparently common to staphylococci and certain other bacterial species. A sensitizing substance common to S. aureus and S. albus strains has been demonstrated (59, 64, 94), which is apparently present also in streptococci, pneumococci, and B. subtilis, but not in C. diptheriae, S. citreus, M. lysodeikticus, or gram-negative bacteria (34, 64, 78, 93, 95), with the exception of one Pseudomonas strain (93). Antibodies to the sensitizing substance were demonstrated in the sera of three patients with glandular fever (23), which is interesting in relation to the antigenic similarity demonstrated between staphylococci and L. monocytogenes ((100) see above). Rantz et al. (92, 93) have payed particular attention to the heterophile sensitizing antigen in their extensive study, and named it NSS, i.e., nonspecies-specific substance.

Neter and Gorzynski (64) obtained higher titers by indirect staphylococcal hemagglutination when the erythrocytes were treated with enzymes. The difference in titer between enzyme-treated and nontreated erythrocytes was thought to be due to incomplete antibodies. Sensitizing material was present in varying quantities in different strains of staphylococci.

In this connection it should be mentioned that Kourilsky et al. (54), by means of the immune-adherence test, demonstrated an antigen-antibody system which was different from the agglutininogen-aggutinin system. The specific receptor was present only in human erythrocytes and the active substance was considered to be a lipopolysaccharide or a polysaccharide.

When the properties of the different substances which have been described as giving cross-reactions, either by precipitation or by hemagglutination, are compared, it may seem improbable that only one heterophile antigen should be present in S. aureus. It is striking, however, that the species reactivity of the "nucleoprotein" of Lancefield and others is very similar to that of the erythrocyte-coating substance described above. Certainly related species such as staphylococci, streptococci, and pneumococci may have more than one antigen in common, and it cannot be denied that the "nucleoprotein" and the sensitizing substance show significant differences. But it should be remembered that the conditions for characterization of the substance have been rather unsatisfactory.

This is a very interesting and important field for further study. It will be necessary to make comparative studies of heterophile substances by different antigen-antibody reactions, and special attention should be given to the comparison of the heterophile "nucleoprotein," the heterophile phosphate of McCarty, and the heterophile sensitizing substance.

F. Agar Precipitation

The double diffusion technique in agar provides a very refined way of separating different antigen-antibody systems. Investigations have shown that in culture filtrates or extracts of S. aureus, a large number of bands, each probably
representing a distinct antigen-antibody system, can be demonstrated. Some of the bands are due to toxin-antitoxin systems, others apparently to bacterial antigen-antibody systems (4, 42, 74, 80, 105). The number of bands depends upon whether bacterial filtrates or extracts are used (4, 11). A correlation has further been demonstrated between the number of lines and the virulence of the staphyloccocal strains.

Certainly the mere demonstration of a number of bands in a staphyloccocal system has in itself a very limited value unless the bands can be identified. Stern and Elek (105) demonstrated a single band in acid extracts of _S. aureus_ cell walls which was apparently due to a group antigen, whereas the endoplasm showed a number of bands which seemed to represent both group- and type-specific antigens. Jensen (42, 43) extracted from _S. aureus_, by means of phosphate buffer or heating to 100°C, a substance which formed a very strong band in agar. Precipitating antibodies to this substance were found in very high titers in all normal human sera examined. Personal investigations ((74) and _unpublished data_ ) have shown that a number of bands, some of which are closely situated, are common to strains of _S. aureus_. The identity of these group bands is unknown. The description and pictures, presented by Jensen, give the impression that the group band, which is very wide, must consist of at least two or three separate systems.

The antigens forming the group bands of _S. aureus_ do not seem to be present in _S. albus_, although we have occasionally observed weak bands common to the two groups. The observation by Haukenes (_unpublished data_ ) of a band common to _S. aureus_, _B. subtilis_, and an _Alcaligenes_ sp., also indicates that the heterophile antigens present in _S. aureus_ can be studied by means of the agar precipitation technique.

It was shown by this technique that certain antigens are removed by washing the staphylococci (Haukenes, _unpublished data_ ) and that unwashed bacteria, disrupted in a frozen condition in a bacterial press, form the best material if one is to obtain a complete antigenic picture.

By comparing the bands with agglutination systems using whole sera or the described factor sera, we have been able to identify some of the agglutinogens as distinct bands. Furthermore, staphyloccocal extracts have been compared by the ring test and by agar precipitation, and the latter method has proved very useful in following the purification of extracts.

However, for various reasons, it seems impossible to demonstrate all agglutinogen-agglutinin systems by agar precipitation, or to identify all precipitation bands by agglutination. Thus the number of staphyloccocal antigens is still greater than shown by either of the two methods. The separation of the e antigen into two distinct antigens (see above) illustrates this fact. It was by means of agar precipitation that the existence of two antigens was first suspected. One of the antigens (n) formed a characteristic, sharp line in agar, whereas the other antigen (e) formed no line.

