A STUDY OF THE MICROORGANISMS OF THE GENERA STAPHYLOCOCCUS AND MICROCOCCUS

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INTRODUCTION

The microorganisms of the genera Micrococcus and Staphylococcus belong to the family Micrococcaceae, which is defined in Bergey's Manual of Determinative Bacteriology (2) as follows:

Family Micrococcaceae

Cells in their free condition spherical; during division somewhat elliptical. Division in two or three planes. If the cells remain in contact after division, they are frequently flattened in the plane of division, and occur singly, in pairs, tetrads, packets or irregular masses. Motility rare. Endospores probably absent. Produce abundant surface growth on ordinary media. Metabolism complex, usually involving the utilization of the amino acids and carbohydrates. Many species form lemon-yellow, orange or red pigment. Aerobes, facultative anaerobes and anaerobes. Generally Gram-positive.

Genus I. Micrococcus

Facultative parasites or saprophytes. Cells in plates or in irregular masses (never in long chains or packets). Generally Gram-positive. Growth on agar usually abundant, some species form no pigment but others form yellow, or less commonly, orange or red pigment. Dextrose broth slightly acid, lactose broth generally neutral. Gelatin frequently liquefied, but not rapidly.

Genus II. Staphylococcus

Usually parasitic, cells occur singly, in pairs and irregular groups, or rarely in packets. Usually Gram-positive. Growth fair to good on the surface of artificial media. As a rule carbohydrates are fermented with the formation of acid. Gelatin commonly liquefied. Nitrites may or may not be produced from nitrates. Produce hemolysis on blood agar. Pigment white, orange, or less commonly yellow.

Genus III. Gaffkya

Facultative parasites or saprophytes. In animal body and special media occur as tetrads. In ordinary media cells may occur in pairs and irregular masses. White to pale yellow.

Genus IV. Sarcina

Facultative parasites and saprophytes. Cells occur in regular packets. Yellow or orange pigment usually formed. Slightly acid to dextrose.

This study was limited to a collection of Gram-positive, mass-forming cocci belonging to the first two genera. The objective was the establishment of standards for the recognition of individual species and for their systematic classification. Of the large number of species currently listed there is considerable difference of opinion as to the taxonomic value of some of the tests by which they are recognized. Moreover, a disproportionate emphasis has been placed on the means by which pathogenic staphylococci may be distinguished from all other related species, and the group as a whole has received inadequate attention. In this work it was decided to apply particular methods of examination, including antigenic analysis, to a broader

group of microorganisms than most investigators have dealt with. It may be stated in anticipation that the investigation has not progressed as far as was originally planned.

HISTORICAL SURVEY

In 1878 Koch noted micrococci in pus; two years later Pasteur cultivated them in liquid medium; and by 1884 the systematic study of the micrococci began when Rosenbach (48) differentiated between those producing white pigment and those producing orange pigment, naming them Staphylococcus pyogenes albus and Staphylococcus pyogenes aureus respectively. He considered them both pathogenic. The next year Passet (44) added a yellow pigmented micrococcus to the list, namely, Staphylococcus pyogenes citreus. Flugge (22) used pigmentation as the basis of classification in 1886, and as early as this the relationship between pigment and pathogenicity was being considered. Liquefaction of gelatin was also used as a distinguishing characteristic by Flugge, by Migula (39) in 1900, and by Chester (11) in 1901. In 1903 Moberg and Unna (40) divided the micrococci into twenty-three groups by the size of cells and cell masses, chromogenesis, action on milk and liquefaction of gelatin. From this time on the characters used in dividing this group of organisms became progressively diversified. Some resistance to the tendency to increase the number of species has been evident from the earliest days. For example, in 1903 Neisser and Lipstein (42) expressed the view that the micrococci were all the same, the white forms being derived from the coloured forms under the influence of environment.

Kolle and Otto (36) first reported the use of serology for the differentiation of the micrococci in 1902, when they were

able to distinguish between pathogenic and non-pathogenic forms by agglutination tests. Their criterion for pathogenicity was apparently habitat.

In 1903 Loeb (37) reported the ability of the broth cultures of Staphylococcus aureus to coagulate goose plasma. Subsequently the plasma coagulating property of staphylococci was almost universally accepted as a distinguishing characteristic of the pathogenic forms though its use was neglected for several years.

Thus, at this early date, the ground was prepared for all the work which has followed. These developments have been discussed under three main headings.

A. Chromogenesis in Taxonomy

The pigments of the micrococci have attracted attention in a taxonomic sense since the time of Rosenbach (48).

In 1908 Dudgeon (17) reported his conclusion that chromogenesis was the only property that could possibly be used for classification, though he did not consider the production of pigment to be a stable characteristic or necessarily indicative of pathogenicity. He emphasized the importance of using freshly isolated strains when determining chromogenesis. In the same year the Winslows (54) distinguished between the red and yellow pigment producers on the one hand and the white and orange coloured forms on the other. With the latter group they included the streptococci and pneumococci. They found the yellow and red group was saprophytic, normally inactive in fermentation and Gram-negative; the white and orange group was parasitic, active in fermentation, and Gram-positive. The staphylococci were classified as Albococci and Aurococci. In 1917 this nomenclature was

changed and the orange and white micrococci were called Staphylococci and Albococci respectively. In 1920, Winslow, Rothberg and Parsons (53) published a study of one hundred and eighty strains of organisms of the genera Albococci and Aurococci in the Winslows' classification. They observed a gradual decrease in the amount of pigmentation, but no sharp lines differentiated individual species. It is interesting to note that they included lemon yellow forms in the Albococci.

Since Dudgeon many investigators have emphasized the changes which dissociation may produce in the colours of a culture, and the care with which the character must be assessed in the recognition of microbial species. At the same time the difficulties can be exaggerated. For example, Pinner and Voldrich (45) reported extensive variation occuring in staphylococci in a paper published in 1932 in which they considered the relationship between pigmentation and pathogenicity. They were able to dissociate typical Staphylococcus aureus into variants, which they designated Staphylococcus roseus, citreus and albus with respect to chromogenesis. The aureus strain was recovered from the albus dissociant by growing in the presence of anti-albus serum. The variants were found to differ from the original in growth intensity, individual morphology, staining reactions, virulence, fermentative and proteolytic action, haemolysin production and agglutination reactions as well as in pigmentation. It is not improbable that correlation of so many properties implies a contaminated culture rather than the appearance of variants in an otherwise pure culture, for other authors have not found pigment variation especially associated with changes in biochemical or serological behaviour (3). It is

abundantly clear, however, that pigmentation, as a single character, has limited reliability.

B. Biochemical Characters in Taxonomy

Gordon (24) studied a large number of orange and white cocci in 1905 and grouped them primarily according to colour. He observed a relationship between the white staphylococci and the streptococci, and concluded that there were nine tests of value in grouping the cocci (especially the white cocci) namely, liquefaction of gelatin, nitrate reduction, reduction of neutral red, action on milk and fermentation of lactose, maltose, glycerin and mannitol. The reduction of nitrates was considered a general characteristic of the white cocci. Andrewes and Gordon (1) used quantitative biochemical tests to study the cocci and in 1905 found that grouping by the method of plotting frequency curves, the peaks of the curve being considered the centre of the species, agreed largely with grouping by cultural reactions.

