HÆMOLYTIC STREPTOCOCCII.

One of the most important duties of the bacteriologist of late years has been to classify the streptococci for the guidance of the clinician.

Sir Frederick Andrewes devoted the last working years of his life to the solution of this problem. He hoped that by making use of modern refined methods of analysis he would be able to secure the identification of given streptococci with particular varieties of disease; but though he devoted seven years of arduous labour to the subject he was unable to attain his object. The results of his investigations have just been published by the Medical Research Council. The work, carried out with meticulous attention to detail, has cleared up many confusions and revealed so many pitfalls in serological studies that we think a short description of the more important studies may be useful to our readers.

Andrewes wrote that "the haemolytic streptococci stand in the forefront of bacteria harmful to mankind." They are of especial danger to the patient because they can invade the lymph-stream and spread widely from the primary focus of infection; they can also invade the blood-stream and cause metastatic infections in different organs. They are found on the mucous surfaces of the healthy body, especially in the nasopharynx, and acting as secondary invaders often cause a fatal result in such diseases as measles and influenza.

The streptococcus was first recognized by Ogston in 1881, and Rosenbach in 1884 gave the name Streptococcus pyogenes to the short-chain coccus he found in suppurative conditions. In 1903, Schottmüller established the haemolytic properties of this coccus on blood-agar plates, and many authorities have employed the term Streptococcus haemolyticus in a specific sense. Andrewes objects to the use of this name as there are other streptococci which haemolyze blood corpuscles and the haemolysis varies in degree. The Str. pyogenes has definite biological characters which enable it to be recognized.

In some septic sore throats, traced to the consumption of milk from infected cows, a streptococcus has been found differing from the Str. pyogenes in the possession of a capsule and growing as large moist colonies on agar plates. Davis gave it the name Streptococcus epidemicus. Andrewes thought it was rash to separate off a species merely by the presence of a capsule. With this exception he considered Str. pyogenes a good species in a natural history sense and as it is a typical haemolytical streptococcus he proceeded to study its haemolytic powers.

Two degrees of haemolysis are now recognized: α-haemolysis, where there is a narrow zone round the colonies, and β-haemolysis, where there is
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extensive clearing around the colonies. *Str. pyogenes* produces β-haemolysis by means of a definite toxin which destroys the limiting membrane of the erythrocytes. The production of this hemotoxin is now considered as the true test of a haemolytic streptococcus.

In order to test for haemolysis, the streptococcus is grown in peptone broth containing ten per cent. of serum for twenty-four to forty-eight hours. When good growth has occurred the tubes are centrifugalized and one drop of a dense suspension of well-washed horse erythrocytes is added to each ten c.c. tube. The tubes are incubated at 37° C. and examined after 20 minutes, 30 minutes, 60 minutes, and then hourly.

Three changes may be observed: haemolysis, reduction, and the formation of methaemoglobin. With an active strain, haemolysis is generally complete in twenty minutes, but it may take two or three days. A second trial may give complete haemolysis, when on the first trial the reaction had been unconvincing. Reduction only takes place in the presence of living cocci and may occur before haemolysis. It is part of the respiratory function of the active coccus and is not due to any product secreted into the culture medium.

The haemolytic streptococci are not so active as many non-haemolytic ones in their ability to form methaemoglobin, and with them it is rarely seen within an hour, though often well marked after four or five hours' incubation. Some of the methaemoglobin formed may be due to acid formation by the cocci.

The haemolysin of *Str. pyogenes* can be filtered off by means of a porcelain filter, but the time taken to pass depends on the permeability of the filter.

Having studied haemolysis in some detail, Andrewes then took up serological studies with his strains, all of which he considered might strictly be classed within the limits of the species *Str. pyogenes*. He had 200 strains in all. Among these, 64 were from scarlet-fever cases, 62 from surgical infections, 63 from puerperal sepsis, and 11 from erysipelas. Each of these was tested for agglutination with sera in his possession, and as regards sugar reactions and haemolytic power.