We feel that agar precipitation is a very convenient and valuable method in staphyloccocal research. However, the method undoubtedly has its limitations. One difficulty is the interpretation of the different bands, because it seems that the specificity of the method is so high that antigen-antibody systems with only minimal differences form their own bands. Small variations in an antigen or antibody, which are probably insignificant when the conventional antigen-antibody reactions are used, may easily take place in response to chemical treatment or even storage. The frequency with which "twin bands" can be produced is in our experience an indication of the "over-sensitivity" of the method.

It is very important that the conditions for reaction of a bacterial extract and an antiserum be thoroughly varied; for example, the concentrations of the two components and the arrangement of the adjacent systems. Absorption tests should also be included. It is our experience that unless these conditions are fulfilled, an antigen-antibody system may easily be overlooked.

**G. Distribution of Antigens and Chemical Structures in the Cell**

The demonstration of a number of different agglutinogens in intact cells of _S. aureus_ suggests that these antigens are situated on the cell surface or penetrate to it from deeper layers. Thus the outer surface of _S. aureus_ must be very complex in its antigenic equipment. Blocking antigens may develop which render the cells more or less inagglutinable by specific antisera (3, 68). The development of blocking antigens and inagglutinability is of regular occurrence,
depending upon the antigenic pattern of a strain (Oeding (72)). According to the general view, the blocking antigen probably forms a surface layer which covers the previously exposed antigens. Repeated washings are of little consequence (72). The surface layer of blocking antigen seems therefore to be firmly bound to the cell and may be formed by the selective development of one particular antigen in comparison to the other antigens present on the surface. The blocking antigen does not seem to be destroyed by autoclaving, which nevertheless makes the strains more agglutinable. The formation of the outer blocking layer is dependent on incubation time, temperature, and probably other factors.

Inagglutinability of staphylococci seems to be more complex and in many ways different from the O inagglutinability caused by the Vi antigen. In staphylococci it is possible that every antigen can act as a blocking substance, provided it is present on the surface in sufficient quantity. Usually, however, the i antigen acts as the blocking substance. Brodie (7) observed that the removal of the antibodies to the heat-stable antigens somewhat enhanced agglutination of the heat-labile antigens. Oeding (72) discussed the possibility that a substance inhibiting agglutination is transmitted from the bacteria to the serum during absorption.

Oeding (68) suggested the way the antigens he described might be arranged, taking agglutination of live and autoclaved bacteria and blocking into consideration. There seems to be no general law as to the arrangement of the different antigens in the cells, although there is undoubtedly a similarity between strains of the same serologic group. One antigen present on the surface and acting as a blocking substance in one strain, may be present deeper in the cell and be blocked by another antigen in a second strain. In some strains the cells seem to be built up of the same antigen, both in the deeper layers and on the surface, as all antigens are active under different experimental conditions. This seemed to be the case in the strains examined by Stern and Elek (105), who apparently found no antigenic difference on agglutination between the whole cell and the cell wall or between the inner and outer surface of the latter. Other strains seem to have layers of different antigens; for example, a surface layer of a blocking antigen, then a layer of two or three other antigens, and occasionally an inner layer of an antigen which can be traced only in certain circumstances which are difficult to predict.

Cohen et al. (11) demonstrated more antibody components in sera against cellular protoplasm than in sera against whole cells. This is difficult to explain unless the ultrasonic treatment used to prepare protoplasm resulted in degradation and formation of new antigenic structures.

Apart from the very rare mucoid variants, staphylococci are not supposed to have true capsules. Lyons (56) claimed that very young cells of S. aureus have capsules containing a type-specific antigen, but this could not be confirmed (52, 103). More recently Price and Kneeland (90) demonstrated capsular swelling in young cells of a strongly mucoid strain of S. aureus 1 to 3 hr after the addition of homologous antiserum. Capsular swelling was also observed in the majority of nonmucoid strains of S. aureus, but not in S. albus strains, when antimucoid serum was added (91). After prolonged immunization even nonmucoid strains produced antibodies which gave swelling with the homologous bacteria. The capsular swelling antibody was apparently common to mucoid and nonmucoid strains.

Although we have not examined the antibody response to very young cultures of S. aureus, the results of Price and Kneeland are difficult to explain in light of our observations that in young bacteria a number of antigens are exposed on the surface. A surface layer of blocking antigen has not yet developed. We have, however, recently observed a substance in 18-hr cultures, by ring test and agar precipitation, which seems to be a heterophile polysaccharide antigen. It is readily removed by washing and seems to constitute a loose surface layer (Haukenes, unpublished data). Although the capsule antigen of Price and Kneeland is described as group specific, there may be a connection between the two observations. Capsular swelling in nonmucoid strains was distinct only after 18 hr contact with antiserum. This might also indicate that the capsular swelling antigen is not exposed on the surface of the live cell, but is brought there by autolysis.