In an extensive survey of the Coccaceae in 1924 Hucker (30) came to the conclusion that these organisms belonged to a single large group that might be divided into genera separated by indefinite border lines. There appeared to be no natural subdivisions. He considered chromogenesis, the action on milk and the ability to reduce nitrates, liquefy gelatin and utilize ammonium phosphate as the sole source of nitrogen the most important criteria for grouping and sub-grouping. Of less importance were the Gram stain, fermentation of carbohydrates, production of ammonia and hemolysin, and habitat. These conclusions were not essentially different from those of Andrewes and Gordon, and the large amount of work done added little to the subject. Indeed, at the present time there is no important surviving test

from this early work with the exception of the well-known relationship between mannitol fermentation and pathogenicity in the staphylococci.

In 1936, Fildes, Richardson, Knight and Gladstone (20) initiated a new phase in these empirical methods of studying the staphylococci. They investigated the nutritional requirements of these organisms and demonstrated that certain essential growth factors were required in addition to the necessary amino acids, inorganic salts and carbohydrates. Fildes and Richardson (19) and Gladstone (23) identified these growth factors as nicotinic acid and thiamin, both necessary in minute quantities. Later Richardson (47) demonstrated that the addition of uracil is necessary for anaerobic growth. More recently Porter and Pelczar (46) have shown certain of the micrococci that fail to grow under these conditions will grow if a small amount of biotin is added. Gladstone was able to grow staphylococci on entirely synthetic medium in 1937 after training the organisms. The growth factors were, of course, present.

It is probable that specific and constant differences may exist in the requirements of different species within these genera. An indication of this is a report by Segal (49) in 1940 of a sharp differentiation between the pathogenic and non-pathogenic staphylococci by their ability to utilize acetyl methyl carbinol as sole source of carbon. Segal deplored the trend to abandon biochemical methods of differentiating the staphylococci, and following the work of Tittsler (51) with acetyl methyl carbinol, tested forty-three strains for ability

to grow on a synthetic media containing only inorganic salts and acetyl methyl carbinol. The results showed that the ability to utilize acetyl methyl carbinol as the sole source of carbon was a characteristic of the non-pathogens, and that the correlation of pathogenicity with this test was more reliable than with liquefaction of gelatin or fermentation of mannitol.

Since Loeb (37) demonstrated the coagulating effect of certain staphylococci on goose plasma, this property has received frequent attention. In 1908 Much (41) repeated the work using rabbit plasma and again described the relationship between the production of plasma coagulase and pathogenicity. In succeeding years this relationship has been continuously emphasized in the literature. Fisher (21) devoted a paper to the plasma coagulating property of staphylococci in 1936 in which he summarized previous work. He concluded that this property occurs most frequently in strains of the aureus group and is most uniformly found in the cultures of virulent strains. According to some it may be obtained in the filtrates of cultures; it is active against the plasma of a variety of animals, but at different rates; it is like thrombin but it is not neutralized by hirudin; most have found it heat stable; it is less active against pure fibrinogen.

Chapman, Berens, Peters and Curcio (9) reported a survey of the properties of the staphylococci in 1934, and concluded that coagulase production was the most important indication of pathogenicity. Hemolysin production and pigmentation were not considered indicative of toxicity if used alone. Coagulase positive organisms were usually pathogenic

without regard to production of hemolysin or colour, but the aureus strains that produced hemolysin were usually pathogenic and the non-hemolytic albus strains were usually non-pathogenic regardless of coagulase. Toxicity (apparently used as equivalent to pathogenicity) could be correlated with a high degree of accuracy with these three tests. In later studies Chapman and his co-workers (6), (7), (8) and (10) reiterated the importance of coagulase, but stressed the necessity for examining other characters in attempting to infer pathogenicity. The most useful adjuncts were hemolysis of rabbit blood agar, chromogenesis and the bacteriostasis by crystal violet or alkaline brom thymol blue.

None of the investigators were as emphatic about the taxonomic value of the coagulase test as Cruikshank in 1937 (16). He found staphylocoagulase constantly present in pathogenic strains and considered it an important factor in pathogenicity. He described it as an enzymic substance, acting in a manner similar to thrombin on the plasma of several animals including man. It is not related to alpha lysin or necrotoxin, but, like them, it occurs only in pathogenic species.

C. Antigenic Structure in Taxonomy

In 1922 Hine (25) studied 90 strains of staphylococci from St. Bartholomew's Hospital in an attempt to make a serological classification. He was conscious of the necessity for such a grouping before any progress could be hoped for in the use of vaccines and serum therapy clinically. He produced agglutinating sera by the intravenous inoculation of rabbits with heat-killed, phenolated suspensions of two strains of the cocci.

Satisfactory sera were prepared in three weeks. Test tube agglutination tests were carried out using heated and phenolated suspensions as antigens, and incubating at 55°C for 20 hours. All strains were also tested for their ability to ferment mannitol and produce pigment. Agglutination tests separated the organisms into two broad groups, one of which was called pyogenes, the other epidermidis. The former group was made up of strains that were orange, white or fawn in colour, all except one of which fermented mannitol. All the strains of the second group produced white pigment and were mannitol negative. Hine was also able to divide the pyogenes group into three serological types and the epidermidis group into two serological types by agglutinin absorption tests. He felt that a satisfactory separation of these organisms by serological methods required the use of agglutinin absorption.

In the same year Julianelle (33) studied a group of hemolytic staphylococci by agglutinin absorption and complement fixation methods, and concluded that there was no relationship between grouping by serological methods and grouping by hemolysin production, colour or biochemical tests. No relationship was found between production of hemolysin and other metabolic activities such as fermentation of carbohydrates, production of ammonia, reduction of nitrates, formation of indol, reduction of methylene blue. Chromogenesis was considered of no significance for classification. Shortly afterwards Hucker and his co-workers (31), (32) corroborated Julianelle's findings. They found fifteen strains of cocci to be all serologically distinct and concluded, furthermore, that there was no relationship between agglutination reactions and other characteristics.

Dudgeon and Simpson (18) in 1928, broke new ground in using the precipitin reaction. With this technique they found evidence of antigenic differences among the staphylococci. This work was followed up by Julianelle and Wieghard (34) in 1933. From staphylococci they extracted a haptene-like polysaccharide and found it to be specific for the homologous strain in precipitation reactions. On this basis they were able to differentiate between a pathogenic type A. and a non-pathogenic type B. Two years later the same authors (35) reported their investigations in detail. Still using the precipitin reaction they were able to divide the staphylococci sharply into two groups on the basis of a specific polysaccharide, which they were able to extract and purify. They found the intravenous inoculation of dead staphylococci into young rabbits produced the best sera for the precipitin test but noted that some rabbits produced no antibodies at all and in all cases the titre of the sera tended to be low. They prepared a purified polysaccharide by extraction with water and repeated precipitation and solution with a variety of reagents. The specific polysaccharide was obtained as a granular precipitate. These polysaccharides were soluble white powders with acid properties, positive to the Molisch test in high dilutions and negative to Fehling's though they could be made positive by hydrolysing. The hydrolysed products would not react with the homologous sera. Type A. and type B. polysaccharides were distinct in their specific rotation, the melting points of their ozazones and the percentage of reducing and fermentable sugars produced by hydrolysis. There were indications that the polysaccharide obtained from type B. staphylococci was related to glucose.

