STUDIES ON THE STREPTOCOCCAL COMPLEMENT-FIXATION TEST

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INTRODUCTION

The classification of the streptococci is of considerable importance both for epidemiological studies and for therapeutic purposes. For long this classification has been a source of interest, and of trouble, to bacteriologists.

Biochemical and morphological criteria often allow of divisions of closely related organisms. Such characters, although frequently variable, are nevertheless valuable -- for example, biochemical differentiation of the coli-aerogenes group; biochemical and morphological differentiation of the three types of _Corynebacterium diphtheriae_. In other groups of organisms, classification by serological methods has proved to be the most useful approach to the problem as in Leptospira and Shigella. The complement-fixation test is considered by many workers to be the most delicate serological test.

The main aim of the present study is to attempt a classification of the streptococci of Lancefield's twelve serological groups (since this work was completed another Lancefield group has been defined) by means of the complement-fixation test. It is hoped that this method

of classification may be an improvement on the precipitin test, commonly in use at present, that at the same time new relationships may be brought to light, that a complete antigenic analysis may be possible, and that refractory strains may be classified.

SECTION 1

HISTORICAL REVIEW

SECTION 1

HISTORICAL REVIEW

THE STREPTOCOCCI

HISTORICAL REVIEW

The great advances made in the Victorian era in the treatment of wounds, accidental or surgical, depended on the discovery of the etiology of infection. As far back as the seventies of last century many investigators observed the occurrence of cocci in inflammatory and septic processes. These cocci differed in size and arrangement, occurring in groups, chains and pairs, but all were at first considered as variations of a single organism. Alexander Ogston (1881), an Aberdeen surgeon, exploring abscesses and other septic foci, discovered cocci in 90% of them. Using methods similar to those of Koch (1878), who had then been working on this subject for some years, he tried to show that these cocci were the actual cause of suppuration. The name Streptococci (attributed to Billroth (1874),) was adopted for the chained cocci. The name Staphylococci was coined by Ogston (1882) for those organisms occurring in groups; he was able to produce lesions with cultures of his cocci.

The modern bacteriology of streptococci may be regarded as beginning with the investigations into the cause of erysipelas. Koch (1881) described and photographed in cases of erysipelas, a chain-forming coccus. Fehleisen (1882, 1883) succeeded in growing the organism in pure culture and with pure sub-cultures induced typical erysipelas in human subjects. The name given to the organism was Streptococcus erysipelatis. Rosenbach, in 1884, found similar cocci in other types of lesion, particularly in those containing pus, and coined the name Streptococcus pyogenes, a nosologic term which has persisted.

For a long time it was thought that there was only one species of Streptococcus, but gradually it was recognized that streptococci represented a genus composed of a large number of species. Since the demonstration that streptococci are not a homogeneous group continued attempts have been made to classify them.

METHODS OF CLASSIFICATION

MORPHOLOGY.

The first efforts at classification of streptococci were based on morphology and in 1891 Lingelsheim described two types; (1) Streptococcus longus, which formed long chains and (2) Streptococcus brevis, which formed short chains. However, classification of streptococci by these

methods was soon abandoned, when it was found that morphology varied with conditions of growth; yet, certain groups and certain types of streptococci are frequently recognizable by their morphology. Thus, the faecal streptococci and Streptococcus lactis (Lancefield's group N) usually occur as diplococci, resembling the pneumococcus (Thiercelin, 1899); and group-M streptococci occur usually as long-chaining cocci.

HAEMOLYSIS.

The ability of some strains of streptococci to lyse red blood cells was first noted by Marmorek (1895). Lysis was found to be caused by a soluble haemolysin elaborated by streptococci. Later, Besredka (1901) obtained cell-free haemolysin by filtering cultures of streptococci grown in heated rabbit's serum. The haemolytic properties of streptococci may be demonstrated by two methods.

A. Whole Blood Plates.

In one of the first serious attempts at classification of the streptococci, Schottmüller (1903a, 1903b) introduced the use of the "whole" blood plate. He described (1) the Streptococcus longus pathog. sive erysipelatos, which had a marked haemolytic action, and usually occurred in long chains; (2) Streptococcus mitior sive viridans, a short-chained coccus producing small colonies, very little haemolysis, and a green discolouration of the medium, ascribed to the formation of

methaemoglobin, and (3) Streptococcus mucosus, rarer than the other two, and distinguished by moist colonies and the presence of a distinct capsule. Str. mucosus, some strains of which are now recognized to be the virulent form of type 111 pneumococcus, was replaced in Schottmüller's classification by Streptococcus anhaemolyticus (saprophyticus) (Mandelbaum, 1907-08; Zangemeister, 1910).

The subject of the action on blood of streptococci was reviewed by Smith and Brown (1915) and later by Brown (1919). Brown stressed the superiority of "shake" cultures poured into petri dishes, with the subsequent development of deep colonies, over plates which had been surface-inoculated. The term viridans, as opposed to haemolytic, was questioned by these workers, because both the so-called viridans strains and the pneumococci produced a degree of haemolysis. Consequently, Brown proposed a classification based on three main types of haemolysis in horse-blood agar. (1) The beta haemolytic type, corresponding to Schottmüller's Streptococcus erysipelatos and forming a wide clear zone of haemolysis. (2) The alpha or viridans type, corresponding to Schottmüller's Streptococcus viridans. In this type the colonies are surrounded by a zone of greenish corpuscles which show partial lysis. Outside this a narrow zone of destroyed corpuscles is also present, but lysis is not so complete as in the haemolytic variety. (3) The gamma type, which causes no haemolysis

and corresponds to Mandelbaum's Streptococcus saprophyticus (anhaemolyticus).

An intermediate type -- the alpha prime type was also described by Brown; this comprises strains intermediate between the haemolytic and the alpha type, with no greenish pigment and no decolourization. The zone of haemolysis although wide is slightly hazy, and the haemolysed zone contains a moderate number of unaltered corpuscles.

B. Tube Haemolysis.

The second method of determining haemolysis is to add a fluid culture of the test strain to a saline suspension of washed red blood corpuscles and observe the resultant effect under suitable conditions.

The factors affecting the production of streptococcal haemolysin were studied in detail by M'Leod (1912), and De Kruif and Ireland (1920). They reported that cultures grown in broth without the addition of serum elaborated little or no haemolysin. Todd (1932), using a yeast medium, and Todd and Hewitt (1932), using a medium containing glucose, sodium bicarbonate and inorganic phosphate, succeeded in obtaining large volumes of haemolysin without the incorporation of serum.

In 1932 Todd demonstrated the antigenic property of streptolysin and later (1938, 1939) showed that haemolytic streptococci from human infections produced two distinct

streptolysins -- O and S, which may be differentiated by immunological means. The O lysin, which exhibits the properties and elementary composition of a protein, is oxygen labile at ordinary temperatures, but can be reactivated by reduction with sodium sulphite. It is destroyed by heat but unaffected by acid. The S lysin, whose chemical nature is still obscure, is not apparently affected by oxidation or reduction, but is extremely sensitive to acid and is very heat labile.

In 1939 Okamoto discovered that yeast nucleic acid caused the formation of a potent haemolysin in cultures of _Str. pyogenes_. The capacity of ribonucleic acid to induce formation of streptolysin S is possessed not only by yeast nucleic acid but also by ribonucleic acid from certain other sources, such as mammalian liver, wheat and bacteria.

O lysin is formed only by strains of Lancefield's groups A, C (human) and G, and is not antigenically specific for any one. Each Lancefield group produces an S lysin which is specific for that group. An antiserum to the S lysin of any single Lancefield group will neutralise only S lysins formed by members of that group. The S lysins can also be differentiated by the action of heat, acid and oxygen (Lancefield, 1940-41). Some strains of group A produce only O and some strains only S lysins. However, most strains of group A

produce both O and S lysins (Herbert and Todd, 1944).

Parallel results are not always obtained by the use of "whole" blood plates and by adding fluid cultures to blood suspensions. Cumming (1927) showed that some strains of streptococci, although lysing the blood in "whole" plates, did not cause the haemolysis of a suspension of blood cells in saline.

Tempting as it may be to base the classification of the streptococci on the criterion of haemolysis, a comprehensive survey of the subject shows that there are reasons why such a classification is not acceptable. These reasons are reviewed briefly. Todd (1928) obtained non-haemolytic variants of beta-haemolytic streptococci by passage through mice. On anaerobic cultivation these forms reverted to beta-haemolytic strains. Fry (1933) showed that some strains which formed alpha-haemolytic colonies on blood agar when incubated aerobically produced beta-haemolytic colonies on the anaerobic cultivation of the blood plates. However, under aerobic conditions, these strains formed a soluble haemolysin. Again, by increasing the oxygen tension Isaacs (1947) changed haemolytic strains of various Lancefield groups into alpha forms; a change associated with the loss of the group-specific carbohydrate.

Stableforth (1932) showed that haemolytic and non-haemo-

lytic streptococci were frequently of the same serological type. Lancefield (1934) reported the loss of the ability of a group-B streptococcus to produce haemolysin without any antigenic change. An epidemic in children caused by haemolytic group-A strains was spread by non-haemolytic streptococci of the same serological group and type (Coburn and Pauli, 1941). Their haemolytic strains changed to non-haemolytic strains on sub-culturing and the non-haemolytic strains changed to haemolytic forms. Non-haemolytic group-A strains have been found by Colebrook and his associates (1942) and Boisvert and Fousek (1943). Penistan (1945) isolated a non-haemolytic streptococcus of Lancefield's group K and stressed the importance from the epidemiological and, possibly, the therapeutic point of view of serological classification rather than haemolytic division.

In the present study of the streptococci a group-M beta haemolytic strain produced on sub-culture both beta-haemolytic and non-haemolytic colonies. The parent strain and the variant were biochemically and serologically identical. A serum prepared with the beta-haemolytic strain precipitated and agglutinated strongly with both the parent and dissociate forms; the serum prepared with the non-haemolytic dissociate precipitated and agglutinated both forms strongly. In addition to this reciprocal behaviour, the identical nature

of the strains was confirmed by agglutinin-absorption and precipitin-absorption tests and complement-fixation tests. The parent strain and the variant remained stable. Another group-M strain changed spontaneously on sub-culture from the beta-haemolytic form to the alpha form. Among the remaining groups "certain non-hemolytic or doubtfully hemolytic strains are sometimes encountered, particularly in Groups H and K." (Lancefield, 1940 - 41).

The problem becomes even more complicated because alpha strains were also found to produce haemolysin. According to Fuller and Maxted (1939), alpha strains produce hydrogen peroxide before the haemolysin with the result that a green zone is formed. If peroxide formation is prevented by anaerobic incubation or is neutralised by catalase, then the haemolysin allowed to act unhindered, lyses the blood, and the colonies are surrounded by the typical beta-haemolytic zone.

The phenomenon of lysis of red blood corpuscles is epitomized by the following quotation:

"From all this it would follow that the appearances of streptococcal colonies on blood media depends on the interplay of a very complex group of bacterial activities, amongst which haemolysin productin, acid production, reducing activities, peroxide production and possibly tryptic and peptic digestion of the blood corpuscles are

the most important, and that the appearances can be modified at will by choosing conditions which will favour more the development of one or other of these activities, and hence the term viridans has little significance unless the conditions of growth are defined." (McLeod, 1929).

Topley and Wilson (1946) make the following tentative classification of the streptococci based on haemolytic activity:

"(1) Haemolytic streptococci.-- These produce beta-haemolysis on blood agar plates. They may be differentiated into two sub-groups: (a) those that produce a filtrable haemolysin, and (b) those that do not. Among strains of Str. pyogenes two variants are known. One produces alpha-haemolytic colonies aerobically but beta-haemolytic colonies anaerobically; it forms a soluble haemolysin in broth aerobically. The other forms completely non-haemolytic colonies, but produces a soluble haemolysin of the O type.

"(2) Streptococci giving alpha-haemolysis.

"(3) Streptococci that have no action on blood media under the usual conditions of testing."

BIOCHEMICAL.

Gordon (1905) focused attention on biochemical tests for classification of the streptococci. Subsequently in a continuation of this work Andrewes and Horder (1906a,

1906b, 1906c) studied strains of pathogenic and "normal" streptococci and divided them into six main groups, basing their classification on the clotting of milk, the reduction of neutral red, growth in gelatin at 20°C, morphology and the fermentation of seven carbohydrates (lactose, saccharose, raffinose, inulin, salicin, coniferin and mannitol).

The six groups were (1) Streptococcus equinus; (2) Streptococcus mitis; (3) Streptococcus pyogenes; (4) Streptococcus salivarius; (5) Streptococcus anginosus and (6) Streptococcus faecalis. A seventh group contained the pneumococci. Another classification based on biochemical reactions and taking into consideration also haemolysis was suggested by Holman (1916). The three substrates he used were lactose, salicin and mannitol. By this method Holman divided the streptococci into eight haemolytic and eight non-haemolytic varieties.

A number of tests advocated for the separation of different types of streptococci may well be considered here. Thus, bovine streptococci are distinguished by their ability to hydrolyse sodium hippurate (Ayers and Rupp, 1922). Animal strains are characterised by the low acidity produced in glucose broth (final pH of 4.2 - 4.8); human strains produce a final pH of 5.0 - 5.6 (Ayers, 1916; Ayers, Johnson and Davis, 1918; Avery and Cullen, 1919; Edwards, 1932). Resistance to heat

has also been used for separation of different varieties of streptococci (Ayers and Johnson, 1914; Ayers, Johnson and Davis, 1918; Orla-Jensen, 1919; Dible, 1921).

Many workers (Kinsella and Swift, 1917; Blake, 1917; Howell, 1918; Hamilton and Havens, 1919; Gordon, 1921; McLachlan, 1927) have shown that fermentative types agree neither with serological classifications nor with the distribution of the types according to disease. Furthermore, as in the case of haemolysis, the fermentative properties of strains exhibit considerable variation (Ritchie, 1908; Walker, 1912 - 13). It is now generally appreciated that for an exact study of the streptococci serological methods should be used. Biochemical tests and haemolysis still are of distinct value, not for the differentiation of streptococci as a whole, but within certain groups (Keogh and Simmons, 1940; Evans, 1936a, 1936b, 1936c, 1937, 1940; Evans and Verder, 1938). For epidemiological study, and for differential association of streptococci with disease conclusive results cannot be obtained without serological methods. "It is a fact that if strains of more than one Lancefield group are classified together on the basis of fermentative properties, the classification is meaningless because those properties rank lower than precipitin reactions in the natural grouping of streptococci." (Evans, 1941).

"When a test is found of value in studying certain types of streptococci it is proper to apply it to all others -- but not at the expense of other reactions which may, in spite of their apparent futility, have special functions when applied to new groups. Trehalose, for example, has outstanding value in its application to the streptococci belonging to the Lancefield group C; but with the streptococci as a whole, exclusive of group C, it is not apparent that its usefulness is greater than that of maltose or sucrose." (Sherman, 1937).

SEROLOGICAL.

Agglutination.

In 1919, Dochez, Avery and Lancefield, using the agglutination reaction and mouse-protection tests, showed that the haemolytic streptococci could be divided into four immunological types and that there were probably at least two others. Similar results were obtained by Havens (1919) and Hamilton and Havens (1919). Further attempts at classification of the haemolytic streptococci using the agglutination reaction were carried out both in this country and America (Bliss, 1920, 1922; Gordon, 1921; Eagles, 1924; Stevens and Dochez, 1924a, 1924b, 1926a, 1926b; Griffith, 1926, 1927; Andrewes and Christie, 1932). This work culminated in the demonstration (Griffith, 1934) that the haemolytic streptococci of

group A (see below) could, by the agglutination test, be divided into twenty-seven serological types. Later Griffith found three other types, and since then new types have been added continually until at present group A alone has been separated into over forty. The agglutination test is beset with technical difficulties due to the autoagglutinability of many strains, the difficulty in preparing suitable antisera and the great extent to which cross-agglutination occurs.

Precipitation.

Lancefield, in a series of studies on the antigenic structure of the streptococci (1925a, 1925b, 1928a, 1928b, 1928c, 1928d, 1928e), applied the precipitin test as a means of classification of haemolytic streptococci. Hot-acid extraction of bacterial cells was the method of preparing the antigen. In 1933 the haemolytic streptococci were divided into five serological groups, each group being distinguished by the presence of a specific soluble substance (polysaccharide), common to all members of any one group, but distinct for each group. Group A was composed of strains from human infections; group-B strains were from bovine and dairy sources; group-C strains from a variety of lower animals; group D from cheese and group E from "certified" milk. The serological division agreed with the distribution of the organisms in nature and also

with their biochemical and cultural characteristics. Cross-precipitation between the groups was negligible. Since 1933 other serological groups have been added -- groups F and G by Lancefield and Hare (1935); groups H and K by Hare (1935); groups L and M by Fry (1941); group O by Boissard and Wormald (1950). The serological entity of Streptococcus lactis was shown independently by Shattock and Mattick (1943 - 44), Sherman et al. (1940) and Seeleman and Nottbohm (1941). Seeleman and Nottbohm assigned the letter L to this group as they were not aware of its appropriation by Fry but because of Fry's work, Shattock and Mattick eventually designated it N.

Although Lancefield's paper (1933) dealt with the serological grouping of the haemolytic streptococci, it must be stressed that included in these groups are strains which are non-haemolytic. Group B is composed mainly of alpha-haemolytic strains. The possession of the group carbohydrate and not the ability to lyse a red-cell suspension is the criterion of this classification.

Using both the precipitin and agglutination reactions, type differentiation within the groups has been made. Two type antigens have been found in group A -- the M and T antigens (Lancefield, 1940). Classification into serological types is based upon the M antigens, irrespective of T antigens,

which often give rise to cross-reactions. Precipitin-test results with the M antigen parallel agglutination results (Krumwiede, 1943).

Complement Fixation.

Earlier workers attempted to classify both the haemolytic and the non-haemolytic streptococci by means of the complement-fixation test. Howell (1918), using bacterial suspensions as antigens, obtained marked cross-fixation between the haemolytic and non-haemolytic streptococci, and concluded that streptococci could not be classified on the basis of the complement-fixation test. Nakamura (1922 - 23) also held this view and stated that the results obtained were dependent om the medium used rather than upon the organisms.

Kinsella and Swift (1917), using an antiformin extract of cells as antigen, found that the non-haemolytic streptococci were not homogeneous. They suggested that this was due to the presence of two separate elements; results of the complement-fixation test depended on these two elements. Some strains possess one of the elements -- the "right hand side", and others possess the "left hand side" element. There was also a mid-zone class of strains showing a relationship to both the left and right-hand groups, and having, it is suggested, both elements. Preparing a similar extract from haemolytic streptococci, Kinsella and Swift (1918) showed, by means of the complement-fixation test, that the haemolytic

streptococci represented a homogeneous group of organisms and that they were related to the left-hand group of the non-haemolytic streptococci was shown by Kinsella (1918). In 1925, Hitchcock, using as the extracting agent 4.0% antiformin, as opposed to the 2.0% antiformin used by Kinsella and Swift, confirmed the latter's findings. Previously, Hitchcock (1924b) using 0.8% antiformin had extracted the non-haemolytic streptococci and found them to be a heterogeneous group.

THE COMPLEMENT-FIXATION TEST AND ITS RELATIONSHIP TO THE PRECIPITIN TEST

In 1901 Bordet and Gengou showed that complement was adsorbed by a mixture of bacteria and their homologous antiserum. This adsorption of complement could be observed without the lysis of the bacteria themselves, but by the failure of added sensitized cells to undergo haemolysis. This test constituted the complement-fixation reaction as known today. Widal and LeSourd (1901) confirmed this result and showed that the serum of typhoid patients would bind complement in the presence of a suspension of *Salmonella typhi*. In 1902 Gengou showed that organized material was unnecessary and that soluble proteins with their homologous antisera adsorbed complement. It was

pointed out that the results, which until then had been attributed to the action of anti-complement, were in reality due to the fixation of complement by a mixture of antiserum and its homologous antigen (Moreschi, 1905). In subsequent work Moreschi (1906) and Pfeiffer and Moreschi (1906) advanced the view that complement was taken up by the precipitate formed by the union of antigen and antibody. Moreschi concluded that the precipitates formed had an anti-complementary action. About the same time and independently of Moreschi, Gay (1905a, 1905b) put forward the same explanation and Klein (1905) found the fixation of complement closely related to the formation of a precipitate.

The complement-fixation test, of all serological tests, is probably the most delicate. "The delicacy of the precipitin reaction is great and only exceeded, in certain respects, by complement fixation and the anaphylaxis reaction." (Karsner and Ecker, 1921). With precipitating antisera, complement fixation occurs with much smaller amounts of antigen than is needed for precipitation. Muir and Martin (1906) and Neisser and Sachs (1905, 1906) demonstrated that whereas complement fixation occurred with dilutions of antibody of 10^{-6}, no visible precipitation was obtained; further, the specificity of the antiserum is retained, even

with such dilutions as this. However, the formation of a precipitate does not of necessity imply that complement will be fixed (*vide infra*). Zinsser and Parker (1923a) could not obtain fixation of complement with pneumococcus capsular polysaccharide and specific immune horse serum, although precipitation occurred; but complement was bound when the same antigen was combined with immune rabbit serum. Similar results were observed by Pittman and Goodner for the specific polysaccharide of *Haemophilus influenzae* (1935).

Bordet and Gengou (1903) and Wassermann and Bruck (1906) unsuccessfully attempted the diagnosis of tuberculosis by means of the complement-fixation test, but abandoned the work, and later Wassermann turned his attention to the now classical test for syphilis (Wassermann, Neisser and Bruck, 1906).

Since its introduction the complement-fixation test has found application in numerous fields. Bacterial infections of animals and man have been diagnosed by its use -- gonorrhea, glanders, contagious abortion, bacillary dysentery, whooping cough and white diarrhoea of chicks. Parasitic infections such as malaria, trypanosomiasis and echinococcus disease have been diagnosed using the complement-fixation test, and more recently it has been applied to viral and rickettsial infections. It is particularly applicable in the viral and

rickettsial infections because the antigens are relatively simple to prepare, whereas neutralisation tests are difficult to execute (small pox, rabies, yellow fever, and typhus). The complement-fixation test has also been used in the serological classification of bacteria, as in the case of *Clostridium botulinum*, staphylococci, *Salmonella typhi* and *Corynebacterium diphtheriae* and, as previously mentioned, streptococci.

SECTION 11 -

ANTISERA

ANTISERA

The strains of streptococci used in this study were all of known serological identity.* The original cultures were freeze-dried and from them stock cultures were maintained in Robertson's meat medium. This medium was excellent for maintaining the viability of the organisms. All strains used maintained their viability in this medium for over two years, with the exception of group-M strains which had to be sub-cultured frequently.

PREPARATION OF ANTISERA

VACCINE PREPARATION.

A. Whole Cells.

Most of the antisera were prepared according to the method recommended by Lancefield (1933). A 24-hour lab. lemco broth culture was centrifuged and the supernatant removed. The deposit was suspended in 0.85% saline and after thorough mixing

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* The author thanks Dr. N. Laughton, Birmingham University and Dr. P. M. F. Shattock, Reading University for the streptococcal cultures of known serological identity.

the suspension was again centrifuged; to the deposit now obtained, 1/20th the original volume of 0.2% formal-saline was added. This suspension was left in the ice-chest (1 - 4°C) for 2 or 3 days and then tested for sterility. If sterile the opacity was adjusted by the addition of 0.2% formal-saline to that of tube 7 on Brown's opacity scale.*

B. Extracts.

1. Difficulty was encountered in preparing antisera against strains of Lancefield's groups D and N. Members of these groups are notoriously difficult in this respect (Seelemann and Nottbohm, 1941; Shattock and Mattick, 1943 - 44). Little of the necessary group antigen is at the surface of the streptococcus. An attempt was therefore made to expose the antigen, without its significant alteration, by using a modification of the method of Shattock and Mattick. The centrifuged deposit of a broth culture was washed in saline and extracted with acetone. The extracted organisms were dried *in vacuo* over calcium chloride and thoroughly ground with a mortar and pestle. 0.2% formal-saline was added and the opacity of the suspension adjusted to that of Brown's opacity tube no. 7,

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* The vaccines used in the early part of the work were prepared from an 18-hour 1.0% glucose-broth and not from a 24-hour lab. lemco broth culture. The latter medium was adopted later as it was thought that the acidity produced in glucose broth might be harmful to the antigenic structure of the vaccine. Results obtained, however, did not support this hypothesis.

as for the other groups. This method of preparation proved successful for groups D and N.

2. Extracts of the bacterial cell prepared by treatment with antiformin (pp. 39-41) were also used as vaccines, in the same manner as the whole-broth cultures.

IMMUNIZATION.

Rabbits were immunized according to the following schedule:

First period:

Day 1.	0.5 ml.	vaccine	inoculated	intravenously.
Day 2.	0.75 ml.	"	"	"
Day 3.	1.0 ml.	"	"	"
Day 4.	1.0 ml.	"	"	"
Day 5.	1.0 ml.	"	"	"

Rest one or more weeks.

Second period:

Day 1.	1.0 ml.	vaccine	inoculated	intravenously.
Day 2.	1.0 ml.	"	"	"
Day 3.	1.0 ml.	"	"	"
Day 4.	1.0 ml.	"	"	"
Day 5.	1.0 ml.	"	"	"

The first test-bleeding was done 5 - 10 days after the last inoculation. The sera were tested for precipitins and if these were present further bleedings were carried out. If precipitins were absent the animals were again inoculated with 1.0 ml. of vaccine on each of five successive days, followed 10 days later by a test-bleeding. The time elapsing between each weekly series of inoculations was found to be of no significance. Animals were bled from the ear or by heart

puncture; the blood was centrifuged and the serum removed and stored in the ice-chest without the addition of preservative.

