SEROLOGICAL INVESTIGATION OF THE CORYNEBACTERIA

by

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At present there is neither a general scheme nor are there even well defined criteria which may be followed in placing an organism within the genus corynebacterium. Morphological and physiological studies to date have not provided a satisfactory solution.

The literature is indicative of extensive serological investigations but is, on the whole, concerned with the type species C. diphtheriae. Most investigators have been occupied with typing studies on the 3 cultural groups of C. diphtheriae, gravis, mitis, and intermedius. Only recently has there been a tendency to investigate the nature of the bacillary antigens, but similarly only with regard to C. diphtheriae. For example, Oeding (1950) and Lautrop (1950) have demonstrated heat-stable antigens which are group specific and heat-labile antigens which are type specific in the diphtheria bacillus. Wong and T'Ung (1939,1940) obtained lipoid, protein, and polysaccharide fractions from the diphtheria bacillus by extraction methods. The lipoid and polysaccharide fractions were group specific while the protein was type specific. Cummins (1954) demonstrated a type specific protein and a group specific polysaccharide in the cell wall of C. diphtheriae.

In the same manner studies on the other species of corynebacteria have been directed towards typing of strains within a species. This is exemplified in the investigations with C. pyogenes and C. equi. However, Merchant (1935) demonstrated a close serological relationship between C. renalis and C. pseudotuberculosis. In 1954 Rosenthal and Cox showed antigenic similarities between strains of 2 phytopathogens, C. insidiosum, and C. michiganense.

When the present investigation of the antigenic characteristics of the corynebacteria was undertaken it was hoped that a complete study of

all the recognized members within the genus would be possible. The studies represent an attempt to clarify the existing confusion within the genus as a whole rather than concentrating on a particular species. All the usual difficulties encountered by other workers in using agglutination reactions were also met with in these experiments and a variety of procedures had to be carried out in attempting to overcome them. Therefore the experiments were initiated by using the direct agglutination reaction while trying to prepare stable antigen suspensions. The inconsistent results of these trials and the failure to duplicate the work of Cummins (1954) in preparing suitable cell wall material for agglutination reactions led to the use of extraction procedures to obtain antigenic materials. It was intended to develop some extraction method which would consistently yield antigenic material suitable for serological studies. It was further hoped that studies would allow for a grouping of the members of the genus studied and thereby provide a more valid basis for the classification of these organisms than has hitherto been available.

HISTORICAL REVIEW

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1. Serological Studies Concerned with C. diphtheriae:

The importance of C. diphtheriae as a human pathogen requires no additional emphasis. This organism is also the type species as well as the most thoroughly investigated member within the genus corynebacterium. Therefore in a review of the literature the stress will be placed on C. diphtheriae.

During the early period of investigations the species had not been divided into the 3 sub-types, gravis, mitis, and intermedius; therefore C. diphtheriae is treated as a single entity. Perhaps the first to make use of the agglutination reaction in connection with C. diphtheriae was Nicholas (1896). This author defined two groups, one agglutinable and the other inagglutinable. Langer (1916) also distinguished two groups, an agglutinable and the other completely non-agglutinable. The latter group absorbed specific agglutinins, and thus Langer concluded that all diphtheria strains were identical in their antigenic structure. Similarly, in a study of 206 strains Havens (1920) found two distinct groups with no cross agglutination between them. Durand (1918, 1920) in a survey of 255 strains, after excluding those that failed to form stable suspensions, divided the remainder into 5 groups by absorption methods; while in an analogous investigation including 133 strains, Bell (1922) defined 3 groups. Varying degrees of cross agglutination among 7 types in a study of 89 strains were described by Smith (1923). In an extensive study of strains from cases, contacts, and carriers, Eagleton and Baxter (1923) classified 332 of 348 strains into 10 groups by agglutination and agglutinin absorption. The authors suggested that all strains of C. diphtheriae possessed the same qualitative structure but differed quantitatively in their antigenic constituents. At the

same time, Scott (1923) reported 8 serological groups in a survey of 265 strains.