These polysaccharides did not exhibit measurable antigenicity even when acetylated and since they reacted to a high dilution in precipitation tests were considered to be true haptenes. Skin reactivity was reported and suggested to be type specific as an immediate wheal and erythema were produced. Julianelle and Wieghard also extracted proteins from these organisms which they considered species specific. The protein was antigenic in rabbits and precipitated with the sera prepared by protein inoculation or bacterial inoculation. Skin reactivity of the species specific kind was recorded with delayed inflammation. Neither the protein nor the carbohydrate was toxic.

This work was amplified in 1937 by Thompson and Khorazo (50). In addition to groups A. and B. of Julianelle, Thompson separated two new groups designated C. and BC. 78% of the strains known to be of human origin fell into group A. by the precipitin reaction. It was noted that organisms of the A. group were the most active biochemically and that they gave a much heavier precipitate in the precipitin reaction than did members of the other groups. Organisms falling into groups B. C. and BC. exhibited a tendency to form viscid colonies. No reason for the tendency was found.

Cowan (14) in 1938 further confirmed Julianelle's grouping. On the precipitin reaction he was able to divide 157 strains of staphylococci into four main groups A. B. C. and D. A. and B. were identical with the groups similarly characterized by Julianelle and by Thompson; C. organisms were apparently pathogenic also; D. were sticky white non-pathogens. Cowan suggested the possibility of sub-division of these main groups by agglutination, presumably having in mind the very

successful application to the streptococci. Cowan reported definite correlation between these serological groups and groups formed on the basis of pigment production, fermentation of mannitol, production of hemolysin and coagulase. He noted that all his strains which produced Alpha or Beta hemolysin were coagulase positive and the others were negative.

A year later Cowan (15) had come to the conclusion that precipitin methods were difficult because of the need for paying attention to the optimal ratio principle and the danger of marked cross reactions with crude bacterial extracts. He turned, therefore, to the study of antigenic structure by agglutination. Among the pathogenic staphylococci three main groups I, II and III accounted for more than two thirds of the strains. Saprophytic strains did not cross react with these antisera, and were themselves serologically heterogeneous. The groups A. B. and C. identified by precipitation and the groups I, II and III identified by agglutination did not coincide and Cowan considered this to mean that the antigens responsible for these two immunity mechanisms were not identical. This is an interesting point and throws into important relief the type specific, antigenic protein extracted from staphylococci by Verwey (52) in 1940. This protein was antigenically distinct from Julianelle's type specific polysaccharide but the grouping by precipitation using either the protein or the polysaccharide was the same, indicating that each of the organisms studied possessed both polysaccharide and protein. Verwey was able to prepare his protein and polysaccharide extracts in a highly purified state. He found that the protein contained 14% nitrogen and 0.14% phosphorus.

As distinct from in vitro studies very little accurate work has been recorded on antibacterial immunity in staphylococcal infection. For that reason no use has been made of this approach in a taxonomic sense, although plenty uncritical appraisal of bacterial vaccines in therapy exist in the literature. The only report in the literature of the demonstration of capsules for staphylococci is by Lyons (38) who, in 1937, reported the results of his studies of antibacterial immunity. He felt that definite antibacterial immunity could be demonstrated against staphylococci and that this immunity was type specific. He was also convinced of the presence of a demonstrable capsule on the pathogenic staphylococci and was able to type these organisms by tests similar to the pneumococci "Quellung" reaction. These studies have not been confirmed.

There has been, in general, a swing from agglutination methods to those of precipitation. This was due, in large measure, to the successful application of a number of investigators of a technique which contributed so much to the understanding of other microorganisms such as the streptococci. Nevertheless, there is no unanimity in the matter. For example, Blair and Hallman (5), (3) in 1936 could make no satisfactory grouping of the staphylococci with the precipitin reaction, using as antigen either toxic filtrates or specific soluble carbohydrates. However, they were able to divide the organisms with which they were working into four groups by reciprocal agglutinin absorption tests. Three of these groups were pathogenic and the fourth non-pathogenic. The agreement between pathogenicity determined by this method and by animal inoculations and production of coagulase was good.

It is difficult to assess how much of the difficulty in reconciling the somewhat discordant results of attempted antigenic analysis of the staphylococci is due to dissociation. Certainly most of the studies reported were carried out with cultures collected over a period of years. Hoffstadt and Youmans in 1932 and 1935 (27), (28), Hoffstadt, Youmans and Clark in 1934 (29) and Hoffstadt and Clark in 1938(26) were able to dissociate staphylococcus aureus into several rough or gonidal forms, differing from the mother form in biochemical and antigenic properties. The smooth, rough and gonidal forms were proven to be antigenically different by means of agglutinin absorption reactions, and specific soluble carbohydrates were produced from both the smooth and rough forms. How often these variations occur during subculture on ordinary media in the course of a series of tests is not mentioned.

In summary it is evident that the earlier workers studied collections of Gram-positive mass-forming cocci from all sources and endeavoured to classify them by an increasing variety of tests. In more recent times the tendency has been to limit the scope of surveys and to regard, for example, organisms of this group as staphylococci if they are isolated from human sources, other strains being considered as micrococci. In view of the obvious fact that animals may contaminate their environment, and vice versa, there is a rapidly growing distaste for the use of source and circumstances of isolation as criteria for identification. For that reason it is to be hoped that conclusions reached by studying limited numbers of strains from particular sources will prove applicable to the Micrococcaceae

as a whole. This applies particularly to the serological studies. A large part of the work done on the antigenic structure of these organisms has been concerned with staphylococci from human sources only.

METHODS

Gram Stain:

The morphology, grouping and Gram reaction of the organisms was studied by staining cultures one, three and five days old. The technique used was to fix a smear from an agar slope by heating in the Bunsen flame; when cool the slide was flooded with gentian violet, which was allowed to react for one minute; the gentian violet was then washed off with Gram's iodine and this was left on the slide for one minute; the slide was washed thoroughly with tap water and decolourized with alcohol till no more colour came out; the slide was rinsed with water, blotted lightly and counterstained with dilute carbol fuchsin for fifteen seconds; the slide was then washed thoroughly and allowed to dry.