A serum was considered to be a satisfactory group serum, and consequently suitable for use only if it gave a positive precipitin reaction, not only with the extract of the strain used to prepare it ("homologous extract" in the narrowest sense) but with extracts of other strains of the same Lancefield group. Such sera were obtained for each Lancefield group except group M. In attempting to prepare group-M sera, although as many as five weekly series of inoculations were given, a satisfactory group serum, which would react with extracts of all the group-M strains available (seven), was not obtained. Two "group"-M sera were prepared against different strains and extracts of all the group-M strains gave a positive reaction with one or other of these sera obtained after three weekly series of inoculations. Laughton (1948) recorded a similar finding with group-M strains.

TITRATION OF ANTISERA BY THE PRECIPITIN TEST

Precipitin tests were carried out by Lancefield's method. Several drops of antiserum were placed in a Dreyer's tube and the same volume of undiluted antigen was layered carefully on top. A precipitate visible as a white ring at the interface of antigen and antiserum denoted a positive precipitin test.

At the end of 30 minutes the tube was shaken and placed for 2 hours in the 37°C incubator. The mixture was then again examined for formation of a precipitate; placed in the ice-chest overnight, and re-examined the next morning.

As agreement was always found between the results of the ring test and those obtained after 2 hours' incubation at 37°C and overnight in the ice-chest, the latter tests were discarded and only the ring test was used.

It is well known that in the precipitin test excess of antigen may cause solution of the precipitate. Consequently, if the test described above was negative, it was repeated with the antigen diluted 1/4 and 1/16. If, on the other hand, the result with the undiluted reagents was positive, the antiserum was diluted 1/4 and 1/16 in order to determine an end-point.

TITRATION OF ANTISERA BY THE AGGLUTINATION TEST

As mentioned previously difficulties are often encountered when an agglutination test is attempted with streptococci. Three modifications of the ordinary agglutination technique were used in carrying out the agglutination tests. (1) Cultures were grown at 30°C for 24 hours. Stevens, quoted by Shibley (1924), reports that growth of streptococci at room temperatures minimises granularity. The temperature of 30°C was used in preference to the room temperature of 15 - 18°C

because at room temperature growth was unreliable. In order to obtain more stable suspensions (2) low concentrations of electrolytes were used in putting up the agglutination tests: 0.4% NaCl instead of the customary 0.85% NaCl. (3) N/250 NaOH was used as diluent for the suspension (De Kruif and Northrop, 1923). The actual procedure was as follows: The organism to be tested was seeded into lab. lemco broth and incubated at 30°C for 24 hours, the culture centrifuged and the supernatant discarded. N/250 NaOH was added gently without undue disturbance of the deposit. The fairly thin suspension obtained constituted the antigen. The test was then put up with 0.4% NaCl to give a final antiserum dilution of 1/25; 1/50; 1/125 and 1/250. If necessary the dilutions were also used in powers of 10^1 and 10^2. The results were read after incubation in the water bath at 56°C for 16 hours.

SECTION 111

ANTIGENS

ANTIGENS

INTRODUCTION

In the preparation of a suitable antigen for use in a bacterial complement-fixation test, four main factors must be considered. (1) the potency of the antigen; (2) the specificity of the antigen; (3) the anticomplementary effect which the antigen may have; (4) the ease with which the antigen is made. [illegible] the stability of the antigen; but this is of account only when [illegible] and the antigen remains [illegible]. [illegible] in the [illegible] test and the complement-fixation test for gonorrhea, the antigen is a known constant and the sera vary. However in grouping bacteria by the complement-fixation test, there may be more than one serum, but [illegible] sera are constant and it is the antigens which are variable.

HISTORY

The [illegible]

ANTIGENS

INTRODUCTION

In the preparation of a suitable antigen for use in a bacterial complement-fixation test, four main factors must be considered. (1) The potency of the antigen; (2) the specificity of the antigen; (3) any anti-complementary effect which the antigen may have; and (4) the ease with which the antigen is made. A fifth factor is the stability of the antigen; but this is of account only when sera vary and the antigen remains the same. Thus, in the Wassermann test and the complement-fixation test for gonorrhea, the antigen is a known constant and the sera vary. However in grouping bacteria by the complement-fixation test, there may be more than one serum, but these sera are constant and it is the antigens which are variable.

HISTORY

The earlest antigens used in bacterial complement-

fixation tests were merely suspensions of the bacterial cells, either in the original cultural medium or in saline. Thus Howell (1918) used cultures grown for 18 - 24 hours in dextrose-ascitic broth as streptococcal antigens in the complement-fixation test. The bacteria were removed by centrifugation, washed twice, suspended in saline and heated at 56°C for 30 minutes. With this antigen, cross-fixation occurred between the haemolytic and the non-haemolytic streptococci. Howell concluded that: "No inverse ratio was observed between the fixative power of an antigen and its antiserum. The positive fixations could not be grouped in any way that would justify a classification of streptococci based on the complement-fixation test." Later Howell and Anderson (1920) carried out complement-fixation tests with anti-meningococcal, anti-streptococcal, anti-pneumococcal and anti-typhoid sera, using eight different kinds of antigens, and claimed that saline suspensions were as successful as any other of their antigens. Gunnison and Schoenholz (1927) found that, in the serological classification of Clostridium botulinum by the complement-fixation test, a simple saline suspension was as satisfactory an antigen as the other three kinds of antigens they tried. However, later workers favoured the use of more complex antigens, such as autolyzed material, soluble extracts, or the residue after extraction.

The use of bacterial extracts as antigens commenced with the discovery by Pick (1912) that in young typhoid cultures there was a substance which did not give the ordinary protein reactions, was resistant to heat and proteolytic enzymes, and was soluble in alcohol. This substance gave specific precipitin reactions with immune serum, but it did not incite antibody production when used as an immunizing agent. Dochez and Avery (1917) found in culture filtrates of pneumococci a non-protein substance which reacted specifically with the homologous bacterial immune serum. This they called the soluble specific substance. It was also found in the blood and urine of animals inoculated with pneumococci and in the blood and urine of human beings suffering from pneumonia. This soluble specific substance resisted the action of heat and of proteolytic enzymes. It was precipitated from solution by alcohol.

A similar substance was found by Zinsser and Parker (1923b), continuing the work of Zinsser (1921), in influenza bacilli, pneumococci, staphylococci, tubercle bacilli and typhoid bacilli. This substance was practically free from protein, was thermostable and was precipitated by alcohol. It gave a specific precipitin reaction with the homologous bacterial antiserum, but on injection into animals did not produce any antibody response.

This substance, designated the _residue material_, was claimed by Zinsser and Parker (1923b) to be the same as the soluble specific carbohydrate of Dochez and Avery and as Pick's non-protein substance. Zinsser and Parker suggested that the term _soluble specific substance_ was more accurately descriptive than _residue antigen_ and should be used. In Landsteiner's terminology it was a hapten, not inciting antibody formation when inoculated into animals but precipitating with the homologous antiserum _in vitro_.

Numerous organisms have since been investigated for the presence of a non-protein residue antigen. All have been found to possess one. Mueller and Tomcsik (1924), modifying the method of Zinsser, obtained a complex carbohydrate in yeast; Laidlaw and Dudley (1925) found in tubercle bacilli a residue material which although failing to stimulate antibodies, gave specific precipitation in a dilution of one in 6.4 millions with tubercle antiserum; Furth and Landsteiner (1928) in a study of the antigenic structure of organisms of the typhoid group demonstrated the presence in _Salmonella typhi_, _Salmonella paratyphi_, _Salmonella enteritidis_ and _Proteus vulgaris_ of a substance almost free from protein, rich in carbohydrate and yielding reducing sugars on hydrolysis; these substances, although incapable of inciting antibody formation, reacted specifically with the homologous antisera. Landsteiner and

Levine (1927) had found a similar substance in Vibrio cholerae. Finally Hitchcock (1924a, 1924b), Zinsser and Tamiya (1925) and Lancefield (1928a, 1933) obtained the soluble specific substance or residue antigen in both haemolytic and non-haemolytic streptococci.

The ease with which this residue substance was obtained differed according to the species of organism. Thus, in the pneumococci, the residue substance was found in culture filtrates; in meningococci it was obtained by washing the bacterial surface with neutral isotonic solvents, and shaking. In other organisms such as streptococci, ground or unground bacterial sediments might have to be treated with antiformin.

Zinsser and his associates claimed that the residue material was precipitable and gave a complement-fixation reaction with the homologous antiserum. However, they did not carry out complement-fixation tests with the residue antigens prepared from streptococci. Further, fixation was not obtained with the pneumococcus residue and pneumococcus horse or bovine antiserum, although the pneumococcus antigen was precipitated by both these antisera.

"In closing we would again call attention to the fact that the residue antigens produced as described, both with pneumococci and a great many other bacteria, constitute the most easily managed and convenient antigens for specific

precipitations and complement fixations . . . . The resultant water-clear fluid precipitates powerfully, and possesses none of the disturbing features for complement fixations possessed by bacterial suspensions or extracts." (Zinsser and Parker, 1923a).

Classification of the streptococci, haemolytic and non-haemolytic, by the complement-fixation test, using bacterial extracts as antigens, was attempted by Kinsella and Swift (1917, 1918). It would appear that their antigen, prepared as it was by extracting ground dried bacterial sediment with antiformin and precipitating the protein with acid, was the residue substance of Zinsser.

Later, Howell and Anderson (1920), in studying the suitability of streptococcal antigens for bacterial complement-fixation tests prepared an antigen similar to that of Kinsella and Swift. This antigen, in comparison with seven other antigens tested, was found to be one of the most efficacious, although giving cross-fixation with heterologous antiserum.

Hitchcock in his studies on the non-haemolytic and haemolytic streptococci used as antigen desiccated ground material extracted with slightly weaker (0.8%) antiformin than that used by Kinsella and Swift (2.0%). He also obtained fixation of complement with anti-streptococcal sera. Hitchcock's antigen clearly contained the residue material of Zinsser. In my experiments the antigens used by Kinsella

and Hitchcock caused lysis of red blood corpuscles and it should be noted that although both these workers claimed successful results they used only 2 M.H.D. of complement.

In grouping streptococci by the precipitin test, Lancefield (1933) used for antigens the residue material prepared according to the method of Porges. In this, the bacterial cells are boiled with weak acid (N/20 HCl). The antigen obtained is group-specific and Lancefield claimed that it was identical with the antigen prepared by Hitchcock; although it will be shown in this thesis (pages 150 - 152) that this may not be the case. Further, Lancefield stated that the antigen could be obtained in any quantity only by disruption of the bacterial cell, as the substance was within the cell body. In Lancefield's work rapid continued freezing and thawing of streptococcus cultures and other physical methods did not prove sufficiently potent to release the group antigen, confirming that it is difficult to bring about autolysis of streptococci.

In 1938, Fuller described the preparation of an extract with formamide which was suitable as a precipitinogen for the Lancefield grouping of streptococci with appropriate antisera. This extract was claimed to be more specific than that used by Lancefield, by virtue of the elimination of non-group-specific substances, which are mainly protein in nature. The formamide

used in preparing this antigen destroys the protein and so eliminates the cross-reactions. The value of this method of preparing the grouping antigen is somewhat lessened by its being not suitable for all streptococci, *e.g.*, with group-M strains the high temperatures used destroy the group antigen.

More recently Maxted (1948) has isolated a substance, presumably an enzyme, from *Streptomyces albus*, which causes the lysis of streptococci. These lysates contain the streptococcal C substance and can be used as antigens in the streptococcal precipitin test. Maxted's results were confirmed by Wannamaker *et al.* (1950).

CELLULAR ANTIGENS.

1. SALINE SUSPENSIONS.

In the present work, the first medium used in the production of a cellular antigen was a 1.0% glucose broth of the following composition:

Laboratory lemco	10 gm.
Peptone	10 "
NaCl	5 "
Glucose	10 "
Distilled water to one litre.	

pH = 7.4

The use of a medium containing glucose was favoured because many streptococci are fastidious organisms, difficult to grow, and the presence of glucose in the medium enhances

their growth. "The praises of glucose, as an ingredient in media for growing haemolytic streptococci, have been sung by many workers, and we ourselves would join in the chorus." (Andrewes and Christie, 1932).

At the end of 18 hours' incubation at 37°C the culture was heated at 56°C for one hour. This heating was not to autolyse, but to kill the organisms. The heated culture was centrifuged and the supernatant removed. The deposit was suspended in 0.85% saline and made up to the original volume. This saline suspension constituted the antigen -- S1.

As it was thought that heating the broth culture at 56°C for one hour might cause destruction or alteration of the antigenic properties of the culture, treatment with formalin was used instead of heat. The 18-hour broth culture was centrifuged; the supernatant removed; the deposit suspended in 0.85% saline to the original volume; 0.5% formalin added and the suspension left in the ice-chest until dead (2 - 3 days). This constituted antigen S2.

Growth of the streptococci in 1.0% glucose broth of initial pH 7.4 results in a pH of 4.2 - 5.6 after 18 hours' incubation at 37°C. The former figure is attained by the group-B strains and the latter by group-A strains. To rule out the possibility that the high acidity produced in the glucose broth might cause an alteration in the antigenic

structure of the streptococci, with subsequent failure of the organisms and homologous antiserum to fix complement, a third antigen was prepared.

The strains were grown in the same medium as previously, but without the glucose. In this medium the pH remained constant at 7.4 after 24 hours. The 24-hour culture was heated at 56°C for one hour; centrifuged; the supernatant removed and the deposit suspended in 0.85% saline, to the original volume (antigen S3).

WHOLE-BROTH CULTURES

The first of these antigens was an 18-hour 1.0% glucose-broth culture. The acidity produced in this medium could, of itself, cause lysis of red cells, and before the cultures could be used as antigens they were neutralised by the addition of 0.5 ml. N/3 NaOH per 10.0 ml. of broth. This, both in groups A and B, gave an antigen which was satisfactory, in that by itself it did not cause lysis of red cells. After neutralisation, the culture was heated at 56°C for one hour. This antigen was designated B1, or glucose-broth antigen.

Other broth antigens were prepared by seeding the test strains in (1) ordinary lab. lemco broth; (2) horse-heart digest broth and (3) horse-heart extract broth, and incubating at 37°C for 24 hours. In these media the pH remained constant at 7.4. The cultures were then heated at 56°C for one hour and

constituted respectively antigen B2 or ordinary broth antigen; B3 or horse-heart digest antigen and B4 or horse-heart extract antigen.

Two more types of broth antigens were prepared in order to determine whether (a) glucose was essential for the production of a suitable antigen and whether (b) acidity was necessary or harmful to the production of a suitable antigen. These antigens were prepared by seeding the test strain into six tubes of a medium consisting of salt, peptone and lab. lemco made up in Sorensen's buffer salts. Three of the tubes contained in addition 1.0% glucose (antigen Sg). The tubes without glucose were designated antigen S. In both these sets of media the pH was not altered by the growth of the streptococci and was pH 6.0 in one pair; 7.2 in another pair, and 8.0 in the third pair. After 24 hours' incubation the cultures were heated at 56°C for one hour.

SOLUBLE EXTRACT ANTIGENS

Hitchcock's (1924) antigen.

A 24-hour 1.0% glucose-broth culture was centrifuged and the deposit suspended in 0.85% saline; the saline suspension centrifuged and the supernatant discarded. The packed organisms were desiccated *in vacuo* over calcium chloride. The dry residue was then thoroughly ground up with pestle and

mortar and accurately weighed to the nearest milligram. 0.5 ml. of 0.8% antiformin was then added per mg. of dried organisms. The antiformin suspension was placed in the water bath at 56°C for 10 minutes and shaken vigorously two or three times during the course of extraction. Three drops of 5.0% sodium thiosulphate were then added to the suspension for every 1.0 ml. of diluted antiformin, in order to neutralise the HOCl portion of the antiformin. The suspension was then rapidly cooled to room temperature and to neutralise the NaOH of the antiformin, N/5 H_2SO_4 was added slowly, with constant agitation, until a heavy flocculent precipitate appeared. This precipitate usually occurred at neutrality but if the reaction was still alkaline further acid was added drop by drop until a faint acid reaction appeared. The precipitate was then removed by centrifugation. The clear supernatant fluid was neutralised with a drop or two of N/5 NaOH and saline containing 0.5% phenol was then added, in such volume that one mg. of dried bacterial cells was represented by 10.0 ml. of the final solution. This method was strictly adhered to throughout, and in this way the solutions used were quantitatively comparable. The antigen obtained in this manner was found to be hypotonic and in order to make it isotonic for the haemolytic system used in the complement-fixation test, 0.75 ml. of 12.5% NaCl was added per 10.0 ml. of antigen.

Hitchcock stressed that different lots of antiformin have a different available chlorine content, and that the preparation of a satisfactory extract is more dependent upon this factor than on alkalinity (see pp.173 -175). It was therefore essential to titrate each new batch of antiformin and, because antiformin loses its potency on standing, to titrate at regular intervals any stock antiformin solution. The method used for this titration was that advised by Hitchcock* (1924a).

Fuller's (1938) antigen.

The test strain was seeded into 1.0% glucose broth and incubated for 18 hours at 37°C, at the end of which time the culture was centrifuged and the supernatant removed. 0.1 ml. of formamide was added in place of every 5.0 ml. of the original culture medium. The tube was shaken, the suspension placed in an oil bath, and the temperature raised to 150°C and held for 15 minutes. The tube was cooled gradually and 0.25 ml. of acid-alcohol added. The acid-alcohol was a mixture of 95 parts of anhydrous alcohol and 5 parts of 2N hydrochloric acid. The precipitate which formed was removed by centrifuging;

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* 10.0 ml. of diluted antiformin were measured into a small flask and to this were added approximately 0.25 mg. of solid KI and 1.0 ml. of concentrated acetic acid. The liberated iodine was titrated with N/10 sodium thiosulphate with a weak solution of starch as indicator. If the available chlorine was found to be approximately equivalent to 1.7 ml. of the standard thiosulphate the dilution of antiformin was considered satisfactory for use. If however, the available chlorine was equivalent to a smaller volume of thiosulphate a correspondingly stronger antiformin solution was used.

0.5 ml. of acetone was added to the supernatant. A further precipitate formed which was also removed by centrifugation. The supernatant was discarded and 1.0 ml. of saline and a drop of phenol-red indicator were added to the precipitate, which contains nearly all the group antigen, and the whole was neutralised with a drop of sodium carbonate. The antigen was made isotonic.

Lancefield's (1928a) antigen.

The strain to be tested was seeded into 1.0% glucose broth and incubated at 37°C for 18 hours. The culture was then centrifuged at 3,000 r.p.m. for 15 minutes and the supernatant fluid discarded. 1.0 ml. of N/20 HCl was added in place of each 10.0 ml. of broth and the resultant suspension thoroughly mixed. The suspension was then boiled for 10 minutes with frequent shaking. After cooling, neutralisation was effected by the addition of N/3 NaOH drop by drop until the neutral point was reached, at which point precipitation took place. The precipitate was removed by centrifuging the suspension for 10 minutes at 3,000 r.p.m. The clear supernatant contains the group antigen. This was preserved by the addition of 0.5% phenol-saline -- 0.5 ml. per 10.0 ml. of antigen. The antigen was made isotonic.

Purified antigen prepared by Lancefield's method.

According to Lancefield, the crude HCl streptococcal

extract contains a number of antigenic substances. The nucleoprotein (P), which is species specific, and the type-specific protein (M) are both present in addition to the group carbohydrate (C). In group-B strains the type-specific antigen is a carbohydrate (S) and is also present in the HCl extract. Partial removal of these antigens can be effected by precipitation with alcohol and I therefore proceeded as follows:

One volume of the crude Lancefield antigen prepared by Porges' method was treated with three volumes of absolute alcohol and left in the ice-chest overnight. The precipitate was removed by centrifugation and the supernatant evaporated to dryness on a sand-bath. The dry residue was resuspended in 0.85% saline and made up to the original volume.

Purification of the Lancefield antigen was also attempted by digestion of the extract with proteolytic enzymes as follows:

A 1.0% glucose solution of trypsin was prepared in Sorensen's buffer of pH 8.0. This was sterilized by filtration through a Chamberland L5 filter. 50.0 ml. of an 18-hour 1.0% glucose-broth culture was centrifuged. After removal of the supernatant fluid 1.0 ml. of saline and 1.1 ml. of the trypsin solution were added. The suspension was then placed in the incubator at 37°C for 2 hours. The action of the enzyme was terminated by boiling for 10 minutes. The suspension, to which 5.0 ml. of N/20 HCl were added was then boiled for a further 10 minutes. The procedure was continued as for the normal preparation of the Lancefield antigen.

THE COMPLEMENT-FIXATION TEST

Guinea-pig complement was used throughout the work. Most of this was obtained commercially and was dried material (Lyovac complement). Some was obtained in the laboratory and was either (a) dried; (b) frozen or (c) preserved with salts (Green, 1938). The most constant results were obtained with Lyovac complement. The immune body and the sheep cells were commercial preparations (Burroughs, Wellcome Ltd.). The final cell concentration was 3.0%. The antisera used in the complement-fixation tests were always heated at 56°C for 20 minutes to destroy any naturally occurring complement.

Complement was always titrated prior to use. It was diluted one in four and 0.01 ml., 0.02 ml., 0.03 ml. and 0.04 ml. were added to four tubes containing 0.5 ml. of sensitized cells and 0.5 ml. of saline. A control tube containing 0.5 ml. of sensitized cells and 0.5 ml. of saline, but no complement, was included. The test was incubated at 37°C for one hour at the end of which time the M.H.D. of complement was determined.

In carrying out all complement-fixation tests the following method was used:

The unit of antigen, antibody, saline and sensitized cells was 0.25 ml. These were constant and it was the volume of complement which varied. In the tests, one unit of antigen was placed in each tube, followed by one unit of antiserum and immediately after, the appropriate complement dose. The doses used were 2, 4, 6 and 9 M.H.D. of complement. If 9 M.H.D. of complement were fixed, the volumes of complement were increased and 12, 15, 20, 25 and 30 M.H.D. were used.

Complement-fixation tests were initially carried out at 37°C in the orthodox manner. However, as will be seen from the following chapters, the results were discouraging. The thought occurred to me to apply fixation at 1 - 4°C, as suggested by Jacobstahl (1910) in the Wassermann test, to the complement-fixation test for streptococci. As will be seen subsequently, this gave unexpectedly good results and proved the method of selection and indeed made the investigation possible.

In the standard method (37°C incubation) the reagents were brought to room temperature before being used. The antigen-antibody-complement mixture was incubated at 37°C for 1-1/4 hours and one unit of sensitized cells was then added to each tube. A further period of one hour's incubation at 37°C followed. The tubes were read immediately on removal from the incubator and again after being placed overnight in

the ice-chest. One or two antigen and one or two antiserum control tubes were always set up. Each control tube contained one unit of saline and one unit of antigen or of antibody. Two and 4 M.H.D. of complement or 3 M.H.D. were used in both antigen and antibody controls. In the low-temperature method the reagents were always brought to ice-chest temperatures before being used. The antigen-antibody-complement mixture was held at 1 - 4°C for 18 hours and afterwards placed in the 37°C incubator for 10 minutes. The sensitized cells were then added and the system incubated for a further one hour. The results were read as in the orthodox test.

The following figures are used to designate the degrees of haemolysis:

4 = no lysis, all complement fixed
3 = trace of lysis
2 = distinct lysis
1 = almost complete lysis
0 = complete lysis

SECTION V

COMPLEMENT-FIXATION TESTS WITH ANTIGEN-ANTIBODY-COMPLEMENT INCUBATION AT 37°C

COMPLEMENT-FIXATION TESTS WITH ANTIGEN-ANTIBODY-COMPLEMENT INCUBATION AT 37°C

Complement-fixation tests were carried out with most of the antigens previously described and the homologous antisera. Incubation of the antigen-antibody-complement mixture was at 37°C for 1-1/4 hours, followed by the addition of the sensitized cells and a further period of one hour's incubation. The order of the addition of the reagents was always the same -- antigen followed by antibody immediately followed by complement. When soluble extracts were used as antigens, precipitin tests were carried out in parallel with the complement-fixation tests.

Experiment 1.

A saline suspension prepared from a heated glucose-broth culture was the antigen (antigen S1, p.37). This was prepared from a group-A, type-4 strain. Complement-fixation tests were carried out over a wide antigen-antibody range, which varied from antigen/antibody = 1/8 to antigen/antibody = 10/1.

Fourteen tests were done.

The greatest number of doses of complement fixed was 6, but the average fixation over the 14 tests was only 0.5 M.H.D.

The results obtained did not favour any particular antigen/antibody ratio, the 6 M.H.D. however were fixed using an antigen/antibody ratio of 10/1 (Table 1, p.57).

Table 2, p.58, shows the results of a similar experiment using a group-B strain and its homologous antiserum.

Experiment 2.

Heating the broth culture at 56°C for one hour could cause a destruction or alteration of the antigenic properties of the culture. Consequently, treatment with formalin was used instead of heat, to kill the organisms. A saline suspension prepared from a formalin-treated glucose-broth culture constituted the antigen (antigen S2, p.37). Eight tests were carried out with a group-B strain, over an antigen/antibody ratio extending from 1/1 to 10/1. The average number of doses of complement fixed was 2 M.H.D.; in one case, with equal strength of antigen and of antibody 6 M.H.D. of complement were fixed (Table 3, p.59).

The results show that slightly better fixation is obtained with a formalin-killed, than with a heat-killed antigen, the average fixation with the formalin-killed antigen being 2 M.H.D. However, when 0.5% formalin is added sterility is not obtained for 2 - 3 days and for practical purposes an antigen readily prepared is desirable. As the formalin concentration increases anti-complementary results are obtained (Table 4, p.60).