During recent years some interesting investigations have been made on the chemical structure of the cell wall of S. aureus. According to Stacey (104), stripping with sodium cholate indicates
that the surface material of gram-positive bacteria consists mainly of polysaccharides in partial combination with nucleic acids, mainly of the ribonucleic acid type, and relatively small amounts of protein or fats. It has been established that the cell walls of many gram-positive bacteria possess distinctive properties, that a number of the uncommon constituents of the cells are localized there, that cell wall peptides are composed of a small variety of amino acids, and that amino sugars form important constituents of the cell wall (Salton (97)). The cell walls of gram-positive bacteria are less complex in their amino acid and amino sugar composition than those of gram-negative bacteria.

In *S. aureus*, small amounts of fats are present in the cell wall. Dyar (19) estimated the amount of lipid to be about 10 per cent, present as phospholipid firmly bound to the cell surface. From electrophoretic studies of *S. aureus* before and after treatment with ribonuclease, it appears that nucleic acids are not located on the external cell surface (32).

Park and Strominger (79), having demonstrated that three uridine nucleotides accumulated in a strain of *S. aureus* that was inhibited by penicillin, found a structure analogous to part of the nucleotide in the cell wall. This compound was supposed to be a precursor of the cell wall. A hexosamine identical to that described by Strange and Powell (106) was found in the cell wall and in the nucleotide. This has been shown to be a characteristic component of cell walls of gram-positive bacteria by Cummins and Harris (14). These authors also demonstrated the presence of glucosamine in the cell wall of *S. aureus*, whereas hexoses or pentoses were not detected.

The presence of a carbohydrate on the cell surface of *S. aureus* was demonstrated by Dyar (19) and Webb (112). Serologic studies show that antigenically active carbohydrates are present on the surface of *S. aureus* (68).

Webb (112) and Salton (96) conclude from the results of enzymatic lysis of *S. aureus* that protein material is not normally exposed on the cell surface. Unheated bacteria are not lysed by trypsin whereas heated bacteria are lysed, especially when first treated with lysozyme. This conclusion is, however, based on the examination of only two staphylococcal strains and may possibly be valid for some strains and not for others. Agglutination of live staphylococci has shown antigens of protein nature to be present on the cell surface, a typical example being the *e* antigen of Oeding (68). There is, therefore, hardly any doubt that protein antigens are frequently or regularly exposed at the cell surface. Mitchell and Moyle (63) are of the opinion that there is a preponderance of protein in the envelope of *S. aureus*.

A number of amino acids have been demonstrated in whole cells of *S. aureus*. Alanine, glutamic acid, and lysine are present in high concentrations in the cell walls of staphylococci and other gram-positive bacteria (14, 79). In addition, glycine seems to be regularly present in the cell wall of *S. aureus*, and possibly certain other amino acids in lower concentrations (14, 41, 63). Serine has been found, apparently in higher concentration in *S. albus* than in *S. aureus* strains (14). Park and Strominger (79) found that 45 per cent of the alanine and 92 per cent of the glutamic acid present in the cell wall of *S. aureus* was in the D form, and similar findings are reported by Salton (97).

Mitchell and Moyle (62) found that in the cell wall of *S. aureus* there is an excess of organic phosphate, the bulk of which is present in an easily hydrolyzed glycerophosphate compound. Three-quarters of the weight of the cell wall fraction was estimated to be a glycerophosphate-protein complex (63). The presence of "excess phosphate" in all the gram-positive organisms examined, but not in the gram-negative ones, suggests that glycerophosphate compounds are common structures of the cell walls of all gram-positive organisms.

McCarty (58) recently demonstrated a polymer of glycerophosphate with serologic activity in various gram-positive bacteria. The characteristics of the purified antigen have been described previously. The antigenic substance was, however, not present in the cell wall fraction, and it appears that it accounts for only a part of the "excess phosphate" demonstrated by Mitchell and Moyle.

### H. Animal Strains

The majority of the studies on the antigenic structure of *S. aureus* have been performed on strains of human origin and little is known of the animal strains. However, it seems to be established that animal coagulase-positive staphylococci have their own antigenic patterns and few antigens in common with human strains.
(28, 61, 83). In absorbed or unabsorbed sera produced against human staphylococci, only a small number of animal strains are agglutinated, and if so usually weakly. Cowan (12), using precipitinogens prepared according to Julianelle and Wieghard (47), found animal strains to belong mainly to group A. This should indicate that some of the animal staphylococci have polysaccharide A in common with human pathogenic staphylococci. However, as pointed out above, the precipitinogens used were probably rather impure, and the cross-reactions may well be due to other common antigens.

Grün (28) found the antigens described by Oeding (66) infrequently represented in animal strains. He demonstrated a new antigen (I) present not only in animal staphylococci but also in human strains. This antigen was heat-stable and might be blocked in 24-hr cultures.

Pattison and Matthews (80) attempted to differentiate animal coagulase-positive staphylococci by different antigen-antibody reactions. The presence of multiple common lines on agar precipitation and the difficulty of absorption indicate extensive sharing of antigens in strains of widely different origin, as has been found also in human staphylococci. The slight antigenic differences observed may be related to host species.