Growth on Agar Slants and in Peptone Broth:

The nature of the growth on agar slants and in peptone broth at 37°C was recorded. The peptone broth was prepared by heating finely minced fresh beef heart, from which the fat had been removed, in distilled water at 75-80°C for 1 1/2 hours, (1 litre of water to 1 pound of meat) and then filtering through paper pulp in a Buchner funnel under slight suction.

1% Difco Proteose Peptone and .25% NaCl, .02% KCl, .01% CaCl were added. The reaction was adjusted to pH 8.5 to phenol red with 10 N NaOH and heated at 120°C for 20 minutes to precipitate the phosphates and then filtered through paper pulp. The reaction was adjusted to pH 7.2 to phenol red with N HCl and again heated to 120°C for 20 minutes and filtered. The reaction was finally adjusted to pH 7.2 and the broth was tubed or bottled and sterilized by autoclaving at 120°C for 20 minutes.

The agar was prepared in the following way:
A meat infusion was prepared and filtered as for the broth; fibre agar sufficient to give a final concentration of 1.5% was washed by soaking in distilled water for an hour and then squeezed as dry as possible in muslin; the agar shreds were then melted in 1/10 the volume of the meat infusion to which was added Proteose Peptone, NaCl, KCl and CaCl to give final concentrations as in the broth; this was added to the bulk of the meat infusion which was heated between 60 and 80°C; it was then set aside to cool overnight in order to adsorb the accessory growth factors; next day it was melted at 100°C and the reaction was adjusted to pH 8.5; it was heated in the autoclave for 20 minutes at 120°C to precipitate phosphates; finally it was filtered through paper pulp, the reaction adjusted to pH 7.2, then tubed, sterilized by autoclaving and sloped.

Growth at Various Temperatures:

Ability to grow at 10, 25, 37 and 45°C was tested by inoculating agar slopes and incubating at the various temperatures.

Production of Pigment:

Pigment production was determined in the growth on agar slopes after incubation at 37°C and room temperature. Pigment was recorded as white, orange, yellow and variations thereof as it appeared on the slope to the naked eye. More delicate gradations of colour, or the production of colour on specialized media, were not considered practical in view of the instability of the pigment producing characteristic of these organisms and the doubtfulness of its import.

Biochemical Reactions:

All strains were tested for their ability to ferment mannitol, glucose and lactose at 37°C. Most of the organisms were also tested for their action on sucrose, levulose, arabinose, raffinose, salicin and inulin. The tests were observed for a period of three weeks. Unless otherwise stated the organisms were inoculated in duplicate into the test media from a saline suspension of the overnight growth on an agar slope. The media were prepared by dissolving 1% Difco Bacto-Tryptone in distilled water, adjusting the pH to 7.0 (phenol red indicator), heating, filtering and readjusting the pH to 7.0. The required sugar was added to a concentration of 0.5%, and 0.1% of a 1.6% alcoholic solution of brom cresol purple as indicator. The whole was tubed and autoclaved at 120°C for 20 minutes.

Action on Litmus Milk:

The action on litmus milk was recorded for three weeks. The litmus milk was prepared by removing the fat from milk by centrifugation, adding 2% of a 2% solution of azolitmin, tubing and sterilizing by autoclaving at 120°C for 20 minutes. The changes were recorded as acid, acid clot, basic, and later

digestion or decolourization were noted.

Liquefaction of Gelatin:

Ability to liquefy gelatin was tested by making stab inoculations into gelatin dissolved in peptone broth. 14% Ayerst, McKenna & Harrison sheet gelatin was dissolved in peptone broth at a temperature below 50°C by slow addition of NaOH. The reaction was kept at pH 7.2 and when it remained stable for 1/2 hour the gelatin was tubes and autoclaved at 120°C for 20 minutes. The gelatin tests were incubated at room temperature and changes were recorded for 100 days.

Coagulase:

The ability to coagulate rabbit plasma was determined by inoculating rabbit plasma with a loopful of the overnight growth on an agar slope and incubating at room temperature. Readings were made after four hours and overnight. The plasma was prepared by adding 20 cc sterile rabbit blood to a flask containing 2 cc of 4% potassium oxalate, centrifuging and collecting the plasma with sterile precautions. The plasma was diluted 1:4 with saline and distributed in 0.5 cc quantities in small bore tubes.

Production of Hydrogen Sulphide:

Ability to produce hydrogen sulphide was determined by making stab inoculums into peptone agar containing 0.5% lead acetate and 0.25% sodium thiosulphate. The lead acetate and the thiosulphate was added sterilly to the molten agar and the medium tubed sterilly. The test was incubated for 3 weeks at 37°C.

Nitrate Reduction:

Nitrate agar was prepared containing 15 grams agar

shreds, 3 grams beef extract, 3 grams proteose peptone and 2 grams potassium nitrate in 1000 cc distilled water. The agar was washed in the usual way and dissolved in the water containing the peptone and beef extract by heating at 120°C for 20 minutes in the autoclave. The mixture was then filtered through paper pulp and the nitrate added. The medium was tubed, sterilized by autoclaving and sloped. The organisms were inoculated in triplicate and one tube was tested on each of the first, third and fifth days. The presence of nitrite was shown by the appearance of a red colour when one drop of each of the following solutions was added: (a) 8 grams sulphanilic acid in 1 litre 5N acetic acid; (b) grams alpha naphthylamine in 1 litre 5N acetic acid.

Production of Indol:

The organisms were tested for ability to produce indol by inoculating into 3% tryptone water and incubated at 37°C. Production of indol was determined by adding the following solutions: (1) 1 gram para-dimethylamidobenzaldehyde in 95 cc absolute alcohol, concentrated HCl 20 ccs. (2) Saturated solution of potassium persulphate (5 grams in 200 cc distilled water).

V.P. Reaction and Utilization of Phosphate:

These tests were carried out as described in the literature in media that did not contain the necessary growth requirements for culturing the micrococci. O'Meara's V.P. medium and the ammonium phosphate liquid medium described by Hucker (30) were used. Neither test was repeated with the accessory growth factors present.

Utilization of Acetyl Methyl Carbinol as Sole Source of Carbon:

The ability to utilize acetyl methyl carbinol as sole

source of carbon was tested by two methods. The first was the use of Tittsler's synthetic medium, advocated by Segal (49). This medium lacked the accessory growth factors and other essential metabolites. The second method of testing for the utilization of the carbinol was to incorporate the substance into a synthetic medium containing every requirement for the aerobic growth of staphylococci except a source of carbon. As a control a similar medium containing glucose in place of acetyl methyl carbinol was employed (19), (20) & (23).

1. Tittsler's Synthetic Medium (51)

Sodium ammonium phosphate	1.5 grams
Potassium dihydrogen phosphate	1.0 gram
Magnesium sulphate	0.2 gram
Acetyl methyl carbinol	2.0 grams
Distilled water	1000 ccs

The pH was adjusted to 6.8 - 7.0, the medium tubed and sterilized in the autoclave at 120°C for 20 minutes.