Experiment 3.

It was thought that the fall of pH in glucose-broth cultures might account for the lack of consistency in the results so far obtained. Consequently the strains were now grown in the same medium as previously used, but without the glucose. In this medium the pH remained constant at 7.4 over a 24-hour period. A saline suspension obtained from a heated culture was used as antigen (antigen S3, p.38). Eight tests were done, the antigen being diluted one in two, and the antibody one in twenty. The average fixation was 2 M.H.D. of complement (Table 5, p.60).

Experiment 4.

The fixation so far obtained with saline suspensions as antigens was not very great and was not consistent. The small amount of complement fixed when saline suspensions are used may be due to failure to expose the antigen, which participates in the reaction. The inconsistency of the results might be due to a peeling off from the bacterial cell of antigenic material, which is then discarded in the culture supernatant.

These two possibilities suggested the use of whole-broth cultures as antigens. The first medium used was the 1.0% glucose broth. It was thought that although the acidity produced by the action of the streptococci on the glucose

substrate could cause destruction of the antigenic material, yet it might be that the developed acidity could assist the liberation of antigen from the bacterial cells into the surrounding medium.

The antigen used was antigen B1 (page 38) prepared by heating and neutralising a glucose-broth culture. Ten tests were done. The antigen was diluted one in five and the antibody dilution was one in twenty. Group-A, type-1, and group-B strains of streptococci were used. The average fixation was 3 M.H.D. of complement, and the maximum fixation was 6 M.H.D. of complement (Table 6, p.61).

Although the results were more satisfactory than in previous experiments it was still considered that the acidity might be harmful to antigen production; consequently the following experiment was carried out to determine whether (a) the glucose was essential or (b) acidity was required for the production of a suitable antigen.

Experiment 5.

The antigens prepared were antigens S (Sorensen's) and Sg (Sorensen's glucose, p.39) of pH 6.0, 7.2 and 8.0. The strain used was group A, type 1. The antigens were diluted one in two and the antiserum one in twenty.

The results in Table 7 (page 62) show that little difference was observed in the number of doses of complement fixed

by each of the six antigens. If the mere presence of glucose had been conducive to antigen production, more antigen would have been found in cultures of glucose medium, which would have been shown by greater fixation of complement, than in those grown in medium devoid of the carbohydrate. This was not so. If the acidity had acted destructively on the cell, with release of antigen, or alternatively had destroyed the antigen, different results would have been observed according to the different pH of the cultures.

Experiment 6.

The antigen used in this experiment was antigen B2 (page 39). This was prepared by heating a 24-hour lab. lemco culture, and diluting it one in two. The antiserum was diluted one in twenty. Fifty-three tests were done with this antigen. Strains of Lancefield's groups A, B, G and L were used. Over the 53 tests the average fixation was 4.5 M.H.D. of complement; the maximum fixation was 9 M.H.D. This occurred only once (Table 8, p.63). The average fixation of 4.5 M.H.D. of complement was quite high, but the results were not consistent and varied from day to day.

Experiment 7.

The following experiment was designed to compare the effect of using different media in preparing the antigen. At the same time it was hoped to determine if the lack of

consistency in results so far obtained was due to a variation in the amount of antigen elaborated in the media. If this were so, would more antigen be forthcoming with prolonged incubation?

A group-A, type-3 strain was grown at 37°C in ordinary lab. lemco broth and 1.0% glucose broth. Portions were withdrawn from both cultures at the end of (1) 18 hours; (2) 42 hours; (3) 48 hours and (4) 120 hours. These portions were heated at 56°C for one hour. The glucose-broth aliquots were neutralised with N/3 NaOH before heating (0.5 ml. per 10.0 ml. of culture). At the end of 120 hours the lab. lemco culture was still viable but at the end of 42 hours the glucose-broth culture was dead. The glucose-broth culture was diluted one in five. The growth in lab. lemco broth being poor, it was diluted one in two. These constituted the antigens and were tested with the homologous antiserum diluted 1/20.

The results in Table 9 (page 64) show that growth in lab. lemco broth in all cases produced a better antigen than growth in glucose broth, although the increase in the quantity of complement fixed was not great -- generally about 2 M.H.D. Prolongation of incubation up to 48 hours gave increased fixation of complement.

Experiment 8.

It was now decided to test a medium which would give better growth of the test strains than lab. lemco broth and at the same time maintain a constant pH. Both horse-heart extract and horse-heart digest fulfil these qualifications.

The group-A, type-3 strain was seeded into 1.0% glucose broth, horse-heart extract and horse-heart digest media. Growth in the three media was excellent and the aliquots

were each diluted one in five. Even at this dilution the horse-heart extract was anti-complementary. With a 1/15 dilution, the lowest dilution of horse-heart extract which did not give anti-complementary results, the problem arose as to whether the antiserum should still be diluted 1/20, or whether the 1/4 ratio of antigen/antiserum should be maintained by diluting the antiserum 1/60. Parallel tests were carried out with the horse-heart extract antigen diluted 1/15 and the antiserum diluted 1/20 and 1/60 (Table 10, p.65). The results show that the differences in fixation with the two dilutions are not significant.

Complement-fixation tests were now put up with horse-heart extract, horse-heart digest and 1.0% glucose-broth (neutralised as before) cultures as antigens. In all tests the glucose-broth antigen was the best and differences between the results obtained with the extract and digest antigens were not significant, although the former appears to be slightly superior to the latter. Prolonged incubation beyond 24 hours did not result in increase in the fixing ability of the antigens (Table 11, p.66).

Experiment 9.

It was stated by Lancefield (1936), that complement fixation does not occur when her carbohydrate group substance is used as antigen in complement-fixation tests with streptococcal antisera. I took the view however that Lancefield's

success in using residue antigens in grouping the haemolytic streptococci, the specificity of these antigens, the ease of their preparation and their potency when used as precipitins, all pointed to residue material as a suitable antigen in the streptococcal complement-fixation test. This view was strengthened by the relationship, according to the unitarian theory of Zinsser, of precipitins and complement-fixing antibodies.

Twenty-nine complement-fixation tests were carried out using Lancefield's antigen (page 42). The strains used were of groups A, B and K. The antigen/antibody ratio ranged from 100/1 through 1/1 to 1/2. The number of doses of complement fixed differed from test to test, but over the 29 tests the average fixation was 1.5 M.H.D. of complement. In three tests 6 M.H.D. of complement were fixed. The antigen/antibody ratio most favourable for fixation seemed to lie in the region of 10/1 -- an antigen excess (Table 12, p.67). Precipitin tests with the undiluted antigen and antiserum were always positive.

Experiment 10.

Failure to obtain good fixation of complement, although precipitation occurred, suggested that some masking factor might be present. Lancefield antigens of groups A and B were purified by alcohol precipitation of the protein (pages 42 - 43). Although these antigens gave positive precipitin results with the homologous antisera, complement-fixation tests resulted in the fixation of small quantities of complement (Table 13, p.68).

Experiment 11.

In this experiment the Lancefield antigens were purified by treatment with trypsin (page 43). Little or no complement was fixed: precipitin tests were positive (Table 14, p.69).

The results of the experiments agree with those of Lancefield that although the carbohydrate is suitable for grouping streptococci by means of the precipitin test, it is unsuitable for use in streptococcal complement-fixation tests.

Experiment 12.

Six complement-fixation tests were put up using Fuller's antigen (pages 41 - 42). Group-A and group-B strains were used. The antigen/antibody ratio extended from 10/1 to 1/1. In no case was there any fixation of complement although precipitin tests with the undiluted reagents were always positive (Table 15, p.70).

Experiment 13.

Using Hitchcock's antigen (pages 39 - 41), 72 complement-fixation tests were set up. These tests were performed with Lancefield's groups A, B, E, L, F and K antigens, using a wide antigen/antibody range. The antigen/antibody ratio ranged from 40/1 through equal concentrations of both antigen and antibody to 1/50. The over-all average number of doses of complement fixed was 3.5 M.H.D. In 11 tests the antigen with its specific immune serum fixed 9 M.H.D. of complement, the greatest amount of complement used. No particular antigen-antibody ratio favoured

fixation (Table 16, p.71). Results varied from day to day. Precipitin tests were always positive with the undiluted reagents.

SUMMARY

Complement-fixation tests were done using whole-broth cultures, cellular antigens and soluble extracts as antigens. Fixation was carried out at 37°C for 1-1/4 hours. The results obtained were not very consistent and it was clear that no matter what antigen was used, large amounts of complement were not consistently fixed. This is in accordance with the findings of other workers. The best results were obtained using the soluble antigen prepared by the method of Hitchcock; Lancefield's crude soluble antigen also gave some fixation. Using cellular antigens, saline suspensions were much less satisfactory than whole-broth cultures. This might be attributed to the discarding of antigenic material in the supernatant fluid. Of the various media used in preparing a broth antigen: 1.0% glucose broth, ordinary lab. lemco broth, horse-heart extract broth and horse-heart digest broth, the best results were obtained with ordinary lab. lemco broth, followed by 1.0% glucose broth and horse-heart extract broth, and lastly horse-heart digest broth.

Prolongation of the period of incubation of the cultural medium beyond 48 hours did not increase the quality of the antigen, nor did it result in any marked increase of antigenic quality over 24 hours' incubation.

THE RESULTS OF COMPLEMENT-FIXATION TESTS WITH ANTIGEN-ANTIBODY-COMPLEMENT INCUBATION AT 37°C

Table 1

Experiment 1. Saline suspensions from glucose-broth cultures as antigen.

Antigen S1* (group A, type 4). Undiluted.
Antiserum rabbit no. 50. Homologous. Diluted 1/10.
Complement 2, 4, 6 and 9 M.H.D. used in test; 2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

Antigen-antibody-complement mixture held 1-1/4 hours at 37°C.

Results**

Test				Controls			
				Antigen		Antibody	
4	4	4	0	0	0	0	0

Interpretation: 6 M.H.D. of complement fixed. Controls satisfactory.

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* The different types of antigen are defined on pp. 36 - 43.

** For explanation of symbols see p. 46.

Table 2

Experiment 1. Saline suspensions from glucose-broth cultures as antigen.

Antigen S1 (group B, strain 80RH). Undiluted.
Antiserum rabbit no. 22. Homologous. Diluted 1/10.
Complement 2, 4, 6 and 9 M.H.D. used in test; 2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

Antigen-antibody-complement mixture held 1-1/4 hours at 37°C.

Results.

Test				Controls			
				Antigen		Antibody	
4	0	0	0	0	0	0	0

Interpretation: 2 M.H.D. of complement fixed. Controls satisfactory.

Table 3

Experiment 2. Formal-saline suspensions of glucose-broth cultures as antigen.

Antigen S2 (group B, strain 80RH).
Antiserum rabbit no. 22. Homologous.
Complement 2, 4, 6 and 9 M.H.D. used in test; 2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

Antigen-antibody-complement mixture held 1-1/4 hours at 37°C.

(1) antigen undiluted; antiserum diluted 1/10
(2) antigen diluted 1/8; antiserum diluted 1/10
(3) antigen diluted 1/10; antiserum diluted 1/10

Results.

	Test				Controls			
					Antigen		Antibody	
(1)	4	0	0	0	0	0	0	0
(2)	4	0	0	0	0	0		
(3)	0	0	0	0	0	0		

The same test with antigen and antiserum both diluted 1/5.

	Test				Controls			
	4	4	4	0	0	0	0	0

Interpretation: With different dilutions of antigen and antibody different amounts of complement are fixed. Controls satisfactory.

Table 4

Experiment 2. The anti-complementary effects of formalin.

Antigen S2 (group B, strain 80RH).

Each tube, including the control tube, contains one unit of antigen and one unit of saline, and in addition 3 M.H.D. of complement. Tube 1 also contains 0.25% formalin; tube 2 contains 0.5% formalin; tube 3 contains 0.75% formalin; tube 4 contains 1.0% formalin and tube 5 contains 1.25% formalin. The control tube contains no formalin. At the end of 1-1/4 hours one unit of sensitized cells is added to each tube. The unit is 0.25 ml.

Results.

Test					Control
0	0	4	4	4	c

Interpretation: Increasing the concentration of formalin progressively decreases the lytic action of complement.

Table 5

Experiment 3. Saline suspensions from lab. lemco broth-cultures as antigen.

Antigen S3 (group A, type 1). Diluted 1/2.
Antiserum rabbit no. 55. Homologous. Diluted 1/20.
Complement 2, 4, 6 and 9 M.H.D. used in test; 2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

Antigen-antibody-complememt mixture held 1-1/4 hours at 37°C.

Results.

Test				Controls			
				antigen		antibody	
4	2	0	0	0	0	0	0

Interpretation: 2 M.H.D. of complement fixed. Controls satisfactory.

Table 6

Experiment 4. Glucose-broth cultures as antigen.

Antigen B1 (group A, type 1). Diluted 1/5.
Antiserum rabbit no. 55. Homologous. Diluted 1/20.
Complement 2, 4, 6 and 9 M.H.D. used in test; 2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

Antigen-antibody-complement mixture held 1-1/4 hours at 37°C.

Results.

Test				Controls			
				Antigen		Antibody	
4	4	0	0	0	0	0	0

Interpretation: 4 M.H.D. of complement fixed. Controls satisfactory.

Table 7

Experiment 5. The effects of glucose and pH on antigen production.

Antigens S and Sg (group A, type 1). Diluted 1/2.
(1) Sorensen's buffer pH 6.0 + 1.0% glucose
(2) " " pH 6.0
(3) " " pH 7.2 + 1.0% glucose
(4) " " pH 7.2
(5) " " pH 8.0 + 1.0% glucose
(6) " " pH 8.0
Antiserum rabbit no. 80. Homologous. Diluted 1/20.
Complement 2, 4, 6 and 9 M.H.D. used in test; 2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

Antigen-antibody-complement mixture held 1-1/4 hours at 37°C.

Results.

	Test				Controls			
					Antigen		Antibody	
(1)	4	4	4	4	4	0	0	0
(2)	4	4	4	4	4	0	0	0
(3)	4	4	4	0	0	0	0	0
(4)	4	4	4	0	0	0	0	0
(5)	4	4	4	0	0	0	0	0
(6)	4	4	3	0	0	0	0	0

Interpretation: Neither glucose nor different pH appears to affect antigen production although the results with antigens (1) and (2) are difficult to interpret because of the anti-complementary effects of the antigen and because no end-point is reached.

Table 8

Experiment 6. Lab. lemco broth cultures as antigen.

Antigen B2. Diluted 1/2.
(1) group B, strain 80RH.
(2) group A, type 1.
(3) group L, strain LXB.
Antisera homologous. Diluted 1/20.
(1) rabbit no. 75.
(2) rabbit no. 17.
(3) rabbit no. 25.
Complement 2, 4, 6 and 9 M.H.D. used in test; 2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

Antigen-antibody-complement mixture held 1-1/4 hours at 37°C.

Results.

	Test				Controls			
					Antigen		Antibody	
(1)	4	4	4	4	0	0	0	0
(2)	4	4	0	0	0	0	0	0
(3)	4	4	0	0	0	0	0	0

Interpretation: In this experiment an average of 6 M.H.D. of complement were fixed. Controls satisfactory.

Table 9

Experiment 7. Comparison of glucose- and lab. lemco broth cultures as antigens and the effect of different incubation periods on the quality of the antigen.

Antigen group A, type 3. (1) B2. Diluted 1/2.
(2) B1. Diluted 1/5.
Antiserum rabbit no. 55. Homologous. Diluted 1/20.
Complement 2, 4, 6 and 9 M.H.D. used in test; 2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

Antigen-antibody-complement mixture held 1-1/4 hours at 37°C.

Results.

	Test				Controls			
					Antigen		Antibody	
After 18 hours' incubation of cultures.								
(1)	4	0	0	0	0	0	0	0
(2)	4	0	0	0	0	0	0	0
After 42 hours' incubation of cultures.								
(1)	4	2	0	0	0	0	0	0
(2)	4	0	0	0	0	0	0	0
After 48 hours' incubation of cultures.								
(1)	4	4	3	0	0	0	0	0
(2)	4	4	2	0	0	0	0	0
After 120 hours' incubation of cultures.								
(1)	4	4	3	0	0	0	0	0
(2)	4	4	0	0	0	0	0	0

Interpretation: Antigens from lab. lemco broth cultures are slightly superior to those prepared from glucose broth. In both cases prolonging the incubation period up to 48 hours results in a more potent antigen.

Table 10

Experiment 8. Comparison of antiserum dilutions.

Antigen B3 (group A, type 3). Diluted 1/15.
Antiserum rabbit no. 55. Homologous. (1) Diluted 1/20.
(2) Diluted 1/60.
Complement 2, 4, 6 and 9 M.H.D. used in test; 2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

Antigen-antibody-complement mixture is held 1-1/4 hours at 37°C.

Results.

	Test				Controls			
					Antigen		Antibody	
(1)	4	4	0	0	0	0	0	0
(2)	4	4	0	0	0	0	0	0

Interpretation: Different antiserum dilutions do not affect the amount of complement fixed.

Table 11

Experiment 8. Comparison of different media used in preparing antigens and the effect of different incubation periods on the quality of the antigen.

Antigen group A, type 3. (1) B3. Diluted 1/15.
(2) B1. Diluted 1/5.
(3) B4. Diluted 1/5.

Antiserum rabbit no. 55. Homologous. Diluted 1/20.
Complement 2, 4, 6 and 9 M.H.D. used in test; 2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

Antigen-antibody-complement mixture held 1-1/4 hours at 37°C.

Results.

	Test				Controls			
					Antigen		Antibody	
After 24 hours' incubation of cultures.								
(1)	4	0	0	0	0	0	0	0
(2)	4	4	0	0	0	0	0	0
(3)	4	2	0	0	0	0	0	0
After 72 hours' incubation of cultures.								
(1)	4	0	0	0	0	0	0	0
(2)	4	4	0	0	0	0	0	0
(3)	4	2	0	0	0	0	0	0
After 120 hours' incubation of cultures.								
(1)	4	4	0	0	0	0	1	0
(2)	4	4	2	0	0	0	1	0
(3)	4	2	0	0	0	0	1	0

Interpretation: Glucose-broth antigens are superior to horse-heart extract broth antigens and horse-heart digest broth antigens. Prolonged incubation beyond 24 hours does not result in any marked increase in the potency of the antigens.

Table 12

Experiment 9. Lancefield's extract (C) as antigen.

Antigen group B, strain 80RH. Lancefield preparation (C).
(1) Undiluted
(2) diluted 1/2
(3) diluted 1/3
(4) diluted 1/4
(5) diluted 1/6
(6) diluted 1/8
(7) diluted 1/10
(8) diluted 1/15
(9) diluted 1/20

Antiserum rabbit no. 22. Homologous. Diluted 1/10.
Complement 2, 4, 6 and 9 M.H.D. used in test; 2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

Antigen-antibody-complement mixture held 1-1/4 hours at 37°C.

Results.

	Test				Controls			
					Antigen		Antibody	
(1)	4	4	4	2	2	0	2	0
(2)	4	2	0	0	2	0	2	0
(3)	4	2	0	0	0	0	2	0
(4)	4	2	0	0	0	0	2	0
(5)	4	2	0	0	0	0	2	0
(6)	3	2	0	0	0	0	2	0
(7)	3	2	0	0	0	0	2	0
(8)	3	2	0	0	0	0	2	0
(9)	3	2	0	0	0	0	2	0

As above, but (a) antiserum diluted 1/30 and antigen undiluted;
(b) antiserum diluted 1/100 and antigen undiluted.

(a)	4	4	3	0	2	0	0	0
(b)	3	0	0	0	2	0	0	0

Interpretation: The greatest amount of complement is fixed with the strongest antigen concentration. Antigen dilutions of 1/2 or greater result in little (2 M.H.D.) complement being fixed. Diluting the serum also decreases the amount of complement fixed.

Table 13

Experiment 10. Purified (alcohol) Lancefield extract (C) as antigen.

Antigens Lancefield purified preparation (C). (1) group A, type 1; (2) group A, type 4; (3) group B, strain 80RH.
(a) diluted 1/10; (b) undiluted.
Antisera. Homologous. Each antiserum tested with the homologous antigen. (1) rabbit no. 55; (2) rabbit no. 50; (3) rabbit no. 22. Diluted 1/10.
Complement 2, 4, 6 and 9 M.H.D. used in test; 2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

Antigen-antibody-complement mixture held 1-1/4 hours at 37°C.

Results.

	Test				Controls			
					Antigen		Antibody	
(1a)	0	0	0	0	0	0	0	0
(1b)	4	3	0	0	0	0		
(2a)	4	0	0	0	0	0	0	0
(2b)	4	2	0	0	0	0		
(3a)	3	0	0	0	0	0	0	0
(3b)	4	0	0	0	0	0		

Interpretation: Only small amounts of complement are fixed and with increasing antigen dilutions the amount of complement fixed decreases.

Table 14

Experiment 11. Purified (tryptic digest) Lancefield extract (C) as antigen.

Details as in experiment 10, table 13 (page 68) but extract purified by tryptic digest and not by alcoholic precipitation.

Results.

	Test				Controls			
					Antigen		Antibody	
(1a)	0	0	0	0	0	0	0	0
(1b)	0	0	0	0	0	0		
(2a)	0	0	0	0	0	0	0	0
(2b)	3	0	0	0	0	0		
(3a)	0	0	0	0	0	0	0	0
(3b)	0	0	0	0	0	0		

Interpretation: No complement, or very little complement, fixed.

Table 15

Experiment 12. Fuller's extract as antigen.

Antigen group B, strain 80RH. (1) Undiluted; (2) diluted 1/5 and (3) diluted 1/10.
Antiserum rabbit no. 22. Homologous. (a) Diluted 1/5 and (b) diluted 1/10.
Complement 2, 4, 6 and 9 M.H.D. used in test; 2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen or of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

Antigen-antibody-complement mixture held 1-1/4 hours at 37°C.

Results.

	Test				Controls			
					Antigen		Antibody	
(1a)	0	0	0	0	0	0	0	0
(2a)	0	0	0	0	0	0	0	0
(2b)	0	0	0	0	0	0	0	0
(3b)	0	0	0	0	0	0	0	0

Interpretation: No complement fixed.

Table 16

Experiment 13. Hitchcock's extract as antigen.

Antigen group A, type 1.
(1) undiluted (2) diluted 4/5
(3) diluted 2/5 (4) diluted 1/5
(5) diluted 1/25 (6) diluted 1/250
Antiserum rabbit no. 77. Homologous.
(a) undiluted (b) diluted 1/5
(c) diluted 1/6 (d) diluted 1/10
(e) diluted 1/50
Complement 2, 4, 6 and 9 M.H.D. used in test; 2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

Antigen-antibody-complement mixture held 1-1/4 hours at 37°C.

Results.

	Test				Controls			
					Antigen		Antibody	
(1a)	4	1	0	0	0	0	0	0
(1c)	4	4	4	2	0	0	0	0
(1d)	4	2	0	0	0	0	0	0
(2e)	4	4	0	0	0	0	0	0
(3a)	4	4	0	0	0	0	0	0
(3b)	4	4	0	0	0	0	0	0
(4b)	4	0	0	0	0	0	0	0
(5b)	0	0	0	0	0	0	0	0
(6b)	0	0	0	0	0	0	0	0

Interpretation: The amounts of complement fixed differ according to the different antigen and antiserum dilutions used. The results suggest that there may be an optimal antigen-antibody ratio for maximal fixation.

SECTION V1

THE EFFECT OF THE TIME INTERVAL BETWEEN THE ADDITION OF THE REAGENTS ON THE COMPLEMENT-FIXING POWERS OF THE MIXTURE

THE EFFECT OF THE TIME INTERVAL BETWEEN THE ADDITION OF THE REAGENTS ON THE COMPLEMENT-FIXING POWERS OF THE MIXTURE

Dean (1911 - 12, 1916 - 17), Goldsworthy (1928) and Goodner and Horsfall (1936) all stressed the loss of fixing ability of an antigen-antibody mixture with any increase in the interval elapsing before the addition of complement. Experiments conducted by these workers showed clearly that as little as ten minutes' delay in adding complement to an antigen-antibody mixture resulted in much less complement being fixed than if it was added immediately. The union of antigen and antibody proceeds very rapidly, occurring within a few seconds in many systems which take several minutes to precipitate. Mayer and Heidelberger (1942) showed that with pneumococcal polysaccharide and the homologous antiserum the first stage of the reaction went to 90% completion in less than three seconds at 0°C.

It was just possible that in the present work the interaction of antigen and antibody might be so rapid that although complement was added immediately after the addition of antibody

to antigen, sufficient time had already elapsed to prevent complement being adsorbed by the antigen-antibody mixture, thus accounting for the poor results so far obtained. An experiment was therefore done with a Lancefield preparation of a group-A, type-1 strain and the homologous antiserum, both diluted one in six. In this experiment (1) the antibody was added to the antigen followed immediately by the complement and (2) the antigen was placed in the tubes followed by the complement and then the antibody. In neither (1) nor (2) did fixation of complement occur (experiment 14, page 74).

SUMMARY

When the antigen-antibody-complement mixture was held at 37°C, the presence of complement at the time of the addition of the antibody to the antigen did not result in complement being fixed.

Table 17

Experiment 14. The effect of the time interval between the addition of complement to the antigen-antibody mixture.

Antigen Lancefield preparation. Group A, type 1. Diluted 1/6.
Antiserum rabbit no. 75. Homologous. Diluted 1/6.
Complement 2, 4, 6 and 9 M.H.D. used in test; 2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

Antigen-antibody-complement mixture held 1-1/4 hours at 37°C.

1. Antigen followed by antiserum then complement.
2. Antigen followed by complement then antiserum.

Results.