I. "Normal" Antibodies

It has been repeatedly observed that nonimmunized rabbits may have circulating antibodies that react with pathogenic staphylococci. The titer of the normal antibodies and its consequence for immunization and agglutination have been estimated very differently. Most authors have found only low titers of "normal" antibodies and used a serum dilution of 1:5 or 1:10 after immunization. Mercier et al. (61) are of the opinion that "normal" antibodies may confuse agglutination and prefer a serum dilution of 1:50.

In this laboratory, "normal" antibodies, reacting with our type strains of S. aureus, are found regularly in rabbit sera. The titers are usually low (1:5 to 1:20) but titers up to 1:50 are not infrequent. In the routine agglutination test, absorbed rabbit antisera are used in 1:10 dilution and no disturbing effect due to "normal" antibodies has ever been observed. Probably these "normal" antibodies are directed mainly against the group antigens and therefore eliminated from the immune sera during absorption. Pillet and Orta (86) found "normal" antibodies in their rabbits mainly against types 3, 4, and 8, and to a lesser degree against type 2, and considered them to be directed against a common group antigen present also in animal staphylococci.

"Normal" antibodies against S. aureus present in rabbit sera are considered to be due to previous infections with staphylococci. We do not know whether the rabbit staphylococci are of animal or human origin. This is significant in view of the difference in antigenic structure of the two groups (see above). Most probably the extent of staphylococcal infections and the production of antibodies will differ from one laboratory to another, and that may also be the case with the kind of antibodies produced.

Investigations on the presence of staphylococcal antibodies in human sera have mainly been concerned with antitoxic antibodies and very little with antibacterial antibodies. Rountree and Barbour (94) demonstrated antibodies in human sera to the erythrocyte-coating substance present in pathogenic staphylococci. The antibody was not transmitted from mother to child. Jensen (42, 43) found antibodies in very high titers against one particular staphylococcal substance in all human sera examined, and in human milk. Human γ-globulin has been found to give multiple bands by agar precipitation against extracts of all staphylococcal strains examined (4), which indicates the presence of small amounts of different group antibodies in human sera. Neter and Gorzynski (64) demonstrated "incomplete" antibodies in high titer and "complete" antibodies to staphylococci in low titer in human γ-globulin by hemagglutination.

Rantz et al. (93) found hemagglutination antibodies against the heterophile substance NSS in human sera, and Dudgeon and Bamforth (15) observed that staphylococci were agglutinated by sera from patients with serum sickness. Thus "normal" antibodies reacting with staphylococci are not necessarily specific or due to previous staphylococcal infections.

III. SEROLOGIC TYPING

A. Methods and Techniques

Pathogenic and saprophytic staphylococci were differentiated serologically by Kolle and Otto (53) and later workers by agglutination in
sera against representative strains of the two
groups. The method was improved by the intro-
duction of polyvalent *S. aureus* sera (9, 67). A
differentiation can also be achieved using the
precipitation technique of Julianelle and Wieg-
hard (12, 47, 107). The coagulase test has,
however, made serologic methods for the charac-
terization of pathogenic strains superfluous.

Until 1939 only scattered attempts had been
made to classify *S. aureus* by serologic methods.
It was realized that although precipitation could
separate saprophytic from pathogenic staphy-
lococci, a type division of the latter was not
possible with this technique (12). The comple-
ment fixation test was also shown to be unsuit-
able, although Seedorf (99), using absorbed sera,
was able to distinguish 13 types among strains of
*S. aureus*.

In nearly all experiments in which a type dif-
ferentiation of *S. aureus* was obtained, agglutinin
absorption was used. When agglutination was
done in tubes, from 3 to 7 types or groups were
reported; one or two of them contained nearly
all typable strains while a great number of strains
remained untypable (35, 44, 114).

1. Cowan's method. Cowan (12, 13) introduced
the slide agglutination technique, which gives
more distinct reactions than tube agglutination
(36, 65). Boiled bacteria were used for the pro-
duction of antisera, for absorption, and for agglutination. By means of absorbed and un-
absorbed sera, Cowan classified strains of *S.
aureus* into three types and an "atypical" group.
Although similar results had been obtained by
earlier authors (see above), Cowan's method was
the first which proved to be serviceable for the
classification of *S. aureus*.

By means of this technique, the number of
types was later extended. In addition to Cowan's
3 main types, Gillespie *et al.* (24) registered 6
subtypes and Christie and Keogh (10), 6 types.
Hobbs (36) added 4 more types to the 9 of
Cowan, and Christie and Keogh, the total num-
ber of types thus amounting to 13. However,
Mercier *et al.* (61) and Andersen and Heilesen
(3) found no evidence of more than 3 types
among the heat-killed international type strains,
and considered further division to depend ex-
clusively on quantitative differences.

Because of considerable antigenic overlapping,
the technique and the determination of types
were in many respects difficult. In particular
absorption presented a serious problem. First,
the absorbed sera tended to have low titers,
and second, in spite of this, cross-reactions were
usually found, *i.e.*, pure type sera had not been
obtained. Indeed, Fillet and Orta (87) claimed
to have obtained pure sera for all of Cowan's,
and Christie and Keogh's 9 types, but the pro-
cEDURE was difficult and there was no proof that
the sera were monovalent.