2. Synthetic Medium of Fildes, Knight, Richardson & Gladstone (19), (20), & (23).

A sterile solution of the necessary amino acids was prepared by dissolving them in distilled water and filtering through a Seitz filter. The amino acids were dl-valine, l-leucine, glycine, l-proline and l-aspartic acid each in concentration M/750 on the basis of the optically active isomer; dl-methione, dl-phenylalanine, d-arginine, HCl and l-histidine. HCl each in concentration M/2000 and l-tryptophane M/1000 and l-cystine M/2500 on the same basis.

The dl compounds were synthetic and the optically active ones highly purified. The pH was adjusted to 7.4.

Solutions of thiamin M/500,000 and synthetic nicotinic acid M/5,000 were prepared and sterilized by autoclaving.

A solution containing ferrous ammonium sulphate M/1000 and magnesium sulphate M/300 was prepared, the pH adjusted to 7.4 and sterilized by autoclaving.

Solutions of K_2HPO_4 M/12 and KNO_3 M/25 were also prepared and sterilized by autoclaving.

A solution of glucose M/4 was prepared and sterilized by filtration.

A sterile solution of acetyl methyl carbinol M/4 was prepared by dissolving in distilled water and filtering.

The solutions were made up in large quantities and mixed in the following proportions, using sterile precautions, then tubed sterilly.

Amino acid solution	50%
Thiamin solution	5%
Nicotinic acid solution	5%
Sulphate solution	5%
Potassium nitrate solution	5%
Glucose solution or acetyl methyl carbinol	5%
Sterile distilled water	25%

All the apparatus used in this experiment (flasks, measuring cylinders, pipettes, test tubes) were specially cleaned by rinsing the clean laboratory apparatus with tap

water, allowing it to drain and then coating it with chromic acid cleaning fluid. The chromic acid was completely removed by washing four times in tap water and twice in distilled water. The pipettes and flasks were sterilized and the test tubes plugged and sterilized. The size of the inoculum was controlled by suspending in saline the organisms from an 18 hour growth on agar to give a concentration of 100,000 cc (Brown's Tubes) and using 0.1 cc of the suspension. Two tubes of each of the three media mentioned were inoculated with each organism and were incubated at 37°C for 3 weeks.

Serological Studies:

The strains used for the production of antisera in rabbits were:

S MX (Thompson's type B, not pathogenic.)
S 32 (Thompson's type C, not pathogenic.)
S 13 (Julianelle's type A, Thompson's type A, a pyogenes strain.)
S 41 (Cowan's type D, not pathogenic.)
S 34 (Cowan's type B, not pyogenes.)
S 80 (Cowan's type C, pyogenes.)
S BC (Thompson's type BC, not pathogenic.)
S 33 (Cowan's type A, said to be related to Julianelle's type A, pyogenes strain.)

Vaccines were prepared in the following way:

Strains were prepared by sub-culturing daily for three days on agar to insure that the strain was in an active growth phase. From such a young culture (12 - 18 hours) inoculations were made into broth distributed in Roux flasks in 200 cc quantities for maximum aeration. The broth cultures were incubated at 37°C

for 18 hours. The suspension was killed by adding 1% formalin and heating at 65 - 70°C for 1/2 hour, and then centrifugated, and the supernatant fluid was removed. The organisms were re-suspended in saline plus 0.1% formalin to a strength of 5000 x 10^6 per cc by Brown's tubes. The suspensions were cultured for sterility and stored in the cold.

Young rabbits 2-3 kilograms in weight were used for the preparation of antisera. The route of injection was the marginal ear vein (Julianelle and Wieghard (35). Two rabbits were used for each strain.

Inoculations were made on three successive days in alternate weeks. The dosage was increased gradually from 500 x 10^6 per kilo to 4000 x 10^6 per kilo. The rabbits were bled before the series of inoculations began, to test for natural antibodies, and after nine weeks were bled before the three daily injections. These inoculations were kept up for a year and a half, with rest periods, in an attempt to get a serum with high titre antibodies for the precipitin reaction.

Antigens for the precipitin reaction were prepared by two methods: The first by extracting with N/16 HCl and boiling, then neutralizing with N/16 NaOH (Julianelle & Wieghard) (34): The second by extracting with distilled water in the autoclave for 20 minutes at 120°C. The latter extract was concentrated by precipitating in acid solution with 5-6 volumes of 95% alcohol. Cowan (14) precipitated with alcohol and purified by reprecipitation with acetic acid. In this work no precipitate was obtained in neutral solution with alcohol, so a few drops of

glacial acetic acid were added to the distilled water extract. This caused a certain precipitation which was removed by centrifugation. The supernatant was then precipitated by adding 5-6 volumes of alcohol. This precipitate was also separated by centrifugation and taken up in a small amount of saline and used as the antigen for the precipitin reaction. The acetic acid precipitate was also tested for ability to cause precipitation with homologous serum. As there was considerable cross reaction between the strains tested in this way, quantitative tests were attempted by preparing large quantities of distilled water extracts, precipitating as above, drying and weighing the precipitates and making solutions of known concentration.

In the precipitin test the sera were titrated against homologous antigens to determine the titre and then constant antibody titrations were performed with all the antigens. The test tube method was used and the reaction was incubated for 4 hours at 56°C and read again after being left at room temperature overnight.

The same sera were used for agglutination tests. Slide agglutination technique was used. At first the antigens were prepared by growing in .05% glucose broth for 3-4 hours, killed by heating in a water bath to 100°C, cooled anc centrifuged and the organisms resuspended in a small volume of broth. Cowan, 1939) (15). Later agglutination tests were performed by washing an 18 hour growth from an agar slant with saline and killing by boiling for 5 minutes. After comparing the results obtained by using these two methods the saline suspension of an agar culture was used exclusively. The sera were diluted 1 in 5

with physiological saline and titrations were carried out against the homologous strain and each of the other strains used for preparation of sera. Halving dilutions of the sera were used and a saline control included in each titration. One drop of the suspension and one drop of the serum dilution were mixed on a large glass plate, so that several tests could be carried out at once. Pasteur pipettes of approximately the same bore were used to distribute the reagents. The slide was rocked for two one-minute periods and the final reading was taken after ten minutes by microscopic examination.

Absorptions were necessary to make the sera specific and in every case massive absorbing doses were required. The overnight growth on large agar plates was suspended in a 1 to 5 dilution of the serum to be absorbed and incubated for 1 1/2 hours at 37°C, with frequent shaking. The organisms were then removed by centrifugation and the serum tested for the presence of heterologous antibodies. In the beginning heat-killed organisms were used for absorption but because of the poor results live organisms were subsequently used. Absorption with more than one strain was necessary in most cases and the antisera against S MX, S 13 and S 34 could not be made specific even with repeated absorptions. It was considered that the long series of injections in preparing the sera might have rendered them non-specific and so fresh sera were prepared against these three strains using the same method but bleeding the rabbits after six weeks. The sera were titrated and absorbed in the same manner as before.

After the sera had been made specific an attempt was made to group all the strains available by their reactions to

the specific sera in the agglutinin test. Suspensions of the organisms were prepared as for the antibody titrations and tested with a 1 in 5 dilution of each of the absorbed sera.