With the recognition of the 3 cultural types of C. diphtheriae, gravis, mitis, and intermedius by Anderson et. al. (1931) another phase of investigation is initiated. In this period the various sub-types are surveyed independently rather than the species as a whole.

An early worker, Ewing (1933) serologically grouped 106 gravis strains of C. diphtheriae and concluded that they fell into 4 distinct types which she termed A, B, C, and D. Cross reactions were observed between members of gravis, mitis, and intermedius groups. In a study of 15 intermedius strains, 13 fell into 2 serological types termed A and B. The 2 remaining strains were singletons.

Murray (1935) also carried out serological studies on the cultural groups of C. diphtheriae. He attempted a classification of some 250 strains. Among 78 gravis strains, 3 serological types were demonstrated; 4 serological types in 102 intermedius strains, and in 70 mitis strains 4 types were reported.

The classical work of Robinson and Peeney (1936) included an extensive serological survey of strains of the gravis group. In this study 739 strains were examined using the agglutination reaction. The strains were divided into 5 types: I, II, III, IV, and V. Types I, II, III, IV, corresponded to Ewings A, B, C and D types respectively.

Hewitt (1947) also contributed to the serological typing of C. diphtheriae. In his discussion grouping is the separation into the gravis, mitis, and intermedius forms, and typing is the further differentiation of strains by serological procedures such as agglutination. In the 170 gravis strains tested Hewitt distinguished 13 specific types.

Of these, the first four types were found to be identical with types I, II, III, and IV of Robinson and Peeney and Ewing's types A, B, C, and D respectively. Type 5 is also Robinson and Peeney's type V. The remaining strains were uncommon strains, mostly singletons, of various origins.

Among the 57 intermedius strains examined 4 types were found which seemed to contain 3 agglutinogens designated m, n, and v. The strains are divided into 2 main types, each having 2 sub-divisions.

The division of mitis strains is complicated by the profusion of types and by the sharing of common antigens by a number of types. A total of 150 strains was tentatively allocated to about 40 different types.

More recent experiments constitute another phase of investigation in attempting to characterize chemically the antigenic components of diphtheria bacilli.

Lautrop (1950) attempted to investigate the occurrence of heat-labile and heat-stable antigens in C. diphtheriae. The results of these experiments indicated the existence of 2 antigens differing in heat sensitivity. The author demonstrated that the heat-labile antigen inhibits the agglutination of the heat-stable antigen in a serum prepared by immunization with an autoclaved culture. A formalin treated culture containing both the heat-labile and heat-stable antigen does not agglutinate in the serum prepared against an autoclaved culture, but, an autoclaved culture, in which the heat-labile antigen has been destroyed agglutinates strongly. Thus, the heat-labile antigen masks or prevents the reaction of the heat-stable O antigen and may be said to cause an O inagglutinability. Lautrop calls the heat-labile antigens K antigens,

and describes them as somatic surface antigens causing O antigen inagglutinability of living or formalin treated cultures. Cells containing K antigen are agglutinable in antisera prepared against living or formalin treated suspensions. The heat-stable antigens are named O antigens and are characterized as somatic antigens which withstand heating for 2 hours at 127°C. From the results of absorption tests the author concludes that type specificity depends on the presence of a substance corresponding to the K antigen, which is therefore considered the type specific antigen of the diphtheria bacillus. The O antigens are regarded as group antigens.

In a parallel study, Oeding (1950), also investigated the thermostable and thermolabile antigens in C. diphtheriae. The author demonstrated that diphtheria bacilli, after heating to 100°C for 2 1/2 hours, lose a greater or smaller part of their ability to agglutinate in homologous immune sera produced by injections of formalin killed bacteria. The 3 groups gravis, mitis, and intermedius behave differently, the gravis antigens almost completely losing their agglutinability, whereas the agglutinability of mitis and intermedius strains was only insignificantly reduced. The author concludes that 2 different antigens exist in the diphtheria bacillus, one