Hobbs (36) considered the strong reactions
to be due to major, and the weak reactions to
minor, agglutinogens. According to her, there
may either be a large number of types capable of
recognition by specifically absorbed sera, or
only a small number of specific types around
which many strains showing minor variations
from the main type are grouped.

Strains were classed by the pattern of their
reactions (10) into types and subtypes, *e.g.*,
1a (24), or types 1 to 13 (36).

Cowan's method has been used by a number of
authors and, although it has not been widely
adopted in routine epidemiologic investigations,
some reports have given encouraging results
(1, 21, 24, 37).

In an epidemic of pemphigus neonatorum,
Andersen (2) was unable to obtain a satisfying
classification of the strains by means of Cowan's
method. However, when rabbits were immu-
nized with trypsin-digested vaccines of selected
strains, and trypsin-digested bacteria were used
for absorption and agglutination, three antigens
(*a, b*, and *c*) were demonstrated. A specific
pemphigus serum was produced by means of
which the epidemic could be followed.

2. Oeding's method. In an epidemic of mastitis,
Oeding (65), by means of agglutinin absorption,
obtained a serum containing the antibody spe-
cific for the epidemic strain. Formalin-killed
bacteria were used for the production of rabbit
immune sera and for absorption, and agglutina-
tion was performed on slides with live 18-hr-old
bacteria.

Selected strains were now studied by means
of cross-absorption, on the basis of which an
antigenic scheme was established (66). Ten
antigens were determined and given letters from
*a* to *k*. One of them (*d*) was common to all yellow
strains, and 2 (*f, g*) gave very weak antibodies.
The resulting 7 antigenic factors were considered
to be distinct and the corresponding, apparently
monovalent, factor sera were used for the sero-
logic typing of pathogenic staphylococci. Strains were designated by the factor sera in which they agglutinated, the antigenic patterns being, for example, abc, or that of the mastitis strain, ae.

When it was discovered that antigens were regularly blocked in 18-hr-old live bacteria, the strains were also examined after autoclaving and designated by their antigenic patterns both in the live and autoclaved condition (69).

Agglutination of live bacteria of the 13 international type strains in “group” sera (a, b, c, e) showed that Cowan’s types I, II, and III had the antigenic patterns abc, ab, and abc, respectively. These patterns were found to be widely distributed and, together with pattern abce, were considered to represent serologic groups. The remaining international type strains showed only minor differences, no more than shown by a number of random strains. When the additional type sera (h, i, k) and examination of autoclaved bacteria were included, a better differentiation of strains was obtained. It was concluded that a limited number of serologic groups exist, which can be further differentiated into a large number of patterns or types. Further investigations showed that blocking and O inagglutinability increase with the duration of incubation and at temperatures below 37 C (Oeding (72)). In 5-hr-old agar cultures the maximal number of antigens and the strongest agglutinations were obtained. The typing of S. aureus is therefore now done with live 5-hr-old bacteria incubated at 37 C. The presence of an i antigen is checked in 18-hr-old cultures, as this antigen may develop slowly. Typing of autoclaved bacteria is not performed except in spontaneously agglutinating strains, which are usually typable after autoclaving.

Recent investigations in this laboratory have, however, shown that in certain instances an antigen may be discovered only after autoclaving.

Grün (29) and Grün and Kühn (31) considered the use of 5-hr-old cultures for agglutination a great advantage. This modification increased the number of antigens recorded and reduced the number of nontypable strains.

Oeding’s serologic classification has proved to be of definite value in epidemiologic investigations (75, 77, 102). Brodie et al. (7, 8) used the type strains of Hobb’s (36) for the production of factor sera, but Oeding’s nomenclature. Immunosization was performed with bacteria grown on 8 per cent agar. The modifications introduced by Brodie et al. may explain certain discrepancies with regard to Oeding’s results. Nevertheless, very satisfying results were found in epidemiologic investigations. Löfkvist (57) and Vischer (110) both examined strains from epidemics of mastitis serologically. Though Oeding’s principle was followed, they used their own strains for the production of type sera. Löfkvist found the serologic method of value, whereas Vischer’s results were less promising.

3. Technical problems. It is a general experience that adequate techniques are essential for successful serologic typing of staphylococci. A concluding review of the main technical problems will therefore be necessary.

Strains: It is important that well characterized type strains be used for serologic investigations of staphylococci, otherwise results will be difficult to compare. The strains should be kept under controlled conditions. In this laboratory monthly subcultures are made on agar stabs. If any doubt should arise as to the identity or characteristics of a strain, it is replaced with a lyophilized culture.

There seems to be a common view that staphylococci are antigenically very mutable. This is not our experience. Although one or two of our type strains have shown certain antigenic changes in the course of several years in the laboratory, all other strains have kept their characteristics remarkably well. Pillet et al. (61, 87) found Cowan’s 3 type strains to be stable, and apart from two strains which lost their agglutinability but recovered it after passage in rabbits, their own strains showed no change in antigenic characteristics. Grün (25) and Grün and Kühn (31) found that strains did not change their antigenic patterns over a longer period. Variants which differed from the mother strains in pigment, hemolytic activity, or sensitivity to antibiotics (105) retained their serologic types. Provided the type strains are adequately controlled, these observations should insure a sound basis for serologic classification.