RESULTS

Forty-nine strains of organisms were studied. All were Gram-positive, mass-forming cocci and, while there were individual differences in morphology and degree of Gram-positiveness, none could be considered of significance for classification. All grew readily on nutrient agar and in broth, and there were considerable differences in the general appearance of the cultures. None of the yellow or very viscid forms were in the coagulase positive group. No other generalization could be made regarding chromogenesis or colony consistency. Forty-one organisms were tested on nutrient agar and all grew well at 37°C and 25°C. Fifteen organisms gave a slight growth at 45°C and twenty-seven grew at 10°C, but the ability to grow at these temperatures was not related to other properties.

Pigment:

Of the forty-nine strains studied, seventeen were orange, one was cream to orange, one cream to yellow, eleven cream, fourteen white and five yellow, (Table 1.). There was in general good correlation between aureus pigment, a positive coagulase test and pathogenicity presumed on source. However, three white and eight cream pigmented strains fell into the same group. It suggests that the presence of orange pigment usually coincides with pathogenicity, but that its absence, even in strains from animal sources, does not testify to a saprophytic origin.

Biochemical Reactions:

All those strains of known pathogenic origin or isolated from pathological lesions were coagulase positive and fermented mannitol. Every strain that produced coagulase fermented mannitol but two strains that did not produce coagulase also fermented mannitol. One of these, S 41 (Cowan's type D), was known to be a non-pathogen, the other was M. liquefaciens (NTC 2654). No other test substance was correlated to a significant degree with other attributes of the organisms, Table 1.). Forty-one of the forty-nine strains fermented lactose and thirty-nine fermented glucose. None of the organisms fermented inulin, salicin, raffinose or arabinose. The fermentation of sucrose, glycerol and levulose followed quite closely the activities of the same organisms on lactose and glucose.

Thirty-one of the organisms tested liquefied gelatin. The rates and extent of liquefaction varied extensively. In some cases complete liquefaction was noted in 2 days, in others liquefaction was not complete until the 60th day. Other organisms liquefied from 25% - 95% of gelatin in from 6 - 60 days, (Table 1.). The property was more frequently present in presumably pathogenic organisms than in the saprophytes, but even this was not invariable. None of the organisms grew in Tittsler's synthetic medium in which acetyl methyl carbinol was the sole source of carbon. Thirty-four strains grew on the complete synthetic medium of Fildes, Knight, Richardson & Gladstone in which glucose was the carbon source. Twenty-five strains grew on the medium of Fildes, Knight, Richardson & Gladstone with acetyl methyl carbinol substituted for glucose.

One of the organisms grew on the acetyl methyl carbinol and did not grow on the glucose medium. Lack of correlation between ability to use acetyl methyl carbinol as sole source of carbon and other properties is shown in Table 1.

None of the organisms produced hydrogen sulphide or indol.

Thirty-two organisms gave hemolysis on human blood agar and twenty-seven on sheep blood agar. Eleven of the organisms that produced hemolysis on sheep blood agar did not do so on human blood agar, and six of the organisms that were hemolytic on sheep blood agar were negative on human blood agar. These hemolytic activities, particularly on sheep's blood, correlated well with the coagulase reaction and mannitol fermentation. This is shown clearly in the following table.

	Mannitol +	Sheep Hemolysin +	Human Hemolysin +	Gelatin Liquefaction +
Coagulase + 28	28	24	22	26
Coagulase - 21	2	3	8	8

Serological Results:

The sera prepared in rabbits by a series of inoculations extending over eighteen months were low in titre and very slow to react in the precipitin reaction. The titres ranged from 1:3 to 1:16. No precipitation was observed in 4 hours at 56°C while the overnight precipitation was scanty but could be read.

The antigen prepared by extracting with N/16 HCl did

not react in a satisfactory manner. The acetic acid precipitate of the water extract was not as reactive as the alcohol. Moreover the former could not be redissolved after drying and so could not be used quantitatively. The alcohol precipitate, as described under methods, was therefore used exclusively as antigen in the precipitin tests. The solution of the antigen was positive to the Molisch test. The antisera were used in a dilution which gave a satisfactory precipitate on titration with homologous antigen. The antigens were dissolved in saline, and halving dilutions, starting at 1:2000 were mixed with the constant antibody dilution. These results are shown in Table 2. The highest dilution in which the antigen reacted with each sera is indicated. The dilutions at which the sera were used are also shown.

The results for the agglutinin reactions of the sera prepared against eight strains of staphylococci and suspensions of these strains is shown in Table 3. Sera specific for the homologous strains were prepared by absorptions as follows:

Serum against S 32 absorbed with a large dose of S 13
and a small dose of S MX;

Serum against S 41 absorbed with a large dose of S 34
and a small dose of S 80;

Serum against S 80 absorbed with a large dose of S 33
and a small dose of S MX;

Serum against S 33 absorbed with a large dose of S 80
and a small dose of S 34;

Serum against S BC absorbed with a large dose of S 80

The MX, S 13 and S 34 sera could not at first be made specific by absorption. In some cases homologous agglutinins were removed more readily than those against the absorbing strain. It was found, however, that when animals were given only a short course of immunization MX and S 13 sera were readily purified by absorption with a massive dose of S 80. No specific S 34 serum was prepared, for S 80 always removed all the agglutinins. The titre of every serum was greatly decreased by absorption.

Seven specific sera were available for the agglutinin reaction. These sera all appeared different though those against S 13 and S 33 showed some similarity. The serum against BC (Thompson's strain) did not react with Thompson's MX (Type B) and the MX sera did not react with the BC suspension. S 32 (Thompson's type C) and BC did cross-agglutinate also several other strains agglutinated with both MX and BC sera. To avoid confusion the following notation has been used for the specific antigens in the various types:

MX ---- a; S 32 ---- b; S 13 ---- c;

S 41 --- d; S 80 ---- e; S 33 ---- f;

BC ---- bx.

Of the forty-one other strains tested by the agglutinin reaction only sixteen could be typed. All of those that were agglutinated by the sera against the pathogenic strains (antigens c, e and f) were in the coagulase positive, mannite positive group and none of the strains agglutinated by the sera against the non-pathogens were in this group, (Table 1.).