	Test				Controls			
					Antigen		Antibody	
(1)	3	0	0	0	0	0	0	0
(2)	1	0	0	0	0	0	0	0

Interpretation: The presence of complement when the antigen and antibody were mixed did not increase the amount of complement fixed.

SECTION V11

ANTIGEN-ANTIBODY RATIOS IN THE COMPLEMENT-FIXATION TEST

WHEN CARRIED OUT AT 37°C

ANTIGEN-ANTIBODY RATIOS IN THE COMPLEMENT-FIXATION TEST WHEN CARRIED OUT AT 37°C

It seemed possible that the antigen-antibody ratios which I used might have been unsuitable for complement fixation. Indeed precipitation occurred only with equal volumes of undiluted antigen and antibody. If we accept the theory that complement fixation is the result of the precipitate formed by the union of antigen and antiserum adsorbing complement and that such a reaction obeys the usual physical-chemical laws, it is clear that the optimal ratio for precipitation is not necessarily the optimum ratio for complement fixation. The amount of complement fixed will be proportionate to the surface area formed by the interaction of antigen and antibody. Any method which will delay precipitation and the aggregation of floccula will result in the complement being presented with a greater area for a longer period. Also, a fine precipitate will have a greater surface area than large floccula, and in consequence, will adsorb more complement.

Muir and Martin (1906) showed that, in the presence of a

constant amount of antiserum, there was a particular amount of antigen which gave maximal complement fixation and another particular volume which gave maximal precipitation. In a series of experiments Dean (1911 - 12, 1916 - 17) showed that considerably less antigen was required for maximal complement fixation than was needed for maximal precipitation.

However, the optimal antigen-antibody ratios for precipitation and for complement fixation have ranged from an antigen excess to an antibody excess, varying from system to system, and dependent, among other factors, on the potency of the reagents.

Goldsworthy (1928) noted that with weak antisera which formed precipitates with antigens relatively slowly, the antigen-antibody proportions were similar for optimal precipitation and for maximal complement fixation. With very rapidly particulating antisera proportionately less antigen was required for maximal fixation than for optimal precipitation. He explained this by stating that a precipitate is formed in three stages. (1) The nascent stage in which there is no visible precipitate. (2) The opalescent-turbid period and (3) a final period where the precipitate is visible and where ultimately large floccula fall to the bottom of the tube. Any method which will delay the occurrence of the third stage will assist in the fixation

of complement. A mixture of an antigen and potent antibody containing the correct proportions for rapid particulation will not of necessity give the maximum fixation of complement. Goldsworthy agrees with Dean (1911 - 12, 1916 - 17), that a slight excess of antibody will give maximum fixation, but only if complement is present at the time of contact of antigen and antibody. If there is a delay in adding the complement to the system it is preferable to have an antigen excess in which case the turbid phase of precipitation persists over a considerable period, and consequently allows fixation to proceed still.

With type-1 specific carbohydrate of pneumococcus, Brown (1934 - 35) and later Goodner and Horsfall (1936) observed the relative proportions of antigen and antiserum giving maximal precipitation and complement fixation to be similar. A corresponding result might be expected with the soluble specific substance of streptococci and the homologous antiserum, but it is worthy of note that an excess of antigen may dissolve the precipitate and that the precipitin test should be done with equal volumes of antigen and antibody and if positive results are not obtained, the test should be repeated with 1/4 and 1/16 dilutions of antigen. In some systems an antigen excess may be desirable. Working with particulate antigens Spooner and Bawden (1935) and Platt (1936) found this to be true.

Parker (1923), Morgan (1923), Dean and Webb (1926), Rice (1942, 1943b) and Rice and Sickles (1942) all stressed the importance of optimum antigen-antibody ratios in serum reactions.

Bearing all these views in mind it seemed possible that failure to obtain fixation of complement might be due to failure to strike the correct antigen-antibody ratio. With such conflicting reports and views as those recorded above it was obvious that an arbitrary experiment would be the best method of determining the antigen-antibody ratio which would give the maximum fixation of complement. Most experiments so far reported have been done over a wide antigen-antibody range, but in many instances the full range within the limits had not been covered.

Experiment 15.

A group-A, type-1 extract prepared by Hitchcock's method and the homologous antiserum were used. Antigen and antiserum dilutions of 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1/5 and 3/10 were prepared. 0.25 ml. was used as the unit of antigen, antibody and haemolytic system. Each antigen dilution was tested with each antiserum dilution and immediately after the addition of the antiserum to the antigen 3 M.H.D. of complement were added to each tube.

The results (Table 18, p.81) show that only in the 1/5 and stronger antiserum concentrations and the 1/10 and lower

antigen dilutions was there any trace of complement fixation. Controls were all negative. The precipitin tests with the undiluted reagents were positive, but all precipitin tests with diluted reagents were negative.

SUMMARY

Failure to obtain the fixation of complement in the experiment carried out so far, does not appear to be due to failure to strike the correct antigen-antibody ratio. Using Hitchcock's antigen over a wide antigen-antibody range and incubating the antigen-antibody mixture at 37°C for 1-1/4 hours little or no complement was fixed at any given ratio. Visible precipitation has until now been observed only with equal volumes of the undiluted reagents. Such a concentration and such a ratio does not give complement fixation. I had hoped that lower concentrations of one or other of the reagents or different ratios would result in a very fine precipitation with consequent complement fixation, but this expectation was not realized.

Table 18

Experiment 15. The optimum antigen-antibody ratio in complement fixation.

Antigen group A, type 1. Hitchcock's preparation.
Antiserum rabbit no. 17. Homologous.
Complement 3 M.H.D. in each tube, both test and controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

Antigen-antibody-complement mixture held 1-1/4 hours at 37° C.

Results.

Antiserum	Antigen 0.0001	0.001	0.01	0.1	0.2	0.3
0.0001	0	0	0	0	0	0
0.001	0	0	0	0	0	0
0.01	0	0	0	0	0	0
0.1	0	0	0	0	0	0
0.2	0	0	0	0	3	3
0.3	0	0	0	3	3	3

Controls, antigen and antibody, of all the above dilutions were negative.

Interpretation: The amount of complement fixed does not appear to depend on the ratio of antigen to antibody. With stronger antigen and antibody concentrations more complement is fixed.

SECTION V111

EFFECTS OF TEMPERATURE OF INCUBATION ON THE COMPLEMENT-FIXATION TEST

EFFECTS OF TEMPERATURE OF INCUBATION ON THE COMPLEMENT-FIXATION TEST

As early as 1910 Jacobstahl pointed out that in the Wassermann test better fixation was obtained at ice-chest temperatures than at 37°C. This work was stimulated by the fact that adsorption of colloids was favoured by low temperatures: as complement fixation represented adsorption on specific precipitates it too might be favoured by low temperatures. Guggenheimer (1911) also found that if ice-chest temperatures were used in the Wassermann test better fixation was obtained than if the tests were carried out at incubator temperature.

Dean (1916 - 17) claimed that any method which delayed the formation of a precipitate aided greater fixation of complement. In typical complement-fixation experiments, using a normal serum and its homologous antiserum, a bacterial extract and its antiserum and alcoholic organ extracts and syphilitic serum, he showed that precipitation

was delayed by ice-chest temperatures. In all cases greater fixation was obtained at ice-chest temperature than at room temperature, which in turn gave greater fixation than incubator temperature. Increased fixation was claimed to be due to precipitation of euglobulin from complement, which is favoured by low temperatures, with its subsequent adsorption on to the antigen-antibody mixture. This precipitation is more complete at 0°C than at 37°C, and any precipitate formed at 0°C is dissolved at incubator temperature. Whereas an antigen-antibody mixture may give no visible precipitate, when complement is added to the mixture a visible precipitate is formed due to the presence of euglobulin. Kahn (1921) using as antigens the pure proteins, phaseolin and edestin, and specific immune rabbit sera obtained greater fixation at ice-chest (8 - 12°C) than at room (18 - 23°C) than at water-bath (37.5°C) temperatures. Kahn and Johnson (1922) using six bacterial antigens (*Salmonella typhi*; *Salmonella paratyphi A*; *Salmonella paratyphi B*; *Brucella abortus*; *Salmonella pullorum*; *Pfeifferella mallei*), found that in all cases, except with *S. pullorum*, better fixation results were obtained at low temperatures. They suggest: "For correct complement-fixation tests, we insist on titrating a number of ingredients, and there is no reason why we should not also determine the proper mode of fixation by special titration."

Rice (1943a), working with pneumococcal antigens and pneumococcal antisera, found that many antisera in the presence of the maximal reaction dose of antigen, fixed two to three times as much complement at 3 - 6°C as at 37°C. In the former case the incubation period was 24 hours; in the latter 1-1/4 hours. Low temperatures favoured the fixation of filterable viruses and their antisera (Bedson and Bland, 1929). In vaccinia and variola complement-fixation tests, Parker and Muckenfuss (1933) found that lower temperatures favoured fixation, and using viral and rickettsial systems, better results are obtained in general with preliminary incubation at ice-chest temperatures than at 37°C.

As the next step in my work, therefore, complement-fixation tests were done at ice-chest temperatures instead of at 37°C. Most workers state that fixation at low temperatures is not complete at the end of 1-1/4 hours and some suggest that maximal fixation occurs after four hours (Kahn and Olin, 1921). Rice (1942) showed that complement fixation is not complete at the conclusion of six hours. Parker and Muckenfuss (1933) prolonged ice-chest incubation for 20 hours in order to obtain maximum fixation and state that fixation is a function of time and not of temperature. This is in agreement with Bedson and Bland (1929). There seemed no harm, and in fact it

was convenient, to prolong to 18 hours the period before the addition of the haemolytic system to the antigen-antibody-complement system. As any free complement might take some time to act upon the sensitized cells added to the mixture, the test was placed in the 37°C incubator for 10 - 15 minutes immediately before the addition of the haemolytic system. It will be shown later that this does not affect the amount, if any, of complement fixed. In all these experiments the reagents were brought to ice-chest temperatures, or to room temperature, if incubation was at 37°C.

Comparison was made of the results of incubating the antigen-antibody-complement mixture for 1-1/4 hours at 37°C and for 18 hours at 1°C using cellular antigens, (both saline and whole-broth cultures), and soluble extracts, (both Hitchcock's and Lancefield's antigens).

The homologous serum was used in all experiments from 16 - 26 reported in this section.

Experiment 16.

The antigen used was S1 (page 37). Using this antigen 2+ M.H.D. of complement were fixed at ice-chest temperature as opposed to complete absence of complement fixation at 37°C (Table 19, p.93).

Experiment 17.

The antigen used was S3 (page 38). Four plus M.H.D. of

complement were fixed at ice-chest temperature as opposed to 2+ M.H.D. of complement at 37°C (Table 20, p.94).

Experiment 18.

The antigen used was B1 (page 38). Six M.H.D. of complement were fixed at ice-chest temperature as opposed to 4 M.H.D. of complement at 37°C (Table 21, p.95).

Experiment 19.

The antigen used was B2 (page 39). Using this antigen 9 M.H.D. of complement (the greatest amount used) were fixed at ice-chest temperature as against 2 M.H.D. of complement at 37°C (Table 22, p.96).

Experiment 20.

Lancefield's soluble extract was used as antigen and 9 M.H.D. of complement (the greatest amount used) were fixed at ice-chest temperature as opposed to the complete absence of complement fixation at 37°C (Table 23, p.97).

Experiment 21.

Hitchcock's soluble extract was used as antigen and although 4 M.H.D. of complement were fixed when incubation of the antigen-antibody-complement mixture was at 37°C, when the mixture was held at ice-chest temperature (1 - 4°C) 9 M.H.D. of complement (the greatest amount used) were fixed (Table 24, p.98).

From all the above experiments it was seen that more

complement was fixed when the antigen-antibody-complement mixture was held for 18 hours at ice-chest temperature (1 - 4°C) than when incubation was at 37°C for 1-1/4 hours. These findings are in accordance with those of Dean (1916 - 17) and Goldsworthy (1928).

A series of experiments was now carried out to determine the velocity of fixation at room, ice-chest and incubator temperatures, and also the time-temperature relationships which gave maximal fixation. In these experiments, the antigens used were prepared by either Hitchcock's or Lancefield's methods, because I thought that I had better results with soluble extracts than with whole cells.

Experiment 22.

A group-A, type-4 antigen prepared by Hitchcock's method and the homologous antiserum were each diluted one in seven. This dilution was chosen in order to obtain an end-point with a reasonable amount of complement. The usual range of complement doses was extended to 30 M.H.D. in the following steps: 2, 4, 6, 9, 12, 15, 20, 25 and 30 M.H.D. The temperatures were ice-chest (2°C), room temperature (16°C) and incubator (37°C). At each temperature, the times allowed for the antigen-antibody-complement mixture to react before the addition of the sensitized cells were: 30 minutes; 1-1/4 hours; 3 hours; 6 hours and 18 hours,

except in the series held at incubator temperature, where due to rapid deterioration of the complement only 30 minutes and 1-1/4 hour periods were used.

From the results in Table 25 (page 99) it will be seen that the velocity of fixation is slightly greater at ice-chest temperatures than at room temperature. A much greater amount of complement is fixed at room temperature than at 37°C and a still greater amount at 2°C. Fixation appears to continue right up to the end of 18 hours at room temperature, whereas at ice-chest temperature it would appear to continue up to the end of 6 hours although in this case the end-point was not reached. In the main these results agree with those of previous workers. It would appear that neither time nor temperature is absolutely critical, and adequate amounts of complement are fixed if a low enough temperature is used for a reasonable period of time.

Experiment 23.

A similar time and temperature experiment was carried out with a group-N strain (strain 201) and the homologous antiserum. In this experiment it was noted that fixation was complete at the end of 6 hours at room temperature. At ice-chest temperature fixation was complete after 12 hours. A greater amount of complement was fixed at ice-chest than at room temperature and once more it was obvious

that time and temperature are not critical (Table 26, p.100).

Experiment 24.

A third set of experiments was done with a group-A, type-1 strain, but the titre of the antiserum was so high, that only in those tests which were incubated for 30 minutes or 1-1/4 hours was an end-point reached (Table 27, p.101). In all other cases 30 M.H.D. of complement, the greatest amount used, were fixed. This result clearly shows that the time and temperature are not critical providing the temperature is low. The result also shows that even at incubator temperature some complement will be fixed if the antiserum is of a high titre.

The scope of this work, as planned, did not include an investigation of the mechanisms underlying the fact that fixation of complement is greater when the antigen-antibody-complement mixture is held at low temperatures than when it is held at 37°C. Nevertheless, one or two experiments were carried out to see if any light could be thrown on the mechanism of the action.

Experiment 25.

This experiment was dsigned to determine if the 10 minute incubation period at 37°C after the antigen-antibody-

complement mixture is held at ice-chest temperature was detrimental to the amount of complement fixed. A Hitchcook preparation of a group-A, type-1 strain and its homologous antiserum were used. Incubation was at 2°C for 18 hours; immediately thereafter the sensitized cells were added. A duplicate set of tubes was set up in the same manner, but in this case after holding at 2°C for 18 hours, the test was incubated at 37°C for one hour before adding the sensitized cells. In both cases 15 M.H.D. of complement were fixed (Table 28, p.102).

Experiment 26.

This experiment was designed to show that fixation still took place when the antigen-antibody-complement mixture was first incubated for one hour at 37°C and then held at ice-chest temperature for 18 hours. After this the sensitized cells were added. The experiment was done in duplicate with a group-A, type-1 Hitchcock's antigen and the homologous antiserum. One set of tubes was held at 2°C for 18 hours; the other was first incubated at 37°C for one hour and then held at 2°C for 18 hours. Both sets were then warmed and the haemolytic system added. In both cases 15 M.H.D. of complement were fixed (Table 29, p.103).

SUMMARY

Complement was fixed by the soluble specific carbohydrate (residue substance) and the homologous antiserum when the antigen-antibody-complement mixture was incubated at low temperatures. The amount of complement fixed was greatest at ice-chest temperature. For complete fixation a period of approximately 6 hours should be allowed, although the exact incubation period is not critical.

The following experiment was done to determine if the order of the addition of the reagents was critical:

Experiment 27.

A Hitchcock's preparation of a group-H strain (Challis) was used as antigen and tested with the homologous antiserum. The order of the addition of the three reagents was varied. In the first case the antigen was placed in the tube, and this was followed by the appropriate complement dose. Thus, when antibody was now added, complement was present at the time of interaction of antigen and antibody. In the second case the complement was not added until after the addition of the antibody to the antigen. In the third case the complement was placed in the tube, then the antigen, and finally the antibody. This once again, ensured that

complement was present at the time of the interaction of the antigen and antibody. The antigen-antibody-complement mixtures were held at 2°C for 18 hours before the addition of the sensitized cells. In all three cases the amount of complement fixed was the same -- 25 M.H.D. (Table 30, p.104).

[illegible] OF EXPERIMENTS ON EFFECTS OF TEMPERATURE [illegible]

ON THE COMPLEMENT-FIXATION TEST

Table 19

[illegible] Saline suspensions prepared from glucose-[illegible] cultures as antigen.

[illegible] group D, strain [illegible]). Diluted 1/5.
[illegible] no. 22. [illegible]. Diluted 1/20.
[illegible] 3, 4, 8 and 9 [illegible] used in tests; 2 and [illegible] controls.

RESULTS OF EXPERIMENTS ON EFFECTS OF TEMPERATURE OF INCUBATION

ON THE COMPLEMENT-FIXATION TEST

[illegible]	Test				Controls			
					[illegible]		[illegible]	
[illegible]	[illegible]	[illegible]	[illegible]	0	[illegible]	0	0	0
[illegible]	[illegible]	[illegible]	[illegible]	0	[illegible]	0	0	0

Interpretation: A greater amount of complement is fixed at 3°C than at 37°C

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* [illegible] rent types of antigen are defined on pp. 35 - 43.

** [illegible] explanation of symbols see p. 46.

Table 19

Experiment 16. Saline suspensions prepared from glucose-broth cultures as antigen.

Antigen S1*(group B, strain 80RH). Diluted 1/5.
Antiserum rabbit no. 22. Homologous. Diluted 1/20.
Complement 2, 4, 6 and 9 M.H.D. used in test; 2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

(1) Antigen-antibody-complement mixture held 1-1/4 hours at 37°C.
(2) Antigen-antibody-complement mixture held 18 hours at 2°C.

Results.**

	Test				Controls			
					Antigen		Antibody	
(1)	0	0	0	0	0	0	0	0
(2)	4	1	0	0	0	0	0	0

Interpretation: A greater amount of complement is fixed at 2°C than at 37°C

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* The different types of antigen are defined on pp. 36 - 43.

** For explanation of symbols see p. 46.

Table 20

Experiment 17. **Saline suspensions prepared from lab. lemco** broth cultures as antigen.

Antigen S3 (group A, type 1). Diluted 1/2.
Antiserum rabbit no. 55. Homologous. Diluted 1/20.
Complement 2, 4, 6 and 9 M.H.D. used in test; 2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

(1) Antigen-antibody-complement mixture held 1-1/4 hours at 37°C.
(2) " " " " " 18 hours at 1°C.

Results.

	Test				Controls			
					Antigen		Antibody	
(1)	4	2	0	0	0	0	0	0
(2)	4	4	2	0	0	0	0	0

Interpretation: **A greater amount of complement is fixed at 1°C than at 37°C.**

Table 21

Experiment 18. Glucose-broth cultures as antigen.

Antigen B1 (group A, type 1). Diluted 1/5.
Antiserum rabbit no. 55. Homologous. Diluted 1/20.
Complement 2, 4, 6 and 9 M.H.D. used in test; 2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

(1) Antigen-antibody-complement mixture held 1-1/4 hours at 37°C.
(2) Antigen-antibody-complement mixture held 18 hours at 1°C.

Results.

	Test				Controls			
					Antigen		Antibody	
(1)	4	4	0	0	0	0	0	0
(2)	4	4	4	2	0	0	2	0

Interpretation: A greater amount of complement is fixed at 1°C than at 37°C.

Table 22

Experiment 19. Lab. lemco broth cultures as antigen.

Antigen B2 (group A, type 3). Diluted 1/2.
Antiserum rabbit no. 55. Diluted 1/20.
Complement 2, 4, 6 and 9 M.H.D. used in test; 2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

(1) Antigen-antibody-complement mixture held 1-1/4 hours at 37°C.
(2) " " " " " 18 hours at 1°C.

Results.

	Test				Controls			
					Antigen		Antibody	
(1)	4	0	0	0	0	0	0	0
(2)	4	4	4	4	0	0	0	0

Interpretation: A greater amount of complement is fixed at 1°C than at 37°C.

Table 23

Experiment 20. Lancefield's extract (C) as antigen.

Antigen Lancefield preparation (C). Group K, strain Turner. Diluted 1/5.
Antiserum rabbit no. 34. Homologous. Diluted 1/5.
Complement 2, 4, 6 and 9 M.H.D. used in test; 2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

(1) Antigen-antibody-complement mixture held 1-1/4 hours at 37°C.
(2) Antigen-antibody-complement mixture held 18 hours at 1°C.

Results.

	Test				Controls			
					Antigen		Antibody	
(1)	0	0	0	0	0	0	0	0
(2)	4	4	4	4	0	0	0	0

Interpretation: A greater amount of complement is fixed at 1°C than at 37°C.

Table 24

Experiment 21. Hitchcock's extract as antigen.

Antigen Hitchcock's preparation. Group A, type 1.
Diluted 1/5.
Antiserum rabbit no. 55. Homologous. Diluted 1/5.
Complement 2, 4, 6 and 9 M.H.D. used in test; 2 and
4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

(1) Antigen-antibody-complement mixture held 1-1/4 hours at 37°C.
(2) Antigen-antibody-complement mixture held 18 hours at 1°C.

Results.

	Test				Controls			
					Antigen		Antibody	
(1)	4	4	0	0	0	0	0	0
(2)	4	4	4	4	0	0	0	0

Interpretation: A greater amount of complement is fixed at 1°C than at 37°C.

Table 25

Experiment 22. Effect of time and temperature of holding antigen-antibody-complement mixture on the amount of complement fixed.

Antigen Hitchcock's preparation. Group A, type 4. Diluted 1/7.
Antiserum rabbit no. 27. Homologous. Diluted 1/7.
Complement 2, 4, 6, 9, 12, 15, 20, 25 and 30 M.H.D. used in test; 2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

(1) Antigen-antibody-complement mixture held 1/2 hour at 37°C.
(2) " " " " " 1-1/4 hours at 37°C.
(3) " " " " " 1/2 hour at 16°C.
(4) " " " " " 1-1/4 hours at 16°C.
(5) " " " " " 3 hours at 16°C.
(6) " " " " " 6 hours at 16°C.
(7) " " " " " 18 hours at 16°C.
(8) " " " " " 1/2 hour at 2°C.
(9) " " " " " 1-1/4 hours at 2°C.
(10) " " " " " 3 hours at 2°C.
(11) " " " " " 6 hours at 2°C.
(12) " " " " " 18 hours at 2°C.

Results.

	Test									Controls			
										Antigen		Antibody	
(1)	4	4	0	0	0	0	0	0	0	0	0	0	0
(2)	4	4	0	0	0	0	0	0	0	0	0	0	0
(3)	4	4	4	2	0	0	0	0	0	0	0	0	0
(4)	4	4	4	4	3	0	0	0	0	0	0	0	0
(5)	4	4	4	4	4	4	1	0	0	0	0	0	0
(6)	4	4	4	4	4	4	3	1	0	0	0	0	0
(7)	4	4	4	4	4	4	4	4	3	1	0	1	0
(8)	4	4	4	3	0	0	0	0	0	0	0	0	0
(9)	4	4	4	4	4	2	0	0	0	0	0	0	0
(10)	4	4	4	4	4	4	2	0	0	0	0	0	0
(11)	4	4	4	4	4	4	4	4	4	0	0	0	0
(12)	4	4	4	4	4	4	4	4	4	0	0	0	0

Interpretation: More complement is fixed at 2°C than at 37°C.
More complement is fixed at 2°C than at 16°C.
More complement is fixed at 16°C than at 37°C.
The velocity of fixation is slightly greater at 2°C than at 16°C.

Table 26

Experiment 23. Effect of time and temperature of holding antigen-antibody-complement mixture on the amount of complement fixed.

Antigen Hitchcock's preparation. Group N, strain 201. Diluted 1/6.
Antiserum rabbit no. 101. Homologous. Diluted 1/6.
Complement 2, 4, 6, 9, 12, 15, 20, 25 and 30 M.H.D. used in test; 2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

(1) Antigen-antibody-complement mixture held 1-1/4 hours at 16°C.
(2) " " " " " 3 hours at 16°C.
(3) " " " " " 6 hours at 16°C.
(4) " " " " " 12 hours at 16°C.
(5) " " " " " 18 hours at 16°C.
(6) " " " " " 1-1/4 hours at 4°C.
(7) " " " " " 3 hours at 4°C.
(8) " " " " " 6 hours at 4°C.
(9) " " " " " 12 hours at 4°C.
(10) " " " " " 18 hours at 4°C.

Results.

	Test									Controls			
										Antigen		Antibody	
(1)	4	4	4	0	0	0	0	0	0	0	0	0	0
(2)	4	4	4	4	0	0	0	0	0	0	0	0	0
(3)	4	4	4	4	4	0	0	0	0	0	0	0	0
(4)	4	4	4	4	4	0	0	0	0	0	0	0	0
(5)	4	4	4	4	4	0	0	0	0	0	0	0	0
(6)	4	4	4	4	.	.	.	.	.*	0	0	0	0
(7)	4	4	4	4	4	0	0	0	0	0	0	0	0
(8)	4	4	4	4	4	4	0	0	0	0	0	0	0
(9)	4	4	4	4	4	4	4	0	0	0	0	0	0
(10)	4	4	4	4	4	4	4	0	0	0	0	0	0

Interpretation: A greater amount of complement is fixed at 4°C than at 16°C. The velocity of fixation is similar at both temperatures.