Rabbit immune sera: Cowan produced rabbit immune sera by the injection of boiled vaccines, thus obtaining antibodies against the heat-stable antigens exclusively. In spite of this simplification, a satisfactory typing scheme was not obtained. A classification which also includes heat-labile antigens (Oeding (66)) is not only possible.
but in our opinion offers better possibilities of a successful result. Immunization should therefore be made with formalin-killed vaccines.

Agglutinin absorption: This should be done with live or formalin-killed bacteria, which give identical results. It is very important that suitable strains are used for absorption. Pillet and Orta (87) used Cowan's type strain I for absorption of all sera except the homologous one. Christie and Keogh (10) and Hobbs (36) emphasized the difficulties of absorption in that too large an absorbing dose of bacteria tended to remove specific as well as nonspecific agglutinins.

These experiments were made with heat-killed bacteria. When formalin-killed or live bacteria are used for absorption, similar difficulties are demonstrated. Successful typing of \textit{S. aureus} cannot be achieved unless sufficiently strong, monovalent sera are obtained. The technique used for absorption is therefore very important.

Pillet \textit{et al.} (85) used quantitative absorption with a defined weight of boiled, dried bacteria for a given amount of serum. According to these authors, absorption is not proportional to the amount of agglutinogen in a strain.

The standard technique of Oeding consists of the absorption of 3 ml serum diluted 1 to 10, with the growth from one Roux bottle of a certain size. This rather empirical technique has worked quite satisfactorily, although absorption has sometimes to be repeated. Brodie (7) preferred repeated absorptions with small portions of bacteria for 30 min each. A similar technique has lately been used by us with good results. Sera are absorbed with defined amounts of wet bacteria in small portions and the titer of specific antibody determined after each absorption. Absorption is considered complete when the specific titer is not significantly reduced compared to the last absorption. As this technique is rather time consuming, a large amount of serum should be produced each time.

As pointed out above, "normal" antibodies will hardly be present in absorbed sera. It should therefore not be necessary to use serum dilutions of 1 to 50 or 1 to 100, as done by Mercier \textit{et al.} (61) or Brodie \textit{et al.} (8). At least, weak antisera should not be diluted more than 1 to 10.

Oeding (66) maintained that a serologic classification of staphylococci should be based upon an antigenic scheme. The use of monovalent factor sera containing defined antibodies will thus make it possible to determine the pattern of the surface agglutinogens present in a strain.

Agglutination tests: Tests should be made on slides which, in contrast to tube agglutination, give clear-cut reactions. Cowan (13) used boiled bacteria, whereas live 5-hr-old cultures are used by Oeding (72). Pillet \textit{et al.} (84) observed that the composition of the culture medium on which bacteria were grown for agglutination influenced the agglutination titer. In a poor medium little agglutinin developed. Grün (26) could not confirm this observation, and cultivation on nutrient agar can be recommended (Oeding (66)).

According to the technique given by Oeding (66), the slides are agitated for some time and then examined with the naked eye. Grün (25) prefers to agglutinate in a moist chamber for 2 hr and to read with a hand lens. We feel that there is a risk of including nonspecific reactions if the reading is too precise.

In our experience any special procedure to avoid spontaneous agglutination is unnecessary as the number of unstable strains is small. When strains showing spontaneous agglutination in live condition are autoclaved, the heat-stable antigens can usually be determined.

\textit{B. Epidemiologic Value}

In the following the value of serologic methods for the typing of \textit{S. aureus} will be analyzed. Consideration will be given both to the results obtained with the method described by Cowan, and to those obtained with Oeding's method. The reports of various authors are, however, not always comparable, due to the use of different typing strains and to technical modifications. Regarding the method of Oeding, his results are only directly comparable to those of Grün. For various reasons special attention will be paid to the reports of these two authors and their coworkers.

1. Reproducibility. If a typing system is to be of any use, the results must be reproducible. The antigenic equipment of the staphylococcal surface is very complex and it appears that the surface is a somewhat whimsical basis for classification. The technical conditions have been discussed above. Here it will only be repeated that agglutination of very young staphylococci offers a great advantage over earlier procedures.
in the effort to demonstrate the total set of agglutinogens. By this procedure strong antigens are easily demonstrated, whereas weak antigens may represent an uncertainty. The determination of the antigenic pattern is not so difficult in an epidemiologic investigation. The epidemic strain may have one weak antigen, which is indicated by parentheses, e.g., the antigenic pattern \( abc(h) \). If a strain has the pattern \( abc \) and is considered on epidemiologic evaluation to be the epidemic strain, the serologic results should be considered affirmative. Reexamination of the strain will usually disclose the weak \( h \) antigen. In the comparison of strains from different materials, e.g., in investigations on frequency distribution, weak antigens certainly represent a difficulty. Similar problems are present in phage typing, and it is a matter of experience to determine a convenient limit of variation.