TABLE I

STRAINS	PIGMENT	Coagulase Production	Haemolysin (Human)	Haemolysin (Sheep)	Fermentation of: Mannite	Fermentation of: Lactose	Fermentation of: Glucose	Gelatin Liquefaction	Action on Litmus Milk	Reduction of Nitrates	Growth on Synthetic Media: Tittsler	Growth on Synthetic Media: Complete with Glucose	Growth on Synthetic Media: Complete with A.M.C.	Type (Agglutination)
S 13 (A. of Julianelle)	Orange	+	-	-	+	+	+	-	AC	+	-	+	+	c,f
B.& H. 17650	Orange	+	+	+	+	+	+	25^{6}	AC	+	-	+	-	f
B.& H. 18176	Orange	+	+	+	+	+	+	100^{60}	AC	-	-	+	+	f
S 80 (C. of Cowan)	White	+	-	+	+	+	+	100^{7}	ACL	-	-	+	+	e
S 33 (A. of Cowan)	Orange	+	+	-	+	+	+	100^{7}	ACL	-	-	-	+	f
Perez (Furuncle)	Orange	+	+	+	+	+	+	80^{6}	-	+	-	+	+	o
CMH 5216 (Blood culture)	Orange	+	+	+	+	+	+	60^{6}	AC(de)	+	-	+	-	c,f,d
Gwatkin 71 (Bovine mastitis)	Cream	+	+	+	+	+	+	85^{6}	ACL	+	-	+	+	e
Gwatkin 96 (Bovine mastitis)	Orange	+	+	+	+	+	+	80^{2}	-	+	-	+	+	c,f,d
Jordan 9	Orange	+	+	+	+	+	+	45^{2}	-	+	-	+	+	f
Albus 43	Cream	+	+	+	+	+	+	-	AC	+	-	+	-	o
Jordan 5	Orange	+	+	+	+	+	+	50^{2}	ACL	+	-	+	+	c,f(ed)
Barrs	Cream	+	+	+	+	+	+	100^{40}	AC	+	-	+	-	o
Gwatkin 197 (Bovine mastitis)	Cream	+	-	+	+	+	+	-	-	+	-	+	-	e
Gwatkin 607 (Healthy udder)	Cream	+	+	+	+	+	+	75^{40}	AC(de)	+	-	+	+	o
24 MA	Cream	+	+	+	+	+	+	100	ACL	+	-	+	+	o
A 932	Orange	+	+	+	+	+	+	100^{65}	-	+	-	+	+	c,f
RVH 4586	Orange	+	+	+	+	+	+	100^{40}	-	+	-	+	+	o
RVH 1469 (Abscess)	Orange	+	-	+	+	+	+	-	AC(di)		-	+	+	o
Nasal pharyngeal swab - cold	Orange	+	-	+	+	+	+	100^{14}	AC		-	+	+	o
RVH (Blood culture)	Cream	+	+	+	+	+	+	100^{6}	A		-	+	+	f
RVH F2287 (Eye wash)	White	+	(+)	-	+	+	+	100^{14}	AC		-	+	-	o
RVH 3061	Orange	+	+	+	+	+	+	100^{6}	ACL		-	+	+	o
Air	White	+	-	-	+	+	+	-	A		-	+	-	o
RVH F 3141 (Susp.of Poisoning)	Orange	+	(+)	+	+	+	+	100^{14}	A		-	+	+	o
RVH 3129 (Blood culture)	Orange	+	+	+	+	+	+	100^{6}	AC(di)		-	+	-	o
RVH 156 (Abscess)	Orange	+	+	+	+	+	+	100^{14}	AC		-	+	+	c,f
UD (Food poisoning strain)	Cream	+	(+)	+	+	+	+	100^{3}	AC		-	+	+	o
MX (D. of Thompson)	White clear	-	+	-	-	+	+	-	AC	-	-	-	-	a
S 32 (C. of Thompson)	White	-	+	-	-	+	+	-	AC	+	-	-	-	b
D 1 (B. of Julianelle)	Cream	-	-	-	-	+	+	-	ACL	+	-	-	-	a,x
NTC 2558 (M.piltonensis)	Cream	-	-	+	-	-	-	-	-	-	-	-	-	o
NTC 193 (M.cereus)	Yellow	-	+	-	-	-	-	90^{27}	-	+	-	+	+	o
S 41 (D. of Cowan)	White	-	-	-	+12	+	+	-	-	+	-	-	-	d
S 34 (B. of Cowan)	White	-	-	-	-	+	-	25^{6}	-	-	-	+	+	o
B. & H. 16635	Yellow	-	+	-	-	-	-	-	B	+	(+)	+	+	o
BC (Thompson)	White	-	+	-	-	+	+	-	ACL	+	-	+	-	b,x
NTC 2654 (M.liquefaciens)	White	-	+	-	+6	+	-	50	AC	-	-	+	+	o
NTC 2677 (M.conglomeratus)	Yellow	-	-	-	-	+	+	-	AC	-	-	-	-	o
NTC 2678 (M.flavus)	Cream to yellow	-	-	-	-	-	-	100^{6}	-	-	-	-	-	o
NTC 2680 (M.luteus)	Deep lemon yellow	-	-	-	-	-	-	100^{60}	-	-	-	-	-	o
NTC 2679 (M.frendleurlichii	Cream	-	-	-	-	-	-	100^{7}	-	-	-	-	-	o
M 30 (Air)	White	-	-	+	+	+	+	-	AC	+	-	-	-	o
M 31 (Air)	White	-	+	-	-	+	+	60^{6}	ACL	+	-	+	-	a
M 13A (Air)	Cream to orange	-	-	-	+	+	+	-	AC	+	-	-	-	a
RVH 4856 (Breast abscess)	White clear	-	+	+	-	-	-	-	AC	+	-	-	-	o
Normal skin	White	-	+	-	-	+	+	-	-	+	-	-	-	a
Normal skin	White	-	+	-	-	+	+	-	-	+	-	-	-	a
Normal skin	Yellow	-	-	-	-	-	-	100^{25}	-	-	-	+	+	o

B. & H. - Breed & Hucker
CMH - Children's Memorial Hospital
RVH - Royal Victoria Hospital
NTC - National Type Collection
\+ - Positive reaction
\- - Negative reaction

AC - Acid, clot
ACL - Acid, clot & liquefaction
AC(de) - Acid, clot & decolourization
AC(di) - Acid, clot & digestion
B - Basic
25^{6} - 25% liquefaction in 6 days (example)

10 of the organisms were not tested for ability to reduce nitrates.

TABLE 2.

IMMUNE SERA AGAINST POLYSACCHARIDE EXTRACTS

Antigens	ANTISERUM							
	MX(1:3)	S32(1:8)	S13(1:17)	S34(1:3)	S41(1:16)	S80(1:8)	S33(1:4)	BC(1:8)
MX	1:128,000	0	0	0	0	0	0	1:16,000
S 32	0	1:16,000	0	1:16,000	0	0	0	1:4,000
S 13	0	0	1:32,000	1:16,000	0	1:4,000	0	0
S 34	0	0	1:4,000	1:64,000	0	0	0	1:8,000
S 41	0	0	1:16,000	1:32,000	1:64,000	0	0	1:8,000
S 80	0	0	1:128,000	1:4,000	0	1:64,000	0	0
S 33	0	0	1:32,000	1:4,000	0	0	1:32,000	1:4,000
BC	0	1:32,000	1:4,000	1:16,000	0	0	0	1:16,000

The figures indicate the highest dilution of antigen which gave a precipitate with the antisera in the concentrations stated.

T A B L E 3.