The first difficulty encountered was the preparation of a stable suspension of streptococci. Following Topley's advice, the suspensions were made from growths on solid media, first dried for an hour or two in the 37° C. incubator. Suspension stability is now considered to be a matter of electrical charge. One may picture bacteria as carrying free electrons on their surfaces, constituting a negative charge, and it is the robbery of these electrons by the kations of the electrolyte which abolishes the charge and allows the attractive forces to gain the upper hand. A stable emulsion is then one in which the negative charges are active and well developed.

Before proceeding to serum agglutination Andrewes made experiments on acid agglutination, as there seems to be a parallelism between acid agglutination and serum agglutination. Sorensen's mixture of sodium...
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acetate and acetic acid was used; it is so well buffered that the addition of a streptococcus suspension in water or saline scarcely affects the pH. The range varied from pH 3.2 to 6.8. It was found that marked agglutination ceased sharply after a pH of 4.4 or sometimes at 4.7. The limit of acid agglutination was very uniform; only three strains were found with no agglutination at all. Two of the strains had a bovine source and failed to give a strict hæmolysis and also fermented raffinose. The third strain had a human source, and in other respects was quite indistinguishable from Str. pyogenes. With this exception acid agglutination was found to give no real help in grouping the hæmolytic streptococci.

In serological studies Andrewes considered it was necessary to have a saline emulsion of streptococci which would remain stable for one and a half to two hours in the water-bath at 55° to 56° C., combined with readiness of specific agglutination with the appropriate serum. He found a growth in glucose broth which was stable, but the response of a growth on glucose agar to specific serum was often so low as to be largely useless. A legumin-agar plate made from the glucose broth culture was, however, found to yield a growth which was stable and agglutinated normally with the homologous serum. So the standard method was to employ glucose broth as the penultimate culture, and then plate from this on to legumin-agar. A convenient density of this emulsion was 300 million per cubic centimetre, and the acidity between pH 6 and 7. The perfect control tube must show no trace of granularity when examined under a hand lens. Normal serum should not be added to the control tube, as it exercises a shielding effect on the action of the electrolyte upon the particles suspended in the fluid and thus might conceal spontaneous agglutination. It is quite a common thing for a series of tubes put up to show an ordinary serum agglutination to appear negative in those containing the strongest dilution of serum, and then to show progressively increasing agglutination as the higher dilutions were reached. A control tube containing only electrolyte shows this up for what it really is—spontaneous agglutination.

In the preparation of the agglutinating serum Andrewes used intravenous injection of an emulsion containing about 3,000 million dead cocci per c.c., the dose rising in a series of twelve injections from 0.1 c.c. at the beginning of the course to 2 c.c. at the end. When proper precautions were taken he did not find the response was generally “group” in character. This might be the case when an animal was immunized with a crude strain of streptococcus without regard to the properties of the individual colony from which the immunizing emulsion was bred. By carefully testing the properties of an individual colony and making a formolized emulsion from it he found that a rabbit immunized throughout with the same emulsion “yielded a serum which reflected with fair accuracy the properties of the cocccus used.”

In agglutinin absorption work with the hæmolytic streptococci the absorbing dose was determined by absorption of a serum with its homo-
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logous coccus in graduated doses. This was found to have manifest advantages over a single dose, and was adopted in all cases.

Andrewes also tested the antigenic constitution of a strain in another way, namely, by the range of absorbing powers which it exhibited amongst a number of other sera. Some of the sera were highly specific, others "group" in character. He found that a group serum was exhausted by other group strains and by any specific strain which possessed as an antigenic component a sufficient amount of a group antigen (which most of them possessed, even so specific a strain as Type II). But a specific serum was unaffected in titre by any purely group strain and by any other specific strain. He pointed out, however, that the conclusions to be drawn from the behaviour of an absorbing strain and absorbed serum are different. A serum is not liable to vary qualitatively, but only to deteriorate quantitatively. But a living coccus is subject to internal mutation in successive subcultures, and therefore we can have no real guarantee that a present subculture is identical in its properties with a stock culture from which it is descended.

In 1922 Miss Cowan, working chiefly with hæmolytic streptococci, isolated what she called "rough" and "smooth" colonies; both were equally hæmolytic, but the smooth colonies were much more virulent than the rough colonies. In 1925 Armstrong isolated a highly virulent streptococcus which also formed rough and smooth colonies, but the rough were more virulent than the smooth. Griffith, Todd and Eagles also described colonies of different kinds formed by hæmolytic streptococci. Eagles found no difference as regards hæmolysis and toxin production between the rough and the smooth colonies, but there was no necessary correlation between virulence and colonial form.

Andrewes studied the serological differences between these different forms of colonies which appeared to have a real existence. He found that in no case was the smooth form agglutinated by the rough serum; while in the case of two strains the rough form was not agglutinated at all by the smooth serum; in another strain the rough form was agglutinated equally by its rough and smooth sera. All the smooth forms of the races studied seemed to have much in common and were agglutinated by any one of the smooth sera. This was not so with the rough forms, which showed a considerable degree of specificity. But further studies by the absorption method showed that a rough form might show group properties and a smooth form might even show greater specificity than a rough form. The smooth forms gave uniform turbidity in liquid media and emulsified well in saline; the rough forms showed the reverse properties.

Andrewes found the rough forms were more virulent. These were the forms which appeared in strains freshly isolated from morbid conditions of the body, but the saline instability rendered it difficult to prepare from freshly isolated strains suitable emulsions for agglutination—a grievous drawback to prompt serological classification.
When making his absorption experiments, Andrewes naturally expected to find the titre of the serum untouched, or reduced, or totally extinguished. In some of his experiments, however, the result of saturation was to raise the initial titre of the serum. This was very frequent with hemolytic streptococci, and occurred in no less than thirty absorption tests after saturation with heterologous streptococci.

Zones of inhibition in precipitin and agglutination are well known, and, since the work of Neisser and Friedmann, have been explained as due to disturbance of the quantitative relations of the colloidal substances concerned. The zones can be removed sometimes by washing with acetone; and also by treatment of the serum with kaolin, after a dose of 0.9 g. per cubic centimetre, an inactive serum would give good results. But quite apart from doctoring with kaolin or doping with acetone, Andrewes found that the titre of a serum for its own coccus could be raised by heavy saturation with a totally heterologous organism. There seemed to be an unspecified removal from the serum of some unknown inhibiting substance. This factor is erratic in its incidence and cannot be foretold. It seems that in an absorption test the very delicate colloidal balance is readily upset by the slightest change in any one of the ingredients.

In 1907 Kruse pointed out that one cannot deduce the identity of two allied organisms unless each is found to absorb the serum of the other. This principle of reciprocal or "mirror" absorptions is well illustrated in the hemolytic streptococci. Andrewes found that only exceptionally are two strains of streptococci serologically identical, and very rarely are they entirely dissimilar. Almost always there is more or less group antigen in common between them; there are even definite hints that more than one group antigen exists.

From his study of the racial inter-relationships of the hemolytic streptococci Andrewes confirmed the individuality of Griffith's scarlet fever Types I, II and III, but Type IV seemed to be mainly a group form. He thought that no one serological form of streptococcus could be credited with the causation of scarlet fever. No distinct types stood out from the miscellaneous assortment of puerperal, surgical, and erysipelas strains of streptococci which he studied, though occasionally representatives of Griffith's scarlet fever Types I and II were found amongst them.

At the end of the summary of his report Andrewes wrote: "A serological study by methods more accurate and quantitative than most workers have used has failed to bring order into chaos. The more one studies hemolytic streptococci the more strongly is the impression gained that they are in a constant state of flux in which it is difficult to find any firm foundation for a permanent systematic classification."
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