The reproducibility of serologic typing can be tested either by repeated examinations on a number of colonies or subcultures of one strain, or by the examination of strains derived from the same source of infection. In investigations of this kind, not only the technical reproducibility but also the stability of a strain must be considered (see above), as well as the possibility of double infection or contamination.

Grün (25, 29) and Grün and Kühn (31), examining the reproducibility of serologic typing by Oeding’s method in subcultures of strains and in variants, found the serologic pattern to be unchanged. Grün points out that the possibility of contamination or double infection should be considered when unexpected results of serologic typing are found in laboratory strains or on epidemiologic examination. The antigenic pattern is considered a stable characteristic and Oeding’s serologic typing has given reproducible results.

A limited number of field investigations has shown that this serologic method gives as stable results as can reasonably be expected (27, 51, 75, 77, 102). When strains from a common source are examined, the antigenic patterns are identical or show only minor differences possibly ascribable to the uncertainty connected with a weak antigen present. The evaluation of the identity of the strains examined was in addition supported by epidemiologic data, antibiograms, and phage typing.

A number of sets of strains have been examined that were isolated from different sites on one person, from different people, or from people and infected fomites in circumstances that made it very probable that all the strains had a common parent. As examples of sets of strains which gave completely identical antigenic patterns can be mentioned 11 strains isolated from different portions of a potato salad responsible for food poisoning (75) and 16 independent strains from 3 outbreaks of food poisoning (77). Kikuth and Grün (51) examining strains of staphylococci isolated from various organs of patients who had died of staphylococcal infections, found identical antigenic pattern in strains from the same patient.

2. Field examinations. In addition to the reproducibility of a typing system, other properties must be present if it is to be of value in epidemiologic investigations. The number of patterns, types, or groups which a method is able to distinguish must not be too small or the certainty with which an epidemic strain can be picked out in patients and carriers will suffer. On the other hand, it should be considered a disadvantage if a system registers a great number of patterns unless they are well characterized and not influenced by technical conditions. Furthermore, the number of untypable strains must not be too large. A method must not be too complicated or it will be of limited practical value.

By the extension of the original 3 serologic groups of Cowan (13) to 9 (10, 24) and later 13 types (36), a serviceable basis for epidemiologic registration was established. The method of Cowan has been used with success in field investigations on cutaneous infections (1, 21, 24, 37) and on various staphylococcal infections (36); however, Andersen (2) was unable to characterize the strains responsible for epidemics of pemphigus neonatorum. Cross-reactions between some of the types were, however, a difficulty in classification (36) and too many strains were untypable. In two series, Hobbs (36) was unable to classify 26 and 20 per cent of the strains, respectively, although the number of untypable strains was reduced by the production of additional sera. The method of Cowan has not been widely adopted for epidemiologic work.

With the method of Oeding, strains are characterized by their antigenic patterns, which give a better differentiation of strains than a system
which is bound to a limited number of types. An epidemic strain can be further characterized by the quantitative appearance of its antigens. In an investigation into the frequency distribution of 239 independent strains, Oeding and Williams (77) found a large number of patterns, although when quantitative variations were not taken into consideration, four of the patterns accounted for 50 per cent of the strains. Similar findings have been made by Grün (29). The method will therefore insure a fairly reliable characterization of an epidemic strain, but should a widely distributed strain be responsible, the possibility of getting a clear picture of the epidemic will, as in all sero-diagnostic work, be reduced.

Attempts to collect strains with related antigenic patterns into types and groups (66, 77) have shown that such a division is mainly of academic interest and should not be applied to epidemiologic typing. It has also been shown that the exclusive use of "group" factor sera (a, b, c, e) (25, 29, 31, 69, 70) does not allow a sufficient number of patterns to be distinguished. Therefore when serology is used alone for the classification of staphylococci, the complete set of factor sera is needed.

The number of strains untypable, either because they have none of the antigens represented in the factor sera or because of spontaneous agglutination, is very small with Oeding's method. About 5 per cent of the strains were found to be untypable by Oeding (70) and Grün (25). When 5-hr-old cultures of 239 independent strains were examined, 19 were untypable, of which the total of 11 spontaneously agglutinating strains could be typed after autoclaving, i.e., only 3 per cent of the strains could not be typed (77). There seems to be a higher proportion of untypable strains from the air, skin, and feces than from pathological material (27, 31).

Comparison of serologic typing, phage typing, and antibiograms has shown that the best results are obtained when two or three of these methods are used simultaneously (75, 77, 102). The use of both phage and serologic methods for routine typing would, however, be too time consuming. A laboratory has to choose one of them, which can easily be combined with examination of the antibiogram. Grün (29) prefers to screen a material by means of antibiogram, after which the suspected strains are examined serologically by means of "group" sera (a, b, c, e) and only when there is still doubt as to the identity, by means of "type" sera (h, i, k, l). Brodie et al. (7, 8) have used the combined characterization of strains by antibiogram and serological type with success.