IMMUNE SERA AGAINST BACTERIAL SUSPENSIONS

Antigens	SERA							
	MX	S 32	S 13	S 41	S 34	S 80	S 33	BC
MX	1:160*	1:20	0	1:10	0	1:10	1:20	1:80
S 32	0	1:40	0	0	0	0	0	1:10
S 13	0	1:80	1:160	1:20	1:10	1:20	1:160	0
S 41	0	0	0	1:20	0	0	1:5	1:10
S 34	1:5	1:10	0	1:40	1:80	1:10	1:5	1:40
S 80	1:40	1:20	1:20	1:80	1:80	1:320	1:320	1:40
S 33	1:20	1:320	1:10	1:320	0	1:80	1:640	0
BC	0	1:80	1:5	1:20	0	1:10	1:20	1:40

* Numbers indicate highest dilution at which sera reacted with corresponding antigen.

DISCUSSION

These results are in general agreement with those of previous workers. In so far as a division of the micrococci into pathogens and saprophytes is concerned, the coagulase reaction in the study achieved perfect correlation. This was due possibly to the small number of strains examined, but nevertheless the conclusion confirms other claims that the plasma coagulation is the best index of pathogenicity. This is probably not mere coincidence, as Cruikshank (16) pointed out, but is the application in vitro of one of the factors involved in invasiveness in vivo. This contrasts with the attempted correlation of particular pigments and pathogenic activity. As Panton, Valentine and Dix (43) showed in 1937 pigment itself plays no part in pathogenicity. They discovered that the orange pigment, considered indicative of pathogenicity in staphylococci, is a harmless substance chemically similar to carotin. The coincidence of orange pigment and invasiveness is far from perfect, but the obvious tendency for the two to occur in the same organism is still striking and puzzling.

Of the other biological characters studied, the ability to ferment mannitol is in closest agreement with the coagulase reaction. Why the enzyme responsible for this fermentation, alone of all those studied, is present particularly in pathogenic strains, is another matter for conjecture. The hemolytic activities and gelatin hydrolysis are less perfectly correlated with plasma coagulating activity. It is highly probable that methods of testing for hemolysis other than by plating on blood agar would vastly improve the relationship.

The species of erythrocyte, and the temperature, the medium, and other conditions of growth, would, by analogy with other genera of microorganisms, profoundly modify the results. These factors were not investigated.

Turning now to one of the newer approaches to the study of the micrococci, the necessity for the presence of the accessory growth factors and the other essential growth requirements must be kept in mind when testing the ability of these organisms to utilize special substances. While none of the strains studied were able to utilize acetyl methyl carbinol in Tittsler's synthetic medium, most of the forms that were able to grow in the complete medium, with glucose as the carbon source, were also able to grow with acetyl methyl carbinol as carbon source, (Table 1.). It is of interest to note that two (no others were tested) of the organisms that failed to grow in the complete medium which contained thiamin and nicotinic acid as accessory growth factors did grow when biotin was added.

Antigenic analysis of these organisms by means of the precipitin test using as antigens acid extracts or the alcohol concentrate of a distilled water extract proved difficult because of the slowness with which the scanty precipitate developed, and the fact that high-titre antisera are difficult to obtain. The latter did not so seriously inconvenience the performance of agglutination tests, where the bulk of the precipitate comes from the antigen and not from the antibody.

Evidence of zoning was seen only infrequently in these precipitin reactions. No explanation is forthcoming for this unless the breadth of the reacting zone was such as to extend

beyond the 1:2000 dilution of antigen in the first tube. The figures denoting the last reacting dilution of antigen may not have much significance from the precipitin point of view, and it is probably better to regard a reaction as being positive or negative only. This would indicate cross-reaction satisfactorily.

Despite the fact that the antisera were used unabsorbed in the precipitin tests and after absorption in the agglutination experiments, the former gave a more clear-cut picture of cross-relationships (Tables 2. & 3.). In general the two techniques gave results more closely in agreement than those achieved by Cowan (14), (15) using the same two techniques over a larger collection of strains.

Finally, some correlation between antigenic structure and biochemical reactions was noted, in that all the organisms that reacted with sera rendered specific for the homologous pathogenic strain were in the coagulase positive group, whereas none of the organisms which reacted with sera made specific against the homologous non-pathogens were coagulase positive. No relation between reactions with unabsorbed sera and pathogenicity could be noted (Table 3.). This is in sharp contrast with the finding of Christie & Keogh (12), (13) in 1940 that coagulase positive organisms reacted specifically with pooled unabsorbed antisera.

This study, like others before it, has revealed a lot of interesting possibilities, but nothing which, as yet, has concrete practical value. It has emphasized again the value of the coagulase reaction and of a number of supporting tests.

CONCLUSIONS

1. The Gram-positive, mass-forming cocci can be divided into two groups by the coagulase reaction, the one group is coagulase positive and is made up of all the normally pathogenic organisms, the other is coagulase negative and is made up of those organisms that are normally non-pathogenic.
2. These groups might be considered genera, namely genus staphylococcus and genus micrococcus, as in the present classification, and production of coagulase appears a simple and definite basis for the division.
3. The division by production of coagulase can be confirmed by other characteristics such as the ability to ferment mannitol and produce hemolysin. The use of sheep blood agar for testing production of hemolysin is indicated by the high degree of correlation between this test and the production of coagulase in comparison with lysis of human blood agar.
4. In view of the information that has been published with regard to the nutritional requirements of these organisms it is very necessary to make sure that these requirements are fulfilled when testing for ability to utilize special substances. Incongruous results might be avoided if small controlled inoculums were used exclusively.
5. The described methods for separating the Gram-positive mass-forming cocci by precipitin or agglutinin reaction are unsatisfactory. The advisability of attempting to obtain a higher titred precipitating serum and, if possible, a more rapidly reading one is indicated by the

difficulties encountered using very slow sera of low titre.

6. The agglutinin reaction shows that these organisms are heterogeneous. Heterologous agglutinins are difficult or, in some cases, impossible to remove by absorption.

7. New methods for the extraction and purification of antigens for the precipitin reaction must be attempted for there is a very real need for some dependable method of typing the Gram-positive mass-forming cocci that occur in infections both for the purpose of attempted serum therapy and of tracing the source of infection.

STRAINS

Most of the strains studied were obtained from Dr. Roy's collection. These included several of the type strains of Julianelle, Thompson and Cowan. It also included named strains from the National Type Collection and several known toxin producers. Other strains were isolated from the air, normal skin and freshly isolated strains from pathological lesions were kindly provided by the clinical laboratory of the Royal Victoria Hospital (Table 1.). The reactions of forty-nine strains are recorded, eighteen strains were lost during subculture over thirty months.

SUMMARY

This study has revealed a sharp differentiation into pathogenic and saprophytic members of the micrococci on the basis of the coagulase reaction. A number of supplementary biochemical tests show fair correlation with the plasma coagulating property. Antigenic analysis gives indication of

serological types, and of agreement between some of these and the coagulase reaction. It would seem that the coagulase test is the easiest and most reliable way of separating the micrococci into two main groups.

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