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* . = not tested.

Table 27.

Experiment 24. Effect of time and temperature of holding antigen-antibody-complement mixture on the amount of complement fixed.

Antigen Hitchcock's preparation. Group A, type 1. Diluted 1/5.
Antiserum rabbit no. 78. Homologous. Diluted 1/5.
Complement 2, 4, 6, 9, 12, 15, 20, 25 and 30 M.H.D. used in test; 2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

(1) Antigen-antibody-complement mixture held 1/2 hour at 37°C.
(2) " " " " " 1-1/4 hours at 37°C.
(3) " " " " " 1/2 hour at 16°C.
(4) " " " " " 1-1/4 hours at 16°C.
(5) " " " " " 3 hours at 16°C.
(6) " " " " " 6 hours at 16°C.
(7) " " " " " 12 hours at 16°C.
(8) " " " " " 18 hours at 16°C.
(9) " " " " " 1/2 hour at 1°C.
(10) " " " " " 1-1/4 hours at 1°C.
(11) " " " " " 3 hours at 1°C.
(12) " " " " " 6 hours at 1°C.
(13) " " " " " 12 hours at 1°C.
(14) " " " " " 18 hours at 1°C.

Results.

	Test									Controls Antigen		Controls Antibody	
(1)	4	4	4	0	0	0	0	0	0	0	0	0	0
(2)	4	4	4	4	0	0	0	0	0	0	0	0	0
(3)	4	4	4	4	4	0	0	0	0	0	0	0	0
(4)	4	4	4	4	4	4	3	3	3	0	0	0	0
(5)	4	4	4	4	4	4	4	4	4	0	0	0	0
(6)	4	4	4	4	4	4	4	4	4	1	0	1	0
(7)	4	4	4	4	4	4	4	4	4	1	0	1	0
(8)	4	4	4	4	4	4	4	4	4	1	0	2	0
(9)	4	4	4	4	4	0	0	0	0	0	0	0	0
(10)	4	4	4	4	4	4	0	0	0	0	0	0	0
(11)	4	4	4	4	4	4	4	4	4	0	0	0	0
(12)	4	4	4	4	4	4	4	4	4	0	0	0	0
(13)	4	4	4	4	4	4	4	4	4	0	0	0	0
(14)	4	4	4	4	4	4	4	4	4	1	0	1	0

Interpretation: More complement is fixed both at 1°C and 16°C than at 37°C.

Table 28

Experiment 25. The effect of incubation at 37°C of the antigen-antibody-complement mixture prior to holding at ice-chest temperature.

Antigen Hitchcock's preparation. Group A, type 1. Diluted 1/5.
Antiserum rabbit no. 80. Homologous. Diluted 1/5.
Complement 2, 4, 6, 9, 12, 15 and 20 M.H.D. used in test;
2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

(1) Antigen-antibody-complement mixture incubated at 37°C for one hour and then placed in ice-chest at 2°C for 18 hours.
(2) Antigen-antibody-complement mixture held at 2°C for 18 hours.

Results.

	Test							Controls			
								Antigen		Antibody	
(1)	4	4	4	4	4	4	0	0	0	0	0
(2)	4	4	4	4	4	4	0	0	0	0	0

Interpretation: Incubating the antigen-antibody-complement mixture at 37°C prior to the holding period of 18 hours at 2°C did not alter the amount of complement fixed.

Table 29

Experiment 26. The effect of incubation at 37°C of the antigen-antibody-complement mixture subsequent to holding at ice-chest temperature.

Antigen Hitchcock's preparation. Group A, type 1. Diluted 1/5.
Antiserum rabbit no. 80. Homologous. Diluted 1/5.
Complement 2, 4, 6, 9, 12, 15 and 20 M.H.D. used in test;
2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

(1) Antigen-antibody-complement mixture held for 18 hours at 2°C and then incubated for one hour at 37°C.
(2) Antigen-antibody-complement mixture held for 18 hours at 2°C.

Results.

	Test							Controls			
								Antigen		Antibody	
(1)	4	4	4	4	4	4	0	0	0	0	0
(2)	4	4	4	4	4	4	0	0	0	0	0

Interpretation: Incubating the antigen-antibody-complement mixture at 37°C subsequent to holding at 2°C for 18 hours did not alter the amount of complement fixed.

Table 30

Experiment 27. The influence of the order of the addition of reagents on the amount of complement fixed.

Antigen Hitchcock's preparation. Group H, strain Challis. Diluted 1/5.
Antiserum rabbit no. 102. Homologous. Diluted 1/5.
Complement 2, 4, 6, 9, 12, 15, 20 and 25 M.H.D. used in test; 2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

(1) Antigen placed in tube first, followed by complement and then antibody.
(2) Antigen placed in tube first, followed by antibody and then complement.
(3) Complement placed in tube first followed by antigen and then antibody.

Antigen-antibody-complement mixture held 18 hours at 2°C.

Results.

	Test								Controls			
									Antigen		Antibody	
(1)	4	4	4	4	4	4	4	4	0	0	0	0
(2)	4	4	4	4	4	4	4	4	0	0	0	0
(3)	4	4	4	4	4	4	4	4	0	0	0	0

Interpretation: The order of the addition of the reagents does not influence the amount of complement fixed.

SECTION 1X

ANTIGEN-ANTIBODY RATIOS AT ICE-CHEST TEMPERATURES

ANTIGEN-ANTIBODY RATIOS AT ICE-CHEST TEMPERATURES

Large amounts of complement are fixed when the antigen-antibody-complement mixture is held at ice-chest temperatures. Therefore, it was now necessary to find the most suitable antigen for the test, and to determine the optimum antigen-antibody ratio. Optimum antigen-antibody ratios were determined for both cellular antigens and soluble-extract antigens.

It does not follow that the antigen-antibody ratio at low temperatures will be the same as that at 37°C. Rice (1942) found that some antisera when tested at 3 - 6°C needed twice as much antigen as at 37°C; other antisera needed four times as much antigen at 3 - 6°C as at 37°C.

SOLUBLE EXTRACTS

Experiment 28.

This was a repetition of experiment 15, (pages 79 - 80, Table 18, p.81) but the reagents were held at 2°C and not at 37°C. The antigen was a Hitchcock preparation of a group-A, type-1 strain and was tested with the homologous antiserum.

Antigen and antiserum dilutions of 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1/5 and 3/10 were prepared. Each antigen dilution was tested with each antibody dilution in the presence of 3 M.H.D. of complement. Incubation was at $2^{o}C$ for 18 hours.

The 3 M.H.D. of complement were fixed by all tubes containing the lower antigen and antiserum dilutions -- 10^{-1}, 1/5 and 3/10 (Table 31, p.112). The 3 M.H.D. were also fixed by an antigen excess -- the 3/10 dilution crossed with the 10^{-2} antiserum dilution.

The test was now extended as shown in Table 32 (page 113). Various antigen-antibody ratios which fixed 3 M.H.D. of complement were tested using four quantities of complement, viz. 2, 4, 6 and 9 M.H.D. In all cases 9 M.H.D. of complement were fixed. Precipitin tests carried out with the undiluted reagents were positive, but those with the diluted reagents were negative.

Experiment 29.

Further antigen-antibody ratio tests were carried out with a group-A, type-1 strain and its homologous antiserum (Table 33, p.114). Dilutions of 1/5, 1/10, 1/15, 1/20 and 1/25 of both antigen and antibody were tested. Ten M.H.D. of complement were added to each tube. The mixtures were held for 18 hours at $1^{o}C$.

The 1/5, 1/10 and 1/15 antiserum dilutions fixed complement with all the antigen dilutions. Precipitin tests were positive with the undiluted reagents only. The test was now extended using 3, 6, 9, 12, 15, 20 and 25 M.H.D. of complement (Table 34, p.115). The same reagents were used and it was found that maximum fixation was obtained with 1/5 antigen and 1/5 antibody dilutions.

Experiment 30.

In this experiment to determine the optimum antigen-antibody ratio Lancefield's antigen was used. The strain was a group-K streptococcus (Turner). The antigen and antiserum dilutions used were 1/5, 1/10, 1/15, 1/20 and 1/25. Each antigen dilution was tested with each antibody dilution. Incubation was at ice-chest temperature for 18 hours. Six M.H.D. of complement were added to each tube. In all cases the 6 M.H.D. of complement were fixed (Table 35, p.116).

The test was now repeated but in each tube 9 M.H.D. of complement were added. Fixation was complete only in the case of the 1/5 antigen dilution tested with the 1/5 and 1/10 antiserum dilutions. These two antiserum dilutions were now crossed with the 1/5 antigen and 2, 4, 6, 9 and 12 M.H.D. of complement added, but in both cases only 9 M.H.D. of complement were fixed (Table 36, p.117). The precipitin

tests were all negative except with the undiluted reagents.

SUMMARY

From the foregoing results it was decided to use in all future work with the residue substance as antigen equal volumes of the same dilution of both antigen and antibody (1/6). Admittedly, both Dean (1916 - 17) and Goldsworthy (1928) favour an excess of antiserum for maximum fixation, but the above findings did not support their suggestions. Another factor against the use of an antiserum excess is that frequently in this work anti-complementary sera were encountered, and their effect is most simply eliminated by dilution. If the dilution of antiserum was great, the antigen dilution would require to be still greater in order to maintain the excess of serum, and with weak serum there would be little fixation. Consequently, most of the work was done with equal volumes of a 1/6 antigen and 1/6 antibody dilution. To avoid anti-complementary effects of antiserum or antigen greater dilutions were used occasionally but the antigen-antibody ratio of 1/1 was always maintained.

CELLULAR EXTRACTS

Most of the work with cellular antigens was done with an antigen-antibody ratio of 10/1. The following experiment (experiment 31) was designed to test the suitability of this ratio.

Experiment 31.

A group-B strain and its homologous antiserum were used. The strain was grown in lab. lemco broth for 24 hours and the culture was then heated at 56°C for one hour. This heated broth culture constituted the antigen. It was diluted 1/2, 1/4, 1/6, 1/8 and 1/10. The antiserum was diluted 1/5, 1/10, 1/15, 1/20 and 1/25. Each antigen dilution was tested with each antibody dilution. Immediately after the addition of the antibody to the antigen 5 M.H.D. of complement were added and the test placed in the ice-chest for 18 hours. All the tubes showed complete fixation of the 5 M.H.D. of complement (Table 37, p.118).

The test was now repeated in the same manner but with 10 M.H.D. of complement. Fixation was complete in all tubes except those containing a 1/10 antigen dilution. These tubes showed only a trace of lysis, indicating marked fixation of complement (Table 37, p.118).

Experiment 32.

A group-A, type-1 strain was grown in lab. lemco broth

for 24 hours at 37°C and the culture was then heated at 56°C for one hour. It was then diluted 1/2 and 1/5 and complement-fixation tests were done with the homologous antiserum diluted 1/20. More complement was fixed when the antigen dilution of 1/2 was used (Table 38, p.119).

SUMMARY

No further antigen-antibody ratio tests with cellular antigens were done, but the dilutions chosen for future use were 1/2 of antigen and 1/20 of antiserum. The reasons for this choice were: (1) it would allow continuity of the work as these were the dilutions used previously, (2) it would save antiserum and (3) greater dilution of the antigen than 1/2 gave a very thin suspension which was not always a potent antigen.

RESULTS OF ANTIGEN-ANTIBODY RATIO EXPERIMENTS

AT ICE-CHEST TEMPERATURES

Table 31

Experiment 28. The optimum antigen-antibody ratio in complement fixation.

Antigen* Hitchcock's preparation. Group A, type 1.
Antiserum rabbit no. 17. Homologous.
Complement 3 M.H.D. in each tube, both test and controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

Antigen-antibody-complement mixture held 18 hours at 2°C.

Results.**

	Antigen					
Antiserum	0.0001	0.001	0.01	0.1	0.2	0.3
0.0001	2	0	1	0	0	1
0.001	0	0	0	0	2	0
0.01	0	0	0	1	3	4
0.1	0	0	3	4	4	4
0.2	0	0	0	4	4	4
0.3	0	0	0	4	4	4

Controls, antigen and antibody, of all the above dilutions were negative.

Interpretation: Fixation occurs in low dilutions of antigen and antiserum and with antigen excess -- 0.3 antigen and 0.01 antiserum.

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* The different types of antigen are defined on pp. 36 - 43.

** For explanation of symbols see p. 46.

Table 32

Experiment 28. The optimum antigen-antibody ratio in complement fixation.

Details as in Table 31 (page 112) but each antigen-antibody cross extended to four tubes containing 2, 4, 6 and 9 M.H.D. of complement.

Results.

Antigen	Antibody	M.H.D. of complement fixed
0.1	0.1	9
0.1	0.2	9
0.1	0.3	9
0.2	0.1	9
0.2	0.2	9
0.2	0.3	9
0.3	0.1	9
0.3	0.2	9
0.3	0.3	9
0.3	0.01	9

Controls, antigen and antibody, of all the above dilutions were negative.

Interpretation: Both with antigen and with antibody excess 9 M.H.D. of complement are fixed.

Table 33

Experiment 29. The optimum antigen-antibody ratio in complement fixation.

Antigen Hitchcock's preparation. Group A, type 1.
Antiserum rabbit no. 80. Homologous.
Complement 10 M.H.D. in each tube of test; 3 M.H.D. in control tubes.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

Antigen-antibody-complement mixture held 18 hours at 1°C.

Results.

Antiserum	Antigen 1/5	1/10	1/15	1/20	1/25
1/5	4	4	4	4	4
1/10	4	4	4	4	4
1/15	4	4	4	4	4
1/20	1	3	3	3	0
1/25	0	2	2	2	2

Controls, antigen and antibody, of all the above dilutions were negative.

Interpretation: Fixation occurs with low dilutions of antigen and antiserum and with antiserum excess -- 1/5 antiserum and 1/25 antigen

Table 34

Experiment 29. The optimum antigen-antibody ratio in complement fixation.

Details as in Table 33 (page 114) but each antigen-antibody cross extended to seven tubes, containing 3, 6, 9, 12, 15, 20 and 25 M.H.D. of complement.

Results.

Antigen	Antibody	M.H.D. of complement fixed
1/5	1/5	25
1/5	1/10	9
1/5	1/15	9
1/10	1/10	9
1/10	1/15	9
1/15	1/10	9
1/15	1/15	9
1/20	1/10	9
1/20	1/15	9
1/25	1/10	9
1/25	1/15	9
1/25	1/20	9

Controls, antigen and antibody, of all the above dilutions were negative.

Interpretation: Maximum fixation is obtained with a 1/5 antigen and a 1/5 antibody dilution.

Table 35

Experiment 30. The optimum antigen-antibody ratio in complement fixation.

Antigen Lancefield preparation. Group K, strain Turner.
Antiserum rabbit no. 34. Homologous.
Complement 6 and 9 M.H.D. used in test; 3 M.H.D. used in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

Antigen-antibody-complement mixture held 18 hours at 1°C.

Results.

		Antigen									
		1/5		1/10		1/15		1/20		1/25	
	M.H.D. of Comp.	6	9	6	9	6	9	6	9	6	9
Antiserum	1/5	4	4	4	4	4	3	4	2	4	2
	1/10	4	4	4	3	4	3	4	2	4	1
	1/15	4	3	4	3	4	3	4	2	4	1
	1/20	4	1	4	1	4	1	4	1	4	1
	1/25	4	0	4	0	4	0	4	0	4	0

Controls, antigen and antibody, of all the above dilutions were negative.

Interpretation: 9 M.H.D. of complement fixed by the same antigen and antibody dilutions and also by an antigen excess -- 1/5 antigen and 1/10 antiserum.

Table 36

Experiment 30. The optimum antigen-antibody ratio in complement fixation.

Details as in Table 35 (page 116) but
(1) antigen diluted 1/5 and antiserum diluted 1/5 and
(2) antigen diluted 1/5 and antiserum diluted 1/10.
Each antigen-antibody cross extended to six tubes containing 6, 9, 12, 15, 20 and 25 M.H.D. of complement.

Results.

(1)	4	4	4	4	1	0
(2)	4	4	4	4	1	0

Controls, antigen and antibody, of the above dilutions were negative.

Interpretation: The same amount of complement is fixed using the same antigen and antibody dilutions and using an antigen excess.

Table 37

Experiment 31. The optimum antigen-antibody ratio in complement fixation.

Antigen B2 (group B, strain 80RH).
Antiserum rabbit no. 81. Homologous.
Complement 5 and 10 M.H.D. used in test; 3 M.H.D. used in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

Antigen-antibody-complement mixture held 18 hours at 1°C.

Results.

		Antigen									
		1/2		1/4		1/6		1/8		1/10	
	M.H.D. of Comp.	5	10	5	10	5	10	5	10	5	10
Antiserum	1/5	4	4	4	4	4	4	4	4	4	3
	1/10	4	4	4	4	4	4	4	4	4	3
	1/15	4	4	4	4	4	4	4	4	4	3
	1/20	4	4	4	4	4	4	4	4	4	3
	1/25	4	4	4	4	4	4	4	4	4	3

Controls, antigen and antibody, of all the above dilutions, were negative.

Interpretation: With increased antigen dilution the amount of complement fixed commences to diminish.

Table 38

Experiment 32. The optimum antigen-antibody ratio in complement fixation.

Antigen B2 (group A, type 1). (1) Diluted 1/2; (2) diluted 1/5.
Antiserum rabbit no. 80. Homologous. Diluted 1/20.
Complement 2, 4, 6 and 9 M.H.D. used in test; 2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

Antigen-antibody-complement mixture held 18 hours at $1^{o}C$.

Results.

	Test				Controls			
					Antigen		Antibody	
(1)	4	4	4	4	0	0	0	0
(2)	4	2	0	0	0	0	0	0

Interpretation: With increased antigen dilution the amount of complement fixed commences to diminish.

SECTION X

FURTHER EXPERIMENTS WITH CELLULAR ANTIGENS, BUT WITH INCUBATION OF THE ANTIGEN-ANTIBODY-COMPLEMENT MIXTURE AT ICE-CHEST TEMPERATURES

FURTHER EXPERIMENTS WITH CELLULAR ANTIGENS, BUT WITH INCUBATION OF THE ANTIGEN-ANTIBODY-COMPLEMENT MIXTURE AT ICE-CHEST TEMPERATURES

Since holding the antigen-antibody-complement mixture at ice-chest temperatures results in more complement being fixed than when the mixture is incubated at 37°C, some experiments previously carried out with whole-broth cultures (B1, B2, B3, Sg and S, pages 38 - 39) were now done at low temperatures; at the same time some new experiments were introduced.

Experiment 33.

Kraus (1897) found that many bacteria when grown in broth elaborated in the medium substances which gave precipitin reactions with appropriate antisera. In 1917 Dochez and Avery made a similar finding with pneumococci. Although it is well known that streptococci do not autolyse, yet I felt that, in discarding supernatants from broth cultures when preparing cellular antigens, I was possibly losing much valuable antigenic material.

To test this hypothesis a group-A, type-1 strain was inoculated into lab. lemco broth, which was incubated at 37°C for 24 hours. The culture was then heated at 56°C for one hour and divided into two portions, one of which corresponded to a whole-broth antigen (B2, page 39). The other portion was centrifuged and the supernatant removed. The deposit was suspended in 0.85% saline and made up to the original volume. There were thus three antigens: (1) the whole-broth antigen; (2) the deposit suspended in saline; and (3) the supernatant. Complement-fixation tests were carried out. The same tests were repeated with a group-G strain. This experiment showed clearly that discarding the supernatant fluid meant the discarding of antigenic material (Table 39, p.126).

Experiment 34.

This experiment was a repeat of experiment 33 but the broth cultures were incubated for 72 hours instead of 24 hours. The results in Table 40 (page 127) again show that discarding the supernatant fluid meant discarding antigenic material. Further, it is seen that prolongation of the incubation period of the culture does not result in any great increase in the antigenic property of the culture.

Comparing the results of Tables 39 and 40 (pages 126 and 127) it will be seen that with the group-A, type-1

strain, when the incubation period of the culture was increased from 24 to 72 hours, the supernatant fraction fixed more complement and the saline suspension fixed less. This is in accordance with the theory that antigen is released into the surrounding medium.

Experiment 35.

This experiment was a repetition of experiment 5 (pages 50 - 51) but carried out at low temperatures. The results (Table 41, p.128) are comparable to those of experiment 5 and show that under the conditions of testing, the antigenic content of the broth was not increased by changing the pH of the medium or by adding glucose.

Experiment 36.

This experiment was designed to determine if prolonged incubation of test cultures resulted in increased antigen elaboration as demonstrated by complement-fixation tests. A group-A, type-1 strain was seeded into lab. lemco broth which was incubated at 37°C for 168 hours. The antigenic content of the culture was traced by withdrawing, heating at 56°C for one hour, and testing aliquots of the culture after 24, 48, 72, 96, 120 and 168 hours' incubation. In each case the cocci were Gram-positive and viable. A similar experiment was done with a group-C and a group-G strain.

As will be seen from the results in Table 42 (pages 129 - 130), prolongation of the incubation time of the cultures did not result in any marked increase in the complement-fixing powers of the antigen. With the group-A, type-1 strain maximum fixation was obtained with an antigen prepared from a 72-hour culture but the results with a 48-hour culture were almost as good. With the group-G strain a 120-hour culture gave the best results, although these results were only slightly better than those obtained with a 24-hour antigen. With the group-C strain there was an increase in the fixing capacity of the antigens up to the 96-hour antigen and after this the fixing property of the antigen decreased. However, the 24-hour antigen was nearly as powerful a fixer of complement when tested with the homologous antiserum as the 96-hour antigen. Further, as previously mentioned, it is generally desirable for practical purposes to use an antigen which may be prepared with a minimum of delay.

Experiment 37.

This was a repeat of experiment 7 (pages 51 - 52) but the antigen-antibody-complement mixtures were held at 1°C for 18 hours. As at 37°C a better antigen was obtained in lab. lemco broth than in glucose broth (Table 43, p.131).

Experiment 38.

This was a repeat of experiment 8 (pages 52 - 53) in which the suitability of different media in preparing an antigen were compared. From Table 44 (page 132) it will be seen that as in the case of incubation of the antigen-antibody-complement mixture at 37°C the glucose-broth culture proved the best antigen. Little difference was noted between the antigenic qualities of the horse-heart digest and horse-heart extract media. Once more it was noted that greatly increased prolongation of the period of incubation of the cultures did not lead to an improvement in the antigens.

SUMMARY

All the experiments described in this section were carried out with cellular antigens at ice-chest temperature.

It was shown that discarding the supernatant meant discarding valuable antigenic material. Further it was found that with whole-broth cultures as antigens, the best medium for antigen production was lab. lemco broth. Next in efficacy was 1.0% glucose broth. Horse-heart extract and horse-heart digest media were alike and inferior in quality.

Prolongation of the incubation period of the culture up to 48 hours usually resulted in a superior antigen, but in some cases this was not so. Even when the complement-fixing powers of the antigen were increased with prolonged incubation of the culture, it is doubtful whether the increased fixation obtained warranted the increased incubation time required.

[illegible]

[illegible]

[illegible]

RESULTS OF EXPERIMENTS WITH CELLULAR ANTIGENS WHEN INCUBATION OF THE ANTIGEN-ANTIBODY-COMPLEMENT MIXTURE IS AT ICE-CHEST TEMPERATURES

Table 39

Experiment 33. The complement-fixing capacity of whole cultures and different culture fractions.

Antigens (1) B2*, (2) B2 centrifuged and the deposit suspended in saline, (3) the supernatant from B2. Group A, type 1. Diluted 1/2. Culture 24 hours.
Antiserum rabbit no. 55. Homologous. Diluted 1/20.
Complement 2, 4, 6, 9 and 12 M.H.D. used in test; 2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

Antigen-antibody-complement mixture held 18 hours at $1^{\circ}C$.

Results.**

	Test					Controls			
						Antigen		Antibody	
(1)	4	4	4	4	0	0	0	0	0
(2)	4	4	2	0	0	0	0	0	0
(3)	4	4	4	2	0	0	0	0	0

Interpretation: The supernatant fluid discarded in making antigen B2 (page 39) contains reacting antigenic material.

The same experiment was repeated using strain G1B of group G and the homologous antiserum from rabbit no. 44.

Results.

(1)	4	4	4	4	0	0	0	0	0
(2)	4	4	1	0	0	0	0	0	0
(3)	4	4	4	0	0	0	0	0	0

Interpretation: As above.

* The different types of antigen are defined on pp. 36 - 43.

** For explanation of symbols see p. 46.

Table 40

Experiment 34. The complement-fixing capacity of whole cultures and different culture fractions.

Details as in experiment 33, Table 39, page 126, but the cultures from which the antigens were prepared had been incubated for 72 hours. Complement doses extended to 15 M.H.D.

Results.

Group-A strain.

	Test						Controls			
							Antigen		Antibody	
(1)	4	4	4	4	4	1	0	0	0	0
(2)	4	2	0	0	0	0	0	0	0	0
(3)	4	4	4	4	1	0	0	0	0	0

Group-G strain.

(1)	4	4	4	4	2	0	0	0	0	0
(2)	4	4	3	0	0	0	0	0	0	0
(3)	4	4	4	1	0	0	0	0	0	0

Interpretation: The supernatant fluid discarded in making antigen B2 (page 39) contains reacting antigenic material. Increasing the incubation time of the culture from 24 to 72 hours results in the supernatant fixing more complement and the saline suspension less.

Table 41

Experiment 35. The effects of glucose and pH on antigen production.

Antigens S and Sg (group A, type 1). Diluted 1/2.
(1) Sorensen's buffer pH 6.0 + 1.0% glucose
(2) " " pH 6.0
(3) " " pH 7.2 + 1.0% glucose
(4) " " pH 7.2
(5) " " pH 8.0 + 1.0% glucose
(6) " " pH 8.0
Antiserum rabbit no. 80. Homologous. Diluted 1/20.
Complement 2, 4, 6 and 9 M.H.D. used in test; 2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

Antigen-antibody-complement mixture held 18 hours at 1°C.

Results.

	Test				Controls			
					Antigen		Antibody	
(1)	4	4	4	4	3	0	0	0
(2)	4	4	4	4	3	0	0	0
(3)	4	4	4	3	0	0	0	0
(4)	4	4	4	3	0	0	0	0
(5)	4	4	4	3	0	0	0	0
(6)	4	4	4	3	0	0	0	0

Interpretation: Neither glucose nor different pH appears to affect antigen production although the results with antigens (1) and (2) are difficult to interpret because of the anti-complementary effects of the antigen and because no end-point is reached.

Table 42

Experiment 36. The effect of different incubation periods on the quality of the antigen.

Antigen B2 (group A, type 1). Diluted 1/2.
Antiserum rabbit no. 55. Homologous. Diluted 1/20.
Complement 2, 4, 6, 9, 12, 15 and 20 M.H.D. used in test; 2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

Antigen-antibody-complement mixture held 18 hours at 1°C.

(1) antigen 24 hour culture
(2) antigen 48 hour culture
(3) antigen 72 hour culture
(4) antigen 96 hour culture
(5) antigen 120 hour culture
(6) antigen 168 hour culture

Results.

	Test							Controls			
								Antigen		Antibody	
(1)	4	4	4	4	3	1	0	0	0	0	0
(2)	4	4	4	4	4	3	0	0	0	0	0
(3)	4	4	4	4	4	4	0	0	0	0	0
(4)	4	4	4	4	4	4	0	0	0	0	0
(5)	4	4	4	4	4	4	1	0	0	0	0
(6)	4	4	4	4	4	4	1	0	0	0	0

The same experiment was repeated using strain G1B of group G and the homologous antiserum from rabbit no. 44.

(1)	4	4	4	4	0	0	0	0	0	0	0
(2)	4	4	4	4	1	0	0	0	0	0	0
(3)	4	4	4	4	2	0	0	0	0	0	0
(4)	4	4	4	4	2	0	0	0	0	0	0
(5)	4	4	4	4	4	0	0	0	0	0	0
(6)	4	4	4	4	4	0	0	0	0	0	0

Table 42 (continued)

The same experiment was repeated using strain E4678 of group C and the homologous antiserum from rabbit no. 53.

(1)	4	4	4	2	0	0	0	0	0	0	0
(2)	4	4	4	2	0	0	0	0	0	0	0
(3)	4	4	4	2	0	0	0	0	0	0	0
(4)	4	4	4	4	2	0	0	0	0	0	0
(5)	4	4	4	3	0	0	0	0	0	0	0
(6)	4	4	4	3	0	0	0	0	0	0	0

Interpretation: Prolongation of the incubation time of the cultures from which antigens are prepared does not result in any marked increase in the complement-fixing powers of the antigen.

Table 43

Experiment 37. Comparison of glucose and lab. lemco broth cultures as antigens and the effect of different incubation periods on the quality of the antigen.

Antigens group A, type 3. (1) B2. Diluted 1/2.
(2) B1. Diluted 1/5.
Antiserum rabbit no. 55. Homologous. Diluted 1/20.
Complement 2, 4, 6 and 9 M.H.D. used in test; 2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

Antigen-antibody-complement mixture held 18 hours at 1°C.

Results.

	Test				Controls			
					Antigen		Antibody	
After 18 hours' incubation of cultures.								
(1)	4	4	4	0	0	0	0	0
(2)	4	4	4	0	2	0	0	0
After 42 hours' incubation of cultures.								
(1)	4	4	4	0	1	0	0	0
(2)	4	4	2	0	0	0	0	0
After 48 hours' incubation of cultures.								
(1)	4	4	4	4	0	0	0	0
(2)	4	4	4	4	0	0	0	0
After 72 hours' incubation of cultures.								
(1)	4	4	4	4	4	2	0	0
(2)	4	4	4	3	4	2	0	0
After 120 hours' incubation of cultures.								
(1)	4	4	4	4	0	0	0	0
(2)	4	4	4	0	0	0	0	0

Interpretation: Lab. lemco broth antigens are slightly superior to those prepared from glucose broth. In both cases prolonging the incubation period up to 48 hours results in a slightly more potent antigen.

Table 44

Experiment 38. Comparison of different media used in preparing antigens and the effect of different incubation periods on the quality of the antigen.

Antigen group A, type 3. (1) B3. Diluted 1/15.
(2) B1. Diluted 1/5.
(3) B4. Diluted 1/5.

Antiserum rabbit no. 55. Homologous. Diluted 1/20.
Complement 2, 4, 6 and 9 M.H.D. used in test; 2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

Antigen-antibody-complement mixture held 18 hours at 1°C.

Results.

	Test				Controls			
					Antigen		Antibody	
After 24 hours' incubation of cultures.								
(1)	4	4	1	0	2	0	0	0
(2)	4	4	4	3	0	0	0	0
(3)	4	4	0	0	2	0	0	0
After 72 hours' incubation of cultures.								
(1)	4	3	0	0	2	0	0	0
(2)	4	4	4	3	2	0	0	0
(3)	4	4	3	0	2	0	0	0
After 120 hours' incubation of cultures.								
(1)	4	4	0	0	0	0	0	0
(2)	4	4	4	2	0	0	0	0
(3)	4	4	0	0	0	0	0	0

Interpretation: Glucose-broth antigens are superior to horse-heart extract broth antigens and horse-heart digest broth antigens. Prolonged incubation beyond 24 hours does not result in any marked increase in the potency of the antigens.

SECTION XI

THE SPECIFICITY OF COMPLEMENT-FIXATION TESTS WITH CELLULAR ANTIGENS

THE SPECIFICITY OF COMPLEMENT-FIXATION TESTS WITH CELLULAR ANTIGENS

The best cellular antigen of those tested was obtained by growing the streptococci in lab. lemco broth for 24 hours at 37°C and heating the cultures for one hour at 56°C (antigen B2, page 39). Such cultures were potent antigens, easily prepared and not anti-complementary. It now became necessary to consider their specificity.

STERILE MEDIA

Experiment 39.

Bliss (1938) found that extracts of streptococci which had been grown in media containing certain peptones gave cross-precipitin tests. I determined if the broth in which I grew my antigens was responsible for pseudo-fixation by diluting sterile lab. lemco broth one in two and testing it with 7 rabbit antisera. In all but one case 2 M.H.D. of complement were fixed (Table 45, p.138).

Experiment 40.

To determine if organisms other than streptococci fixed

complement when tested with streptococcal antisera, antigen B2 (page 39) was prepared from a Staphylococcus aureus strain and tested with both a group-A and a group-B antiserum. The test was carried out (a) incubating the antigen-antibody-complement mixture for 1-1/4 hours at 37°C and (b) holding the mixture for 18 hours at 1°C. Non-specific fixation was noted at both temperatures although it was more marked at ice-chest temperature (Table 46, p.139).

Experiment 41.

A potent group-A, type-1 antigen (antigen B2) was tested with 18 normal rabbit sera. The average fixation at 37°C was approximately 3 M.H.D. of complement and at ice-chest temperatures the average fixation was approximately 4 M.H.D. of complement. Three sera fixed 6 M.H.D. of complement both at ice-chest temperatures and incubator temperature (Table 47, pp.140 - 141). Serum W6 tested with sterile lab. lemco broth diluted 1/2 fixed 6 M.H.D. of complement at 37°C and 6 M.H.D. of complement at ice-chest temperature (Table 48, p.142).

The above experiments show that non-specific fixation or pseudo-fixation of complement, or possibly both, occur when cellular antigens obtained from lab. lemco broth are used. Such fixation does not appear to be due to the presence of streptococcal antibodies in the sera of normal rabbits but

to a factor in lab. lemco broth antigens, which when tested with normal sera fixed complement. Even sterile lab. lemco broth fixed complement when tested with such sera.

CROSS COMPLEMENT-FIXATION TESTS

Experiment 42.

Cross complement-fixation tests were now done with cellular antigens (antigen B2, page 39) of streptococci of several Lancefield groups, and antisera prepared against strains of groups A, B, C, E, K and N. This was merely a random selection of groups necessary because of the shortage of complement at the time. The results are shown in Table 49 (pages 143 - 145). From this table it is evident that clear-cut interpretation of the results is difficult, even although in certain cases it is possible to group correctly an antiserum or an antigen. Thus, antisera of groups B, E and K fixed much more complement when tested with their homologous antigens than when tested with heterologous antigens. Antiserum of group A however, showed fixation not only with the homologous antigen, but also to practically the same end-point with the antigen of group L. Antiserum of group C fixed the same amount of complement when tested with the

antigen of group L, as when tested with the homologous antigen. Antiserum of group N gave cross-reactions to practically the titre of the serum with several antigens.

Antigens of groups B and K showed fixation with their specific immune sera to a much greater extent than with other sera, and this was also found, although to a lesser extent, with antigen of group N. However, antigen of group C fixed the same amount of complement, more or less, irrespective of the serum with which it was tested.

The cross-reactions did not seem to follow any particular pattern. Further, the results could not be allied to those obtained when precipitin tests were carried out with the anti-sera and extracts of the organisms used in the cross-fixation tests. It is possible that some of the cross-fixations noted are due to diffusion of antigens from the streptococcal cells into the surrounding culture media and that we are indeed observing previously unknown serological relationships.

It has already been shown that using cellular antigens some pseudo-fixation occurs, which results in approximately 3 - 4 M.H.D. of complement being fixed irrespective of the antigen and antiserum used. If this could be eliminated the cross-fixation results would be much clearer. Thus, with antisera of groups B and K, fixation would be nearly specific if 4 M.H.D. of fixation were eliminated from each antigen

tested. This is only a hypothesis as absorption tests were not done; in fact, technical difficulties loomed so great that no attempt was made to eliminate the cross-fixation.

SUMMARY

Complement-fixation tests were done with whole-broth cultures as antigens. Pseudo-fixation and non-specific fixation both occurred frequently, interfering with cross-fixation results and rendering their interpretation difficult.

RESULTS OF SPECIFICITY TESTS WITH CELLULAR ANTIGENS

Table 45

Experiment 39. The complement-fixing powers of sterile lab. lemco broth.

Antigen B2 (sterile lab. lemco broth). Diluted 1/2.
Antisera. From 7 immunized rabbits. Diluted 1/20.
Complement 2, 4, 6 and 9 M.H.D. used in test; 2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

Antigen-antibody-complement mixture held 18 hours at 1°C.

Results.**

Antiserum Rabbit no.	Lancefield group	Test				Controls Antigen		Controls Antibody	
55	A	4	1	0	0	0	0	0	0
81	B	4	0	0	0	0	0	0	0
83	E	3	0	0	0	0	0	0	0
15	F	4	0	0	0	0	0	0	0
101	H	4	3	0	0	0	0	0	0
82	K	4	0	0	0	0	0	0	0
25	L	4	0	0	0	0	0	0	0

Interpretation: Sterile lab. lemco broth used in preparing antigen B2 (page 39) fixes complement when tested with antisera prepared against strains of different Lancefield groups.

** For explanation of symbols see p. 46.

Table 46

Experiment 40. Complement fixation with a staphylococcal antigen and streptococcal antisera.

Antigen B2 (Staphylococcus aureus). Diluted 1/2.
Antisera (1) rabbit no. 75. Group-B antiserum. Diluted 1/20.
(2) rabbit no. 80. Group-A antiserum. Diluted 1/20.
Complement 2, 4, 6 and 9 M.H.D. used in test; 2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

(a) Antigen-antibody-complement mixture held 1-1/4 hours at 37°C.
(b) " " " " " 18 hours at 1°C.

Results.

	Test				Controls			
					Antigen		Antibody	
(1a)	3	0	0	0	0	0	0	0
(1b)	4	4	2	0	0	0	3	0
(2a)	3	0	0	0	0	0	0	0
(2b)	4	4	0	0	0	0	0	0

Interpretation: A staphylococcal antigen prepared from lab. lemco broth (antigen B2, page 39) when tested with streptococcal antisera fixes complement.

Table 47

Experiment 41. The complement-fixing powers of normal sera tested with a cellular streptococcal antigen.

Antigen B2 (24-hour lab. lemco broth culture). Diluted 1/2.
Sera from normal (uninoculated) rabbits. Diluted 1/20.
Complement 2, 4, 6 and 9 M.H.D. used in test; 2 and 4 M.H.D. used in controls.

Each tube, except the control tubes contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

(1) Antigen-antibody-complement mixture held for 1-1/4 hours at 37°C.
(2) Antigen-antibody-complement mixture held for 18 hours at 1°C.

Results.

Serum Number		Test				Controls			
						Antigen		Antibody	
2	(1)	4	4	4	0	0	0	0	0
	(2)	4	4	2	0	2	0	2	0
60	(1)	4	0	0	0	0	0	0	0
	(2)	4	3	0	0	0	0	0	0
61	(1)	4	0	0	0	0	0	0	0
	(2)	4	3	0	0	0	0	0	0
137	(1)	0	0	0	0	0	0	0	0
	(2)	4	2	0	0	0	0	0	0
59	(1)	4	2	0	0	0	0	0	0
	(2)	4	3	0	0	2	0	1	0
58	(1)	4	0	0	0	0	0	0	0
	(2)	4	0	0	0	0	0	0	0
1	(1)	3	0	0	0	2	0	2	0
	(2)	4	2	0	0	2	0	2	0
W2	(1)	4	1	0	0	0	0	0	0
	(2)	4	4	0	0	0	0	0	0
W3	(1)	4	0	0	0	0	0	0	0
	(2)	3	0	0	0	0	0	0	0
W4	(1)	4	4	4	0	0	0	0	0
	(2)	4	4	4	0	0	0	0	0

Table 47 (continued)

Serum Number		Test				Controls Antigen		Controls Antibody	
W5	(1)	4	4	0	0	0	0	0	0
	(2)	4	3	0	0	0	0	0	0
W6	(1)	4	4	4	3	0	0	0	0
	(2)	4	4	4	2	0	0	4	0
101	(1)	3	0	0	0	0	0	0	0
	(2)	4	2	0	0	0	0	0	0
56	(1)	4	1	0	0	0	0	2	0
	(2)	4	4	0	0	0	0	4	0
5	(1)	4	0	0	0	0	0	2	0
	(2)	4	0	0	0	0	0	0	0
17	(1)	4	0	0	0	0	0	0	0
	(2)	4	2	0	0	0	0	0	0
44	(1)	2	0	0	0	0	0	0	0
	(2)	4	0	0	0	0	0	0	0
34	(1)	4	1	0	0	0	0	0	0
	(2)	4	4	1	0	0	0	0	0

Interpretation: Cellular streptococcal antigens tested with normal sera fix complement.

Table 48

Experiment 41. The complement-fixing powers of a normal serum and sterile lab. lemco broth.

Antigen B2 (sterile lab. lemco broth). Diluted 1/2.
Serum no. W6 from a normal (uninoculated) rabbit. Diluted 1/20.
Complement 2, 4, 6 and 9 M.H.D. used in test; 2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

(1) Antigen-antibody-complement mixture held 1-1/4 hours at 37°C.
(2) " " " " " 18 hours at 1°C.

Results.

	Test				Controls			
					Antigen		Antibody	
(1)	4	4	4	0	0	0	0	0
(2)	4	4	4	2	0	0	4	2

Interpretation: Sterile lab. lemco broth used in preparing antigen B2 (page 39) fixes complement when tested with a serum from a normal (uninoculated) rabbit.

Table 49

Experiment 42. Cross-fixation with streptococcal antisera and broth cultures as antigen.

Antigens B2 (24-hour lab. lemco broth cultures). Diluted 1/2.

Group A, type 1
Group B, 80RH
Group C, Azgazdarah
Group D, 98D
Group E, EC24B
Group F, 351
Group K, Turner
Group L, LXB
Group M, 205
Group N, 201

Antisera. Homologous to the above. Diluted 1/20.
Complement 2, 4, 6, 9 and 12 M.H.D. used in test; 2 and 4 M.H.D. used in controls.

Each tube except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

Antigen-antibody-complement mixture held for 18 hours at 1°C.

Results.

Antigen   Antiserum group A, rabbit no. 78

Antigen	Test					Controls: Antigen		Controls: Antibody	
A	4	4	4	4	4	1	0	2	0
B	4	4	2	0	0	0	0		
C	4	4	3	0	0	0	0		
D	4	4	0	0	0	0	0		
F	4	4	0	0	0	0	0		
K	4	4	0	0	0	0	0		
L	4	4	4	4	0	2	0		

Table 49 (continued)

Antigen	Antiserum group B, rabbit no. 81								
	Test					Controls			
						Antigen		Antibody	
A	4	4	4	0	0	1	0		
B	4	4	4	4	4	0	0	0	0
C	4	4	4	1	0	0	0		
D	4	4	1	0	0	0	0		
F	4	4	1	0	0	0	0		
K	4	4	0	0	0	0	0		
L	4	4	0	0	0	2	0		
M	4	3	0	0	0	0	0		
N	4	3	0	0	0	0	0		

Antigen	Antiserum group C, rabbit no. 53								
	Test					Controls			
						Antigen		Antibody	
A	4	4	0	0	0	1	0		
B	4	2	0	0	0	0	0		
C	4	4	4	0	0	0	0	0	0
D	4	2	0	0	0	0	0		
F	4	4	0	0	0	0	0		
K	4	4	0	0	0	0	0		
L	4	4	4	0	0	2	0		

Antigen	Antiserum group E, rabbit no. 83								
	Test					Controls			
						Antigen		Antibody	
A	4	4	4	2	0	1	0		
B	4	4	3	0	0	0	0		
C	4	4	4	0	0	0	0		
D	4	3	0	0	0	0	0		
E	4	4	4	4	4	0	0	3	0
F	4	4	2	0	0	0	0		
N	4	2	0	0	0	0	0		

Table 49 (continued)

Antigen	Antiserum group K, rabbit no. 82								
	Test					Controls			
						Antigen		Antibody	
A	4	4	0	0	0	1	0		
D	4	1	0	0	0	0	0		
F	4	2	0	0	0	0	0		
K	4	4	4	4	2	0	0	2	0
L	4	4	0	0	0	2	0		
M	4	2	0	0	0	0	0		
N	4	3	0	0	0	0	0		

Antigen	Antiserum group N, rabbit 101								
	Test					Controls			
						Antigen		Antibody	
A	4	4	4	0	0	0	0		
B	4	4	0	0	0	0	0		
D	4	4	0	0	0	0	0		
F	4	4	2	0	0	0	0		
K	4	2	0	0	0	0	0		
L	4	4	3	0	0	2	0		
N	4	4	4	0	0	0	0	3	0

Interpretation: Some antisera fix greater amounts of complement with homologous antigens than with heterologous antigens. However, clear-cut interpretation of the cross-fixation results is difficult.

SECTION X11

THE SPECIFICITY OF COMPLEMENT-FIXATION TESTS WITH SOLUBLE EXTRACTS AS ANTIGEN

THE SPECIFICITY OF COMPLEMENT-FIXATION TESTS WITH SOLUBLE EXTRACTS AS ANTIGEN

I had now found that streptococcal antigens prepared as described by Hitchcock (1924a) (pages 39 - 41) are suitable for both the precipitin and complement-fixation tests; and I had determined a technique whereby these antigens could be used along with homologous antisera and complement in a complement-fixation test. I next began experiments to determine the specificity of the antigen and antibody. The fixation which had been observed in the preliminary ice-chest experiments with the Hitchcock antigen could be attributed to various reasons -- non-specific factors in the antiformin, a nucleo-protein or other compound common to different bacterial genera, or a common compound possessed by different species of streptococci. The sera themselves might be non-specific. All these hypotheses were tested in turn.

Experiment 43.

This experiment was designed to determine if fixation with the Hitchcock antigen was pseudo-fixation caused by the

extracting antiformin.

5.0 ml. of 2.0% antiformin solution (the available chlorine in solution was equivalent to 1.7 ml. of standard thiosulphate) was heated in a water bath at 56°C for 10 minutes with frequent agitation. The solution was then treated in the same manner as the desiccated, ground, extracted bacteria in Hitchcock's preparation (pages 39 - 41). The final solution was tested with a streptococcal antiserum of group A (Table 50, p.154a).

There was no fixation of complement and precipitin tests were also negative.

Experiment 44.

A culture of Staphylococcus aureus was extracted with antiformin by Hitchcock's method and tested with a streptococcal antiserum of group A. A very small amount of complement was fixed and precipitin tests were negative (Table 51, p.155).

Experiment 45.

Two viridans strains of streptococci (nos. 2046 and 2048) were extracted with antiformin by Hitchcock's method. These antigens were tested with a group-A antiserum. Strain 2048 fixed 2 M.H.D. of complement and with strain 2046 no fixation occurred. Precipitin tests were also negative (Table 52, p.156).

Experiment 46.

A potent Hitchcock antigen of group A, type 1 was tested with 18 normal rabbit sera (Table 53, pp.157 - 158). With no serum was more than 2 M.H.D. of complement fixed. The average fixation at 37°C was approximately 1 M.H.D. of complement and at ice-chest temperatures approximately 1.5 M.H.D. Precipitin tests in parallel were all negative.

Experiment 47.

This experiment was to determine whether complement was fixed by an unabsorbed antiserum and Hitchcock antigens of strains of the same group but of different type. A group-A, type-1 antiserum was tested with antigens prepared from group-A, types 1, 3 and 4 strains. Twenty M.H.D. of complement, (the maximum amount tested) was fixed in every case, and it may be concluded that the reagents used in this experiment were not type specific (Table 54, p.159).

Experiment 48.

Antisera were prepared against all the Lancefield groups (A - N). Hitchcock extracts were prepared from the homologous strains. The strains were:

Group A, type 1	Group G, SHC/188
Group B, 80RH	Group H, Challis
Group C, Azgazdarah	Group K, Turner
Group D, 98D	Group L, LXB
Group E, EC24B	Group M, 205
Group F, 351	Group N, 201

Complement-fixation tests were carried out, crossing each antigen with each antiserum. The full results of these tests are shown in Table 55 (pages 160 - 166). In the main, the reactions are perfectly clear cut, each antigen and its homologous antiserum (except group K) fixing a large amount of complement, and no complement, or only a small amount, being fixed when heterologous antigens and antisera were tested.

However, there were some cross-reactions to what might be considered a significant titre. Thus, the group-H antiserum when tested with the group-M antigen fixed a large amount of complement, and although the amount fixed when tested with the group-G antigen was not so great, it was still significant. Cross-fixation to a relatively high titre occurred between the group-A antiserum and groups E, H, L and N antigens. Group-C and group-D antisera when tested with groups E and H antigens also fixed a considerable amount of complement. However, it must be noted that in every instance, the amount of complement fixed by the homologous antigen and antiserum was greater than that fixed by the heterologous antigen and antiserum; thus, recognition of an unknown antigen or of an unknown antiserum would appear possible. The cross-reactions in no way detract from the specificity of the streptococcal complement-fixation test but merely accentuate the value of the test in detecting minor relationships between the different Lancefield groups. Precipitin tests carried out at the same time as the cross-complement-fixation tests gave essentially similar results on all the main points.

It was still possible that the complement-fixation results were merely manifestations of the particular antigen

and antiserum tested. Other streptococcal antigens and antisera of the same Lancefield groups, prepared in an identical manner, might not mirror the above results. Further cross-complement-fixation tests were therefore done with other antigens and antisera and gave essentially the same results as those already obtained (Tables 55 and 56, pp. 160 - 168).

The failure of the homologous group-K antigen and antiserum to fix complement was unexpected and consequently was further investigated. A Lancefield extract (C) was made of the group-K strain, Turner. Contrary to the negative results with the Hitchcock extract, the Lancefield extract gave a positive precipitin test with the homologous serum. It has previously been mentioned (page 35) that the Lancefield and Hitchcock antigens, although prepared in different ways, both contain the group carbohydrate (C) and are possibly identical. However, the results recorded below cast doubt upon the identity of these two antigens.

Experiment 49.

Hitchcock and Lancefield antigens of a group-K strain, Turner, were tested with the homologous antiserum. Both antigens were prepared from ground desiccated organisms and in each case 1.0 ml. of the final extract was equivalent to 1.0 mg. of dried organisms. With the Hitchcock antigen no

complement was fixed; with the Lancefield antigen 15 M.H.D. of complement were fixed (Table 57, p.169). That the failure of the Hitchcock antigen and homologous antiserum to fix complement was not due to the individual rabbit response was shown by repeating the experiment with another antiserum prepared against the same strain. A similar result was obtained, the Lancefield antigen again fixing 15 M.H.D. of complement and the Hitchcock antigen fixing only 4 M.H.D. of complement (Table 57, p.169).

Experiment 50.

The results of the preceding experiment cast doubt upon the validity of the results in the part of experiment 48 (pp. 148 - 150) in which the group-K antigen or group-K antiserum were used. As the Hitchcock preparation of the group-K strain failed to react with the homologous antiserum it was possible that even although the extract did not react with the sera of other groups, cross-reactions were missed. The antisera used in experiment 48 were therefore each tested with a Lancefield extract of strain Turner. It was found that with this antigen a cross-reaction was obtained with the group-A serum, whereas no reaction was noted when the Hitchcock preparation was used (Tables 55 and 58, pp. 160 - 166 and 170).

Experiment 51.

The results of experiments 48 and 50 (pages 148 - 151) showed that whereas the Hitchcock extract of strain Turner did not react with a group-A antiserum, the Lancefield extract did. This group-A antiserum fixed 30 M.H.D. of complement when tested with the homologous Lancefield and Hitchcock antigens. It seemed improbable, therefore, that the positive reaction between the Lancefield preparation of the group-K strain and the group-A antiserum, as opposed to the negative reaction with this serum and the Hitchcock preparation of the group-K strain, was due to the serum. To test this, the group-A antiserum and Lancefield and Hitchcock extracts of strains of the various Lancefield groups were crossed, and in all cases the results with both antigens were not significantly different (Table 59, p.171).

These experiments indicate that the failure of the Hitchcock extract of the group-K strain, Turner, to fix complement was due to a defective antigen.

Experiment 52.

The setting up of a full complement-fixation test as outlined in experiment 48 (Tables 55 - 56, pp. 160 - 168) is laborious and costly in material. For diagnostic purposes the ideal test is one in which a single tube for

each serum or each antigen being tested is used with a standard amount of complement. A single-tube specificity test was now done. Each antigen and each antiserum used in experiment 48 was diluted one in fifteen; the 12 antigens were each tested with the 12 antisera and 6 M.H.D. of complement were added to each tube. A Lancefield extract of the group-K strain was used in preference to a Hitchcock extract. By using increased antigen and antibody dilutions I hoped that the specificity of the test (with the amount of complement used) would be increased, while at the same time fixation of complement would still occur when the homologous antigen and antibody were mixed. Six M.H.D. of complement was an arbritary figure. The mixture was incubated at 2°C for 4 hours. At the end of this time, after a preliminary "warming-up" period in the 37°C incubator, sensitized cells were added and the test was further incubated for one hour. Controls with 3 M.H.D. of complement were done for each antigen and antiserum. The results in Table 60 (page 172) are clear-cut. Each antiserum fixed complement only when tested with one antigen -- the homologous one.

SUMMARY

Streptococcal complement-fixation occurs when a soluble extract is used as antigen with the homologous antiserum. The

fixation is specific in that it is not found if streptococcal extracts are tested with normal sera or if extracts prepared from streptococcal strains outwith the Lancefield groups (e.g. Streptococcus viridans) are tested with antisera to streptococci within the Lancefield groups.

Cross-complement-fixation tests with the 12 Lancefield groups were carried out. The results were highly specific. With cross-fixation as there was, the amount of complement fixed was not so great as the amount fixed by the homologous antigen and antiserum. It would appear that with the group polysaccharide (residue substance C) as antigen, streptococci of Lancefield groups A - N can be grouped by the complement-fixation test.

RESULTS OF SPECIFICITY TESTS WITH SOLUBLE EXTRACTS AS ANTIGEN

Table 50

Experiment 43. The complement-fixing powers of antiformin.

Antigen. Neutralised antiformin. Diluted 1/6.
Antiserum rabbit no. 78. Group A, type 1. Diluted 1/6.
Complement 2, 4, 6 and 9 M.H.D. used in test; 2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

(1) Antigen-antibody-complement mixture held 1-1/4 hours at 37°C.
(2) " " " " " 18 hours at 1°C.

Results. **

	Test				Controls			
					Antigen		Antibody	
(1)	0	0	0	0	0	0	0	0
(2)	0	0	0	0	0	0	0	0

Interpretation: The antiformin used in preparing the Hitchcock extracts does not, when tested with a streptococcal antiserum, fix complement.

** For explanation of symbols see p. 46.

Table 51

Experiment 44. Complement fixation with a staphylococcal antigen and streptococcal antiserum.

Antigen Hitchcock's preparation. Staphylococcus aureus. Diluted 1/6.
Antiserum rabbit no. 78. Group A, type 1. Diluted 1/6.
Complement 2, 4, 6 and 9 M.H.D. used in test; 2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

(1) Antigen-antibody-complement mixture held 1-1/4 hours at 37°C.
(2) " " " " " 18 hours at 1°C.

Results.

	Test				Controls			
					Antigen		Antibody	
(1)	2	0	0	0	0	0	0	0
(2)	3	0	0	0	3	0	0	0

Interpretation: Little complement is fixed when an extract of a staphylococcus is tested with a streptococcal antiserum.

Table 52

Experiment 45. Complement fixation with *viridans* streptococci and Lancefield group antiserum.

Antigen Hitchcock's preparation. (1) Strain 2046. Diluted 1/6.
(2) Strain 2048. Diluted 1/6.
Antiserum rabbit no. 78. Group A, type 1. Diluted 1/6.
Complement 2, 4, 6 and 9 M.H.D. used in test; 2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

Antigen-antibody-complement mixture held 18 hours at 1°C.

Results.

	Test				Controls			
					Antigen		Antibody	
(1)	4	0	0	0	0	0	4	0
(2)	4	3	0	0	0	0	4	0

Interpretation: Complement is fixed when an extract of a *viridans* streptococcus is tested with a Lancefield group antiserum.

Table 53

Experiment 46. The complement-fixing powers of normal sera tested with Hitchcock's antigen.

Antigen Hitchcock's preparation. Group A, type 1. Diluted 1/6.
Sera from normal (uninoculated) rabbits. Diluted 1/6.
Complement 2, 4, 6 and 9 M.H.D. used in test; 2 and 4 M.H.D. used in controls.

Each tube, except the control tubes contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

(1) Antigen-antibody-complement mixture held for 1-1/4 hours at 37°C.
(2) Antigen-antibody-complement mixture held for 18 hours at 1°C.

Results.

Serum Number		Test				Controls			
						Antigen		Antibody	
2	(1)	4	0	0	0	0	0	0	0
	(2)	4	4	1	0	2	2	4	0
60	(1)	2	0	0	0	0	0	0	0
	(2)	4	2	0	0	0	0	2	0
61	(1)	3	0	0	0	0	0	0	0
	(2)	4	2	0	0	0	0	0	0
137	(1)	2	0	0	0	0	0	0	0
	(2)	2	0	0	0	0	0	0	0
59	(1)	4	2	0	0	2	0	0	0
	(2)	4	3	0	0	2	1	0	0
58	(1)	0	0	0	0	0	0	0	0
	(2)	1	0	0	0	0	0	0	0
1	(1)	2	0	0	0	0	0	0	0
	(2)	4	2	0	0	3	0	0	0
W2	(1)	4	0	0	0	0	0	0	0
	(2)	4	2	0	0	0	0	3	2
W3	(1)	0	0	0	0	0	0	0	0
	(2)	0	0	0	0	0	0	0	0
W4	(1)	0	0	0	0	0	0	0	0
	(2)	4	3	2	0	0	0	3	2

Table 53 (continued)

Serum Number		Test				Controls			
						Antigen		Antibody	
W5	(1)	0	0	0	0	0	0	0	0
	(2)	4	0	0	0	0	0	1	0
W6	(1)	4	0	0	0	0	0	2	0
	(2)	4	4	0	0	0	0	4	2
101	(1)	0	0	0	0	0	0	0	0
	(2)	1	0	0	0	0	0	0	0
56	(1)	0	0	0	0	0	0	0	0
	(2)	1	0	0	0	0	0	2	0
5	(1)	0	0	0	0	0	0	0	0
	(2)	2	0	0	0	0	0	2	0
17	(1)	0	0	0	0	0	0	0	0
	(2)	2	0	0	0	0	0	0	0
44	(1)	0	0	0	0	0	0	0	0
	(2)	0	0	0	0	0	0	0	0
34	(1)	0	0	0	0	0	0	0	0
	(2)	0	0	0	0	0	0	0	0

Interpretation: Hitchcock's antigen and normal sera fix complement.

Table 54

Experiment 47. Is complement fixation type specific?

Antigens Hitchcock's preparation. Diluted 1/6. (1) Group A, type 1; (2) group A, type 3; (3) group A, type 4.
Antiserum rabbit no. 78. Homologous to the group-A, type-1 strain. Diluted 1/6.
Complement 2, 4, 6, 9, 12, 15 and 20 M.H.D. used in test; 2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

Antigen-antibody-complement mixture held 18 hours at 1°C.

Results.

	Test							Controls			
								Antigen		Antibody	
(1)	4	4	4	4	4	4	4	0	0	0	0
(2)	4	4	4	4	4	4	4	0	0	0	0
(3)	4	4	4	4	4	4	4	0	0	0	0

Interpretation: The reagents used in this experiment are not type specific. A group-A antiserum of type 1 fixes antigens prepared from group-A strains of types 1, 3 and 4.

Table 55

Experiment 48. Cross-fixation with streptococcal antisera and soluble extracts as antigen.

Antigens. Hitchcock's preparation. Diluted 1/6.

Group A, type 1	Group G, SHC/188
Group B, 80RH	Group H, Challis
Group C, Azgazdarah	Group K, Turner
Group D, 98D	Group L, LXB
Group E, EC24B	Group M, 205
Group F, 351	Group N, 201

Antisera. Homologous to the above. Diluted 1/6.
Complement 2, 4, 6, 9, 12, 15, 20, 25 and 30 M.H.D. used in test; 2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

Antigen-antibody-complement mixture held for 18 hours at 1°C.

Results.

Antigen Antiserum group A, rabbit no. 78

Antigen	Test									Controls			
										Antigen		Antibody	
A	4	4	4	4	4	4	4	4	4	0	0	0	0
B	4	2	0	0	0	0	0	0	0	0	0		
C	4	4	0	0	0	0	0	0	0	0	0		
D	4	0	0	0	0	0	0	0	0	0	0		
E	4	4	2	0	0	0	0	0	0	0	0		
F	4	2	0	0	0	0	0	0	0	0	0		
G	4	0	0	0	0	0	0	0	0	0	0		
H	4	4	4	0	0	0	0	0	0	4	0		
K	0	0	0	0	0	0	0	0	0	0	0		
L	4	4	2	0	0	0	0	0	0	0	0		
M	2	0	0	0	0	0	0	0	0	0	0		
N	4	4	2	0	0	0	0	0	0	0	0		

Table 55 (continued)

Antigen	Antiserum group B, rabbit no. 81												
	Test									Controls			
										Antigen		Antibody	
A	2	0	0	0	0	0	0	0	0	0	0		
B	4	4	4	4	4	4	4	4	4	0	0	0	0
C	0	0	0	0	0	0	0	0	0	0	0		
D	0	0	0	0	0	0	0	0	0	0	0		
E	0	0	0	0	0	0	0	0	0	0	0		
F	0	0	0	0	0	0	0	0	0	0	0		
G	1	0	0	0	0	0	0	0	0	0	0		
H	1	0	0	0	0	0	0	0	0	0	0		
K	0	0	0	0	0	0	0	0	0	0	0		
L	0	0	0	0	0	0	0	0	0	0	0		
M	0	0	0	0	0	0	0	0	0	0	0		
N	1	0	0	0	0	0	0	0	0	0	0		

Antigen	Antiserum group C, rabbit no. 53												
	Test									Controls			
										Antigen		Antibody	
A	2	0	0	0	0	0	0	0	0	0	0		
B	4	0	0	0	0	0	0	0	0	0	0		
C	4	4	4	4	4	4	0	0	0	0	0	0	0
D	3	0	0	0	0	0	0	0	0	0	0		
E	4	4	2	0	0	0	0	0	0	0	0		
F	4	2	0	0	0	0	0	0	0	0	0		
G	4	2	0	0	0	0	0	0	0	0	0		
H	4	4	3	0	0	0	0	0	0	0	0		
K	2	0	0	0	0	0	0	0	0	0	0		
L	4	3	0	0	0	0	0	0	0	0	0		
M	2	0	0	0	0	0	0	0	0	0	0		
N	4	2	0	0	0	0	0	0	0	0	0		

Table 55 (continued)

Antigen Antiserum group D, rabbit no. 59

Antigen	Test									Controls			
										Antigen		Antibody	
A	3	0	0	0	0	0	0	0	0	0	0		
B	3	0	0	0	0	0	0	0	0	0	0		
C	4	0	0	0	0	0	0	0	0	0	0		
D	4	4	4	4	3	1	0	0	0	0	0	0	0
E	4	4	1	0	0	0	0	0	0	0	0		
F	4	0	0	0	0	0	0	0	0	0	0		
G	2	0	0	0	0	0	0	0	0	0	0		
H	4	4	1	0	0	0	0	0	0	0	0		
K	2	0	0	0	0	0	0	0	0	0	0		
L	4	2	0	0	0	0	0	0	0	0	0		
M	2	0	0	0	0	0	0	0	0	0	0		
N	4	1	0	0	0	0	0	0	0	0	0		

Antigen Antiserum group E, rabbit no. 83

Antigen	Test									Controls			
										Antigen		Antibody	
A	2	0	0	0	0	0	0	0	0	0	0		
B	0	0	0	0	0	0	0	0	0	0	0		
C	1	0	0	0	0	0	0	0	0	0	0		
D	0	0	0	0	0	0	0	0	0	0	0		
E	4	4	4	4	4	4	4	4	4	2	0	0	0
F	0	0	0	0	0	0	0	0	0	0	0		
G	4	0	0	0	0	0	0	0	0	0	0		
H	4	2	0	0	0	0	0	0	0	0	0		
K	0	0	0	0	0	0	0	0	0	0	0		
L	2	0	0	0	0	0	0	0	0	0	0		
M	0	0	0	0	0	0	0	0	0	0	0		
N	3	0	0	0	0	0	0	0	0	0	0		

Table 55 (continued)

Antigen Antiserum group F, rabbit no. 15

Antigen	Test									Controls: Antigen		Controls: Antibody	
A	2	0	0	0	0	0	0	0	0	0	0		
B	0	0	0	0	0	0	0	0	0	0	0		
C	4	0	0	0	0	0	0	0	0	0	0		
D	1	0	0	0	0	0	0	0	0	0	0		
E	4	4	0	0	0	0	0	0	0	2	0		
F	4	4	4	4	2	0	0	0	0	0	0	0	0
G	4	1	0	0	0	0	0	0	0	2	0		
H	4	2	0	0	0	0	0	0	0	2	0		
K	1	0	0	0	0	0	0	0	0	0	0		
L	4	0	0	0	0	0	0	0	0	0	0		
M	0	0	0	0	0	0	0	0	0	0	0		
N	4	0	0	0	0	0	0	0	0	0	0		

Antigen Antiserum group G, rabbit no. 44

Antigen	Test									Controls: Antigen		Controls: Antibody	
A	0	0	0	0	0	0	0	0	0	0	0		
B	0	0	0	0	0	0	0	0	0	0	0		
C	0	0	0	0	0	0	0	0	0	0	0		
D	0	0	0	0	0	0	0	0	0	0	0		
E	0	0	0	0	0	0	0	0	0	0	0		
F	3	0	0	0	0	0	0	0	0	0	0		
G	4	4	4	4	3	0	0	0	0	0	0	0	0
H	4	4	0	0	0	0	0	0	0	0	0		
K	0	0	0	0	0	0	0	0	0	0	0		
L	4	4	3	0	0	0	0	0	0	0	0		
M	0	0	0	0	0	0	0	0	0	0	0		
N	4	0	0	0	0	0	0	0	0	0	0		

Table 55 (continued)

Antigen Antiserum group H, rabbit no. 106

Antigen	Test									Controls			
										Antigen		Antibody	
A	3	0	0	0	0	0	0	0	0	0	0		
B	4	0	0	0	0	0	0	0	0	0	0		
C	4	0	0	0	0	0	0	0	0	0	0		
D	2	0	0	0	0	0	0	0	0	0	0		
E	4	3	0	0	0	0	0	0	0	0	0		
F	4	1	0	0	0	0	0	0	0	0	0		
G	4	4	4	3	0	0	0	0	0	0	0		
H	4	4	4	4	4	4	4	4	2	0	0	0	0
K	2	0	0	0	0	0	0	0	0	0	0		
L	4	4	0	0	0	0	0	0	0	0	0		
M	4	4	4	4	3	0	0	0	0	0	0		
N	4	4	2	0	0	0	0	0	0	0	0		

Antigen Antiserum group K, rabbit no. 82

Antigen	Test									Controls			
										Antigen		Antibody	
A	2	0	0	0	0	0	0	0	0	0	0		
B	0	0	0	0	0	0	0	0	0	0	0		
C	4	0	0	0	0	0	0	0	0	0	0		
D	4	0	0	0	0	0	0	0	0	0	0		
E	0	0	0	0	0	0	0	0	0	0	0		
F	1	0	0	0	0	0	0	0	0	0	0		
G	0	0	0	0	0	0	0	0	0	0	0		
H	3	0	0	0	0	0	0	0	0	0	0		
K	4	0	0	0	0	0	0	0	0	0	0	0	0
L	2	0	0	0	0	0	0	0	0	0	0		
M	3	0	0	0	0	0	0	0	0	0	0		
N	3	0	0	0	0	0	0	0	0	0	0		

Table 55 (continued)

Antigen Antiserum group L, rabbit no. 25

Antigen	Test									Controls			
										Antigen		Antibody	
A	1	0	0	0	0	0	0	0	0	0	0		
B	4	0	0	0	0	0	0	0	0	0	0		
C	3	0	0	0	0	0	0	0	0	0	0		
D	3	0	0	0	0	0	0	0	0	0	0		
E	4	0	0	0	0	0	0	0	0	0	0		
F	3	0	0	0	0	0	0	0	0	0	0		
G	4	3	0	0	0	0	0	0	0	0	0		
H	4	3	0	0	0	0	0	0	0	0	0		
K	2	0	0	0	0	0	0	0	0	0	0		
L	4	4	4	4	4	3	0	0	0	0	0	0	0
M	2	0	0	0	0	0	0	0	0	0	0		
N	4	0	0	0	0	0	0	0	0	0	0		

Antigen Antiserum group M, rabbit no. 102

Antigen	Test									Controls			
										Antigen		Antibody	
A	3	0	0	0	0	0	0	0	0	0	0		
B	3	0	0	0	0	0	0	0	0	0	0		
C	3	0	0	0	0	0	0	0	0	0	0		
D	2	0	0	0	0	0	0	0	0	0	0		
E	4	2	0	0	0	0	0	0	0	0	0		
F	3	0	0	0	0	0	0	0	0	0	0		
G	4	0	0	0	0	0	0	0	0	0	0		
H	4	4	0	0	0	0	0	0	0	0	0		
K	3	0	0	0	0	0	0	0	0	0	0		
L	4	0	0	0	0	0	0	0	0	0	0		
M	4	4	4	4	4	4	4	4	4	0	0	0	0
N	4	0	0	0	0	0	0	0	0	0	0		

Table 55 (continued)

Antigen Antiserum group N, rabbit no. 101

Antigen	Test									Controls Antigen	Controls Antibody		
A	1	0	0	0	0	0	0	0	0	0	0		
B	2	0	0	0	0	0	0	0	0	0	0		
C	1	0	0	0	0	0	0	0	0	0	0		
D	1	0	0	0	0	0	0	0	0	0	0		
E	4	0	0	0	0	0	0	0	0	2	0		
F	1	0	0	0	0	0	0	0	0	0	0		
G	0	0	0	0	0	0	0	0	0	0	0		
H	4	4	4	0	0	0	0	0	0	4	0		
K	0	0	0	0	0	0	0	0	0	0	0		
L	2	0	0	0	0	0	0	0	0	0	0		
M	4	4	0	0	0	0	0	0	0	0	0		
N	4	4	4	4	4	4	4	0	0	0	0	0	0

Interpretation: Each antigen with its homologous antiserum, except group K, fixes a large amount of complement. Cross-reactions, but to a lower titre than that of the serum, are also observed.

Table 56

Experiment 48. Cross-fixation with streptococcal antisera and soluble extracts as antigen.

Details as in experiment 47, Table 55, page 160, with the following exceptions:
The group-C antigen is prepared from strain 4678;
The antisera are from different animals than those used in experiment 47 and the group-C antiserum was prepared against strain 4678.

Results.

Antigen Antiserum group A, rabbit no. 79.

Antigen	Test									Controls			
										Antigen		Antibody	
A	4	4	4	4	4	4	4	4	4	0	0	0	0
B	2	0	0	0	0	0	0	0	0	0	0		
C	4	4	0	0	0	0	0	0	0	0	0		
D	2	0	0	0	0	0	0	0	0	0	0		
E	4	4	0	0	0	0	0	0	0	0	0		
F	2	0	0	0	0	0	0	0	0	0	0		
G	4	4	4	0	0	0	0	0	0	0	0		
H	4	4	4	0	0	0	0	0	0	0	0		
K	4	0	0	0	0	0	0	0	0	0	0		
L	4	4	4	0	0	0	0	0	0	0	0		
M	0	0	0	0	0	0	0	0	0	0	0		
N	4	4	0	0	0	0	0	0	0	0	0		

Antigen Antiserum group C, rabbit no. 62.

Antigen	Test									Controls			
										Antigen		Antibody	
A	0	0	0	0	0	0	0	0	0	0	0		
B	0	0	0	0	0	0	0	0	0	0	0		
C	4	4	4	4	4	0	0	0	0	0	0	0	0
D	1	0	0	0	0	0	0	0	0	0	0		
E	4	4	0	0	0	0	0	0	0	0	0		
F	4	0	0	0	0	0	0	0	0	0	0		
G	0	0	0	0	0	0	0	0	0	0	0		
H	4	0	0	0	0	0	0	0	0	0	0		
K	0	0	0	0	0	0	0	0	0	0	0		
L	2	0	0	0	0	0	0	0	0	0	0		
M	0	0	0	0	0	0	0	0	0	0	0		
N	2	0	0	0	0	0	0	0	0	0	0		

Table 56 (continued)

Antigen Antiserum group K, rabbit no. 34

Antigen	Test									Controls			
										Antigen		Antibody	
A	1	0	0	0	0	0	0	0	0	0	0		
B	4	0	0	0	0	0	0	0	0	0	0		
C	4	0	0	0	0	0	0	0	0	0	0		
D	2	0	0	0	0	0	0	0	0	0	0		
E	4	2	0	0	0	0	0	0	0	0	0		
F	1	0	0	0	0	0	0	0	0	0	0		
G	0	0	0	0	0	0	0	0	0	0	0		
H	2	0	0	0	0	0	0	0	0	0	0		
K	0	0	0	0	0	0	0	0	0	0	0	0	0
L	2	0	0	0	0	0	0	0	0	0	0		
M	2	0	0	0	0	0	0	0	0	0	0		
N	2	0	0	0	0	0	0	0	0	0	0		

Interpretation: Each antigen with its homologous antiserum, except group K, fixes a large amount of complement. Cross-reactions, but to a lower titre than that of the serum, are also observed.

Table 57

Experiment 49. A comparison of Hitchcock's and Lancefield's antigens in the streptococcal complement-fixation test.

Antigen (1) Lancefield's preparation; (2) Hitchcock's preparation. Group K, strain Turner. Diluted 1/6.
Antisera (a) rabbit no. 34; (b) rabbit no. 82. Homologous. Diluted 1/6.
Complement 2, 4, 6, 9, 12, 15 and 20 M.H.D. used in test; 2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

Antigen-antibody-complement mixture held 18 hours at 1°C.

Results.

	Test							Controls			
								Antigen		Antibody	
(1a)	4	4	4	4	4	4	0	0	0	0	0
(1b)	4	4	4	4	4	4	0	0	0	2	0
(2a)	0	0	0	0	0	0	0	0	0	0	0
(2b)	4	4	0	0	0	0	0	0	0	2	0

Interpretation: A Lancefield preparation of strain Turner, group K tested with the homologous antiserum fixes a large amount of complement: A Hitchcock preparation of the same strain tested with the same antiserum fixes little or no complement.

Table 58

Experiment 50. A Lancefield preparation of a group-K strain tested with antisera of Lancefield's groups A - N.

Antigen Lancefield's preparation. Group K, strain Turner. Diluted 1/6.
Antisera. As in experiment 48, Table 55, pages 160 - 166. Diluted 1/6.
Complement 2, 4, 6, 9, 12, 15, 20, 25 and 30 M.H.D. used in test; 2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

Antigen-antibody-complement mixture held for 18 hours at $1^{o}C$.

Results.

Antisera	Test									Controls			
										Antigen		Antibody	
A	4	4	4	0	0	0	0	0	0			0	0
B	0	0	0	0	0	0	0	0	0			0	0
C	4	0	0	0	0	0	0	0	0			0	0
D	3	0	0	0	0	0	0	0	0			0	0
E	0	0	0	0	0	0	0	0	0			0	0
F	0	0	0	0	0	0	0	0	0			0	0
G	3	0	0	0	0	0	0	0	0			0	0
H	0	0	0	0	0	0	0	0	0			0	0
K	4	4	4	4	4	4	0	0	0	0	0	0	0
L	0	0	0	0	0	0	0	0	0			0	0
M	0	0	0	0	0	0	0	0	0			0	0
N	4	0	0	0	0	0	0	0	0			0	0

Interpretation: Antigen-K with its homologous antiserum fixes a large amount of complement. A cross-reaction is observed between antigen K and the antiserum of group A.

Table 59

Experiment 51. A comparison of antigens prepared by Hitchcock's and Lancefield's methods.

Antigens (1) Hitchcock's preparation; (2) Lancefield's preparation. Both diluted 1/6.
(a) group H, strain Challis; (b) group L, strain LXB;
(c) group N, strain 201.
Antiserum rabbit no. 78. Prepared against a group-A, type-1 strain. Diluted 1/6.
Complement 2, 4, 6 and 9 M.H.D. used in test; 2 and 4 M.H.D. used in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

Antigen-antibody-complement mixture held 18 hours at 1°C.

Results.

	Test				Controls			
					Antigen		Antibody	
(1a)	4	2	0	0	0	0	0	0
(2a)	4	1	0	0	0	0	0	0
(1b)	4	4	3	0	0	0	0	0
(2b)	4	4	0	0	0	0	0	0
(1c)	4	4	0	0	0	0	0	0
(2c)	4	0	0	0	0	0	0	0

Interpretation: Similar amounts of complement are fixed whether a Hitchcock's preparation or a Lancefield's preparation is tested with a heterologous antiserum.

Table 60

Experiment 52. A single-tube streptococcal complement-fixation test.

Antigens. Hitchcock's preparation.* Lancefield's groups A - N. Diluted 1/15.
Antisera. Homologous to the above. Diluted 1/15.
Complement 6 M.H.D. used in test; 3 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

Antigen-antibody-complement mixture held for 4 hours at $2^{o}C$.

Results.

Antigens

	A	B	C	D	E	F	G	H	K	L	M	N
A	4	0	0	0	0	0	0	0	0	0	0	0
B	0	4	0	0	0	0	0	0	0	0	0	0
C	0	0	4	0	0	0	0	0	0	0	0	0
D	0	0	0	4	0	0	0	0	0	0	0	0
E	0	0	0	0	4	0	0	0	0	0	0	0
F	0	0	0	0	0	4	0	0	0	0	0	0
G	0	0	0	0	0	0	4	0	0	0	0	0
H	0	0	0	0	0	0	0	4	0	0	0	0
*K	0	0	0	0	0	0	0	0	4	0	0	0
L	0	0	0	0	0	0	0	0	0	4	0	0
M	0	0	0	0	0	0	0	0	0	0	4	0
N	0	0	0	0	0	0	0	0	0	0	0	4

Controls of antigen and antibody containing 3 M.H.D. of complement were negative.

Interpretation: Each antigen and homologous antiserum fixes complement. No cross-reactions are observed.

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* With group K the antigen was not a Hitchcock preparation but a Lancefield extract.

SECTION X111

MISCELLANEOUS FACTORS AFFECTING THE STREPTOCOCCAL COMPLEMENT-FIXATION TEST

MISCELLANEOUS FACTORS AFFECTING THE STREPTOCOCCAL COMPLEMENT-FIXATION TEST

A. TO DETERMINE WHICH PORTION OF ANTIFORMIN IS CRITICAL IN THE EXTRACTION OF STREPTOCOCCI

Experiment 53.

This experiment was designed to determine on which antiformin constituent, the sodium hydroxide or the liquor sodae chlorinatae, the Hitchcock method of extraction of streptococci depends. Two antigens were prepared:

Antigen A. An 0.6% solution of sodium hydroxide was used as the alkali. (This is equivalent to 0.8% antiformin containing 7.5% alkali). 1.0 ml. of this was added to each 2.0 mg. of dried bacteria: the suspension was placed in a water-bath and shaken vigorously two or three times in the course of extraction over a period of 10 minutes at 56°C. The tube was then cooled and the contents neutralised by adding drop by drop N/5 H_2SO_4. The resulting precipitate was removed by centrifugation and the clear supernatant fluid retained. 0.5% phenol-saline was added to the supernatant so that the final volume represented 1.0 ml. per 1.0 mg. of dried organisms.

Antigen B. The second antigen was prepared by adding 1.0 ml. of an 0.05% solution of liquor sodae chlorinatae per 2.0 mg. of dried organisms. This suspension was then heated in a water-bath at 56°C for 10 minutes with vigorous agitation and 3 drops of 5.0% sodium thiosulphate were added to the suspension per 1.0 ml. of liquor sodae chlorinatae. N/5 H_2SO_4 was added to give neutrality. 0.5% phenol-saline was then added to give a final volume such that 1.0 ml. represented 1.0 mg. of dried bacteria.

Complement-fixation tests were done with the above antigens A and B and a Hitchcock preparation, antigen C, in which the extraction is made with whole antiformin.

Table 61 (page 175) shows that the most potent antigen is obtained by extracting the streptococci with antiformin, although good results can be obtained by the use of liquor sodae chlorinatae. Alkaline extraction at 56°C is not sufficient to prepare the residue substance.

SUMMARY

The essential constituent of the antiformin necessary for extraction of streptococci is the liquor sodae chlorinatae. With this alone a good antigen may be prepared although the whole antiformin gives better results. Alkali extraction alone is not efficacious.

Table 61

Experiment 53. A comparison of the effect of different antiformin constituents on the extraction of streptococci.

Antigens* group A, type 1.
A = "NaOH" antigen
B = "Liquor sodae chlorinatae" antigen
C = "Whole antiformin" (Hitchcock) antigen.
All diluted 1/6.

Antiserum rabbit no. 78. Homologous. Diluted 1/6.
Complement 2, 4, 6, 9, 12, 15, 20 and 25 M.H.D. used in test; 2 and 4 M.H.D. used in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

Antigen-antibody-complement mixture held 18 hours at 1°C.

Results.**

Antigen	Test								Controls			
									Antigen		Antibody	
A	4	3	0	0	0	0	0	0	4	0	0	0
B	4	4	4	4	4	4	0	0	4	0	0	0
C	4	4	4	4	4	4	4	4	0	0	0	0

Interpretation: The most potent antigen is obtained by extracting streptococci with antiformin, although good results can be obtained by the use of liquor sodae chlorinatae. Alkaline extraction is not sufficient to prepare the residue substance.

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* Full details of the antigens are given on pages 39 - 41 and 173.

** For explanation of symbols see page 46.

B. THE POTENCY OF STORED ANTIGENS

Kinsella (1918) stated that an antiformin extract of streptococci did not long retain its strength. In my experiments the Hitchcock antigens were prepared with a slightly weaker antiformin solution than that used by Kinsella; it was of interest to test if they had retained potency during storage.

Experiment 54.

A group-A, type-1 antigen which had been prepared three years previously was tested with the homologous antiserum. Table 62 (page 177) shows that 30 M.H.D. of complement, the maximum amount tested, were fixed. Similar results were obtained with old extracts of groups B, C and G antigens.

SUMMARY

Antiformin extracts of streptococci as prepared for this work retain their potency over several years.

Table 62

Experiment 54. The potency of stored antigens.

Antigen Hitchcock's preparation. Group A, type 1. Prepared 3 years previous to this experiment. Diluted 1/6.
Antiserum rabbit no. 78. Homologous. Diluted 1/6.
Complement 2, 4, 6, 9, 12, 15, 20, 25 and 30 M.H.D. used in test; 2 and 4 M.H.D. in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

Antigen-antibody-complement mixture held 18 hours at 1°C.

Results.

Test									Controls			
									Antigen		Antibody	
4	4	4	4	4	4	4	4	4	0	0	0	0

Interpretation: Antiformin extracts of streptococci retain their potency over several years.

C. THE DETECTION OF SMALL VOLUMES OF ANTIGEN OR ANTISERUM BY THE COMPLEMENT-FIXATION TEST

One of the most valuable features of the complement-fixation test is that it serves to detect small volumes of antigen and antibody.

Experiment 55.

Antiserum no. 78 prepared from a group-A, type-1 strain was found to be very potent and was tested in several dilutions with the homologous antigen in various dilutions with different amounts of complement. The results (Tables 63 and 64, pp. 179 - 181) show that, even when both antigen and antibody were diluted one in fifteen, as many as 25 M.H.D. of complement were fixed. Precipitin tests carried out in parallel were positive only with the one in five antigen and one in five antibody dilution.

SUMMARY

With potent antisera, small volumes of antigen or antibody may be detected by the complement-fixation test.

Table 63.

Experiment 55. The detection of small volumes of antigen or antiserum by the complement-fixation test.

Antigen Hitchcock's preparation. Group A, type 1.
Antiserum rabbit no. 78. Homologous.
Complement 10 M.H.D. used in test; 3 M.H.D. used in controls.

Each tube, except the control tubes, contains one unit of antigen and one unit of antibody. The control tubes contain one unit of saline and one unit of antigen or of antibody. At the end of the period denoted one unit of sensitized cells is added. The unit is 0.25 ml.

Antigen-antibody-complement mixture held 18 hours at 1°C.

Results.

Antiserum	Antigen 1/5	1/10	1/15	1/20	1/25	1/50	1/100
1/5	4	4	4	4	4	4	0
1/10	4	4	4	4	4	4	1
1/15	4	4	4	4	4	4	2
1/20	4	4	4	4	4	4	2
1/25	4	4	4	4	4	4	2
1/50	4	4	4	4	4	4	2
1/100	4	4	4	4	4	4	1

Controls, antigen and antibody, of all the above dilutions were negative.

Interpretation: Complement is fixed by high dilutions of antigen and antiserum.

Table 64

Experiment 55. The detection of small volumes of antigen or antiserum by the complement-fixation test.

Details as in Table 63, page 179. Each antigen-antibody ratio was tested with 10, 15, 20, 25 and 30 M.H.D. of complement except in the series in which the 1/100 antigen dilution was used. The volumes of complement in this series were 2, 4, 6 and 9.

Results.

Antigen	Antibody	Number M.H.D. of complement fixed.
1/5	1/5	25
1/5	1/10	25
1/5	1/15	25
1/5	1/20	25
1/5	1/25	25
1/5	1/50	15
1/5	1/100	10
1/10	1/5	25
1/10	1/10	25
1/10	1/15	25
1/10	1/20	25
1/10	1/25	25
1/10	1/50	15
1/10	1/100	10
1/15	1/5	25
1/15	1/10	25
1/15	1/15	25
1/15	1/20	25
1/15	1/25	20
1/15	1/50	15
1/15	1/100	10
1/20	1/5	25
1/20	1/10	25
1/20	1/15	20
1/20	1/20	20
1/20	1/25	20
1/20	1/50	15
1/20	1/100	10

Table 64 (continued)

Antigen	Antibody	Number M.H.D. of complement fixed
1/25	1/5	20
1/25	1/10	20
1/25	1/15	20
1/25	1/20	15
1/25	1/25	15
1/25	1/50	10
1/25	1/100	10
1/50	1/5	10
1/50	1/10	10
1/50	1/15	10
1/50	1/20	10
1/50	1/25	10
1/50	1/50	10
1/50	1/100	10
1/100	1/5	6
1/100	1/10	6
1/100	1/15	6
1/100	1/20	6
1/100	1/25	6
1/100	1/50	6
1/100	1/100	6

Interpretation: Small volumes of antigen or of antiserum may be detected by the streptococcal complement-fixation test.

D. THE ANTIBODY RESPONSE OF RABBITS IMMUNIZED WITH HITCHCOCK'S ANTIGEN

It is generally accepted that the soluble specific substance prepared by the usual laboratory methods is a hapten and lacks the power of stimulating antibody production if used as a vaccine. Gentler methods of extraction which do not depolymerize the acetylated group result in a product with some antigenic power. To determine if the antigen prepared according to the method of Hitchcock could stimulate antibody production, three rabbits were inoculated with a Hitchcock preparation of a group-A, type-1 strain. The usual method of inoculation was followed (page 25). Test-bleedings were done 4, 11, 18, 25 and 32 days after the last inoculation.

No precipitins, agglutinins or complement-fixing antibodies were demonstrable in sera obtained from any of these test bleedings. That the rabbits themselves did not lack the ability to produce antibodies if suitably stimulated was shown by subsequently inoculating them with cellular vaccines. Precipitins, agglutinins and complement-fixing antibodies

were all present in the sera from test bleedings after these inoculations. To ensure that this was not a cumulative effect of the two vaccines, extract and cellular, a group-B strain was used as the cellular vaccine. It was thus seen that failure to produce antibodies when the soluble specific substance was used as vaccine was not due to an inherent defect of the rabbits, but to the fact that the vaccines employed could not stimulate antibody production.

SUMMARY

Hitchcock's antigen used as a vaccine did not stimulate antibody production. Failure to produce antibodies was not due to an inherent deficiency of the rabbits.

SECTION XIV

DISCUSSION

DISCUSSION

Complement fixation has been used successfully in the study of many organisms. If the classical theory is true: that complement is adsorbed to the precipitate formed by the union of antigen and antibody, there seems no adequate reason why the test should not also prove successful in the study of streptococci.

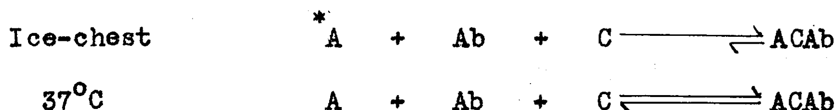
Streptococci are divided into groups A - N on the basis of the precipitin test (Lancefield), which utilizes the interaction of the soluble specific substance (residue or C substance) and antiserum. However, when I tested the same reagents under conditions which gave precipitation, complement was not fixed. There was one important difference, however, in that to prevent anti-complementary results the reagents had to be diluted 1/6. Yet, the most potent reagents used in this work did not give a visible precipitate if diluted more than 1/4. It may be that no precipitate was formed because of the greater antigen-antibody dilutions used -- and that consequently complement was neither adsorbed nor fixed. This appears unlikely, particularly

because the complement-fixation test is probably the most delicate of all serological tests. Further, the lack of a visible precipitate does not necessarily imply the absence of a precipitate; the term visible depends on the conditions under which precipitation or its absence are recorded. Complement-fixation tests in the present work were done in "Turner" tubes of approximately 1/2" diameter and 2" long, and it may be that only under the conditions of testing no precipitate was observed.

It has been shown by many workers, for example, Dean (1916 - 17), Goldsworthy (1928) that the optimum antigen-antibody ratio for the complement-fixation test may not be the same as that for precipitation. The optimum ratios for these two tests vary, depending on the system used and the conditions under which the test is carried out such as temperature. It is possible that the 1/1 ratio which I used in the streptococcal precipitin test is not optimal for the complement-fixation test. However, my tests over a wide antigen-antibody ratio did not give constant fixation at any level and it seems, therefore, that when the soluble specific substance of streptococci is used under conditions similar to those in the precipitin test, complement is not fixed.

That complement was specifically fixed when the physical conditions of my test were altered so that the antigen-antibody-complement mixture was held at 1 - 4°C instead of at 37°C is difficult to reconcile with present theories and must be accepted as a new phenomenon. Two experiments were done in an effort to discover the reasons for the low-temperature fixation. The kinetics and dynamics of the problem remain to be studied and should prove a fruitful field. The most obvious explanation is based on Bordet's and Dean's contention that the precipitate formed by the union of antigen and antibody adsorbs complement, and that any method which will delay the formation of the precipitate should, because of the larger surface area which will result, cause an increase in the volume of complement fixed. At 1°C the collision between the molecules will be less frequent than at 37°C, resulting in a greater surface area and in more complement being fixed.

Fixation at low temperatures and not at 37°C may be explained by theorizing that at low temperatures the dynamic equilibrium of the system is biased towards the combination of antigen, antibody and complement, whereas at 37°C the firmness of union is not so great and that the complex readily dissociates.

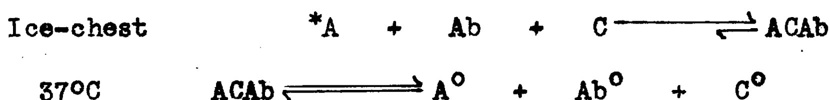


Ice-chest $^{*}A + Ab + C \rightleftharpoons ACAb$

37°C $A + Ab + C \rightleftharpoons ACAb$

The fact that incubation of the antigen-antibody-complement mixture at 37°C, subsequent to its being held at ice-chest temperatures, does not result in disturbance of the low temperature equilibrium, with resulting dissociation, does not necessarily contradict the above hypothesis. The firmness of the union may be such that dissociation of the complex proceeds slowly and that if the 37°C incubation period was extended, would prove to be reversible. However, with increased time there would be increased destruction of complement.

On the other hand, there may be dissociation when the reagents are placed at 37°C subsequent to their being kept in the ice-chest, but the individual constituents of the reaction may have undergone physical changes such as denaturation, for example.



Ice-chest $^{*}A + Ab + C \rightleftharpoons ACAb$

37°C $ACAb \rightleftharpoons A^{o} + Ab^{o} + C^{o}$

The findings may also be interpreted in terms of the Pauling modification (1946) of Ehrlich's theory. In my

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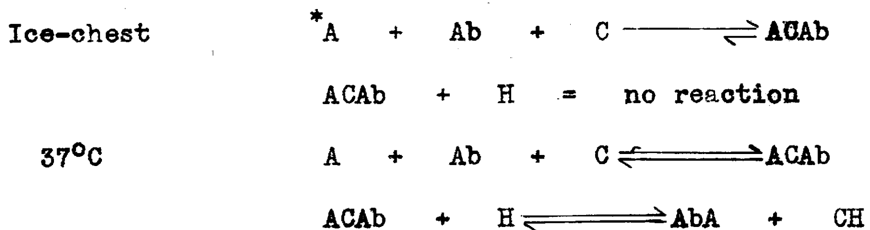
*

A	=	antigen
Ab	=	antibody
C	=	complement
ACAb	=	antigen-antibody-complement complex
A^{o}	=	altered antigen
Ab^{o}	=	altered antibody
C^{o}	=	altered complement

system three complex molecular aggregates participate: (1) the antigen-antibody complex; (2) complement; and (3) sensitized cells -- the indicator system. When preliminary incubation of (1) and (2) is at 37°C it may be that the bond linkage and resulting bond energy is not sufficient for the formation of a stable molecular aggregate, and that on the addition of the third complex, there is dissociation of the antigen-antibody-complement, and that a firm bond is formed between the indicator system and the complement.

Again, when preliminary incubation is at low temperatures the forces of attraction between (1) and (2) are such that the linkage is firm and is not disrupted even when the indicator system is added at 37°C. Such a view is strengthened by the findings of early workers that the affinity of complement for an amboceptor system is greater at 0°C than at higher temperatures.



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*		
A	=	antigen
Ab	=	antibody
C	=	complement
ACAb	=	antigen-antibody-complement complex
H	=	sensitized cells (indicator system)
AbA	=	antigen + antibody
CH	=	complement + sensitized cells

The differences in the results at the two temperatures may be due to the spatial arrangement of the antigen-antibody combination. At 37°C there may be distortion or masking of the complement linkages on the antigen-antibody union, thus excluding complement from the complexity. At ice-chest temperatures on the other hand the spatial arrangement may be of such a nature that complement will fit into the structure. Expressing this spatial hypothesis in the more modern terms of Pauling, and considering the antigen and antibody not as one complex molecular aggregate but as individual molecular structures, it may be that at 37°C, due to the imperfect fit of complement, antigen, and antibody, the linkage bonds are weak and bond energy low. At low temperatures the antigen, antibody, and complement may conform, one with the other, in respect of their dimensions and the spatial arrangement of their atoms.

Approaching the phenomenon of low-temperature fixation as opposed to the absence of fixation at 37°C from a different angle, it is possible that complement contains either an activator or an inhibitor of the streptococcal antigen-antibody system. Presuming the latter, then this inhibitor may act at 37°C but not at ice-chest temperatures. Less likely is the possibility that activation of the system occurs at low temperatures but not at 37°C. A parallel can be drawn

with streptokinase which activates fibrinolysin in human serum and inhibits the development of the reaction if the patient has had a streptococcal infection.

The results were unsatisfactory with whole-broth antigens (lab. lemco broth) even when the antigen-antibody-complement mixtures were held at ice-chest temperatures. There was some fixation, but its specificity was not great if viewed in the light of Lancefield's group classification. Lancefield's division of the haemolytic streptococci into serological groups is based on the presence of a polysaccharide within the bacterial cell. Under normal conditions there is no autolysis of streptococci and the cells must be ruptured by harsh treatments to liberate the group material. Therefore, when whole-broth antigens are used it is most unlikely that the reaction, if any, is an expression of the group-specific central polysaccharide. Possibly the reaction is due to a combination of the whole bacterial cell and antibody. The results will more closely parallel agglutination than precipitation, but with haemolytic streptococci agglutination (i.e. union of antibody and bacterial cell) does not parallel group precipitation (i.e. union of polysaccharide and antibody). This would account for lack of group-specific complement fixation. It is of course possible that the reactions observed represent new relationships more of the order of type than of group.

Experiments, not reported here, were done in which inoculated animals were bled at weekly intervals, and their sera tested for complement-fixing antibodies against both Hitchcock's and whole-broth antigens. When the cellular antigen was used the number of doses of complement fixed by the sera from different bleedings of an animal was practically a constant. No clear interpretation could be made of the curves which resulted from plotting the amount of complement fixed by a cellular antigen either at 37°C or at 1 - 4°C against the time of bleeding. The amount of complement fixed was never great and the difference between fixation at 37°C and cold temperatures was slight. This is contrary to the findings with Hitchcock's antigen, with which a definite rise and fall of complement-fixing antibodies could be traced. Further, with Hitchcock's antigen, much more complement was fixed at ice-chest temperatures than at incubator temperature. All these factors point to fixation by the cellular antigen as not being a true specific fixation. The theory of non-specific fixation is further strengthened by the fact that the sera of one rabbit although giving a positive precipitin test did not fix complement when tested with Hitchcock's antigen at 37°C or at ice-chest temperatures, but did fix complement when tested with a cellular antigen.

That smaller amounts of complement were fixed when the cells

from a culture suspended in saline were used as antigen than when a whole-broth culture was used, suggested that antigenic material was discarded with the supernatant. The supernatants of these cultures did fix complement. Autolysis may occur, but this has already been discussed. However, a sterile lab. lemco broth also fixed complement, and it seems probable that fixation with supernatants may be due to combination of peptone and the antibody. The antisera may contain antibodies for the peptone in culture medium. Examples of culture medium stimulating antibodies in preparing rabbit immune sera are fairly common. Sordelli and Mayer (1931a, 1931b) and Morgan (1936) obtained erroneous results in experiments on bacterial polysaccharides due to the antisera containing antibodies against the carbohydrates in the agar. Goebel (1938) noted that false reactions may be obtained due to a carbohydrate obtained from peptone in culture medium and as has already been mentioned, Bliss (1938) obtained false precipitation due to peptone in the medium. However, the vaccines used in preparing the sera for the present work were made from saline suspensions of washed cultures, although the cultures were grown in the same medium as that used in preparing whole-broth antigens. Further, complement was fixed even when sterile lab. lemco broth was tested with normal sera. This

fixation of complement could be entirely non-specific and attributable to the spatial arrangement or configuration of the peptone. A combination may occur between the peptone and the complement, between the peptone and the antisera, or among all three reagents, and thus false fixation of complement could be explained.

The antigens obtained by Hitchcock and Lancefield treatments, which are respectively an alkali and an acid treatment, are not necessarily identical. Certain chemical groupings attached to the polysaccharides of some strains, or of some groups, are possibly acid labile, whereas others are probably alkali labile. For example, an acetyl group on a group-K strain may be labile to alkali. A corollary may be drawn between these findings and those of Wannamaker _et al._ (1950) that formamide extraction of streptococci does not necessarily parallel extraction with Maxted's _Streptomyces griseus_ enzyme. The practical significance of this finding is that a negative result with an antigen prepared by any one method is not conclusive of the negative identity of the strain in question. Therefore, if the serological identity of a strain is strongly suspected and yet the antigen fails to react with the appropriate serum, various methods of preparing the antigen should be used.

The streptococcal complement-fixation test, although

demanding an exacting technique and necessitating the use of more reagents than the precipitin test, has several definite advantages over the latter test. Its sensitivity far surpasses that of the precipitin test; less antigen and antibody are needed; and as opposed to the precipitin test, the reagents, both antigen and antibody, need not be water clear. Minor or heterologous streptococcal antibodies or antigens need cause no confusion in grouping unknown strains if the complement-fixation test is used. Group antigens and antibodies may be so diluted that complement is fixed only when the homologous system is present, and not when the antigen and antibody are heterologous. Alternatively, by using suitably large amounts of complement, there will be fixation only in the presence of homologous reagents and not when the antigen and antibody are heterologous. In the grouping of unknown strains, dilution of the reagents to an extent which will eliminate cross-reactions and the use of a small amount of complement is probably the best technique, being sparing both in antisera and complement. However, a clearer antigenic picture of a strain will emerge if the reagents are diluted only to the extent which will prevent anti-complementary results, and if several increasing doses of complement are used. With such a technique a complete streptococcal antigenic scheme, similar to that of the Salmonella

may be constructed.

In the typing of streptococci, further work may well show that the complement-fixation test is more efficacious than the agglutination and precipitin tests.

SUMMARY

THE STREPTOCOCCAL COMPLEMENT-FIXATION TEST

Attempts were made to group the haemolytic streptococci of Lancefield's groups A - N with the complement-fixation test. These attempts were unsuccessful when the test was carried out in the conventional way, holding the antigen-antibody-complement mixture at 37°C. When the mixture was held at 1 - 4°C large amounts of complement were fixed and the fixation was specific, the results being parallel with those of the precipitin test. The antigen used in these tests was made by alkaline extraction of cells _ad modum_ Hitchcock (1924a) resulting in a clear fluid containing the group-specific polysaccharide. Similar results were obtained with Lancefield's (1928a) C antigen.

Experiments were also done with cellular antigens, both whole-broth cultures and saline suspensions, but these did not give constant or specific fixation either at 37°C or at ice-chest temperatures. The fixation with these antigens was either a pseudo- or a non-specific fixation.

Experiments were done, both with the cellular antigens and the extracts, over a wide antigen-antibody ratio, and holding the antigen-antibody-complement mixture for various lengths of time at various temperatures. With the soluble extract a 1/1 ratio of a 1/6 dilution of both antigen and antibody held at 1 - 4°C for 18 hours gave good results and was most convenient.

Possible reasons for fixation at low temperatures and not at 37°C are presented. The streptococcal complement-fixation test has certain advantages over the precipitin test: greater sensitivity; use of smaller volumes of reagents that are difficult to prepare; reagents do not have to be water clear; minor cross-reactions are more easily eliminated.

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