As mentioned above, we are of the opinion that as a rule a serologic investigation should be made with the complete set of sera. When an epidemic strain is sufficiently characterized, a simplified serologic screening may be possible for further examinations. Recently, however, new antigens have been described by Grün (28) (I) and Haukenes and Oeding (33) (m, n) and investigations in progress in this laboratory indicate the existence of still more antigens. A typing set of 10 or more factor sera will be too cumbersome for routine examinations. The procedure can be simplified after the principle of Grün (see above), or screening can be done with pools of factor sera. We feel convinced that the description of new antigens in S. aureus will eventually improve serologic classification and explain observations which are difficult to understand today.

3. Correlation to disease. There is no convincing report of correlation between serologic type and type of infection or virulence in staphylococci (29). Andersen (2) isolated a well characterized strain of staphylococci from cases of pemphigus neonatorum in Denmark, and Oeding (65) found a special serologic type responsible for a large number of cases of mastitis in Norwegian hospitals. The penicillin-resistant mastitis staphylococcus was considered to be particularly virulent and to have a special affinity for the lactating mammary gland. However, these staphylococci have also been found in other infections, and pemphigus and mastitis have been caused by other serologic types of staphylococci. Experience in recent years has shown that a certain strain can dominate a locality for some time and eventually be replaced by another strain.

4. Correlation to antibiotic sensitivity. Oeding (66, 70) found that strains having the antigenic pattern abc were more frequently resistant to penicillin than strains belonging to other serologic types. This was observed in different Norwegian hospitals. Pillet et al. (82) observed that strains belonging to serologic type 8 were all resistant to chlortetracycline and oxytetracycline.
These authors (66, 70, 82) found it possible that certain serologic types are particularly likely to develop resistance to antibiotics. When one serologic type shows a higher or lower incidence of antibiotic resistance, this may naturally be connected with the frequency with which strains of this type have been in contact with antibiotics and with the ability of the type to spread in a locality (30, 70). A possible correlation is very difficult to determine and must await further study.

5. Correlation to phage types and groups. A few comparisons of serologic and phage typing have been published, intending to seek a correlation between particular types and groups. Hobbs (36) and Wahl and Fouace (111) found that there was a tendency for strains of Cowan’s types I to III to fall into the later established phage groups I to III. Oeding (89) observed that strains with serologic pattern abc (Cowan’s type III) usually belonged to phage group III.

Oeding and Vogelsang (76) and Pillet et al. (82) found good correlation between serologic pattern abc (Cowan’s group I) and phage group I, although strains belonging to this serologic group were frequently not typable by phages (36, 76).

Oeding and Williams (77) found that all strains lysed by phage 187 possessed the k antigen. Phage 3A strains were often spontaneously agglutinable; such strains in an earlier investigation (76) were frequently connected with phage 70.

It can be concluded from these reports that there is a broad correlation between phage typing and serologic groups, but only a certain tendency for association between types determined by the two methods. In particular there is no example of complete correlation and in some cases the reactions are widely spread (77).

Whereas Pillet et al. (82) believe the receptors for agglutinins to be associated with the receptors for phages, Oeding and Williams (77) conclude that they are different.

IV. Conclusion

It is mainly because hospital infections caused by Staphylococcus aureus have developed into a serious problem during recent years that there has been an increasing interest in this organism. A vast number of publications have appeared which deal with the epidemiologic conditions in staphylococcal infection. It is undoubtedly very important that we should learn the routes of infection and the conditions that exist in our modern hospitals where such infections occur. Although the yellow staphylococcus has always been one of our most important pathogens, it is, however, astonishing how little progress has been made during the last decades in basic research on this organism. We feel that more knowledge of the fundamental characteristics of S. aureus will help us to understand and combat staphylococcal epidemics. It is our impression that this view is today shared by a number of microbiologists who have taken up staphylococcal research on an experimental basis.

For a long time the antigenic properties of S. aureus have been considered very complex and people have therefore felt reluctant to start work in this field. Immunologic and serologic work on staphylococci does certainly present many difficulties but this should stimulate rather than impede the taking up of these important and topical problems. The literature on the antigenic properties of S. aureus is scattered and extends over a long period of time. Our knowledge is so limited that one might well say that a critical review should wait. Nevertheless it was felt that at the present time a discussion of the problems might be a help to those working with or planning to work with staphylococcal immunology and serology.

Many gaps have to be filled before we shall be able to get a clear picture of the immunologic and serologic faculties of S. aureus. It is therefore difficult to say on which points it may be most advantageous to concentrate research at present. We feel that a registration of the antigens of S. aureus, their nature, and their properties should have high priority. As the basic research must be done on a few selected strains it is necessary, if we are to be able to compare the results, for international type strains to be used. These should be kept under controlled conditions to avoid variations in their characteristics. Furthermore, the results obtained with different antigen-antibody reactions should be correlated and the nomenclature of the antigens made uniform. Increased knowledge of the antigens of S. aureus will eventually improve serologic type classification and facilitate work in other fields of staphylococcal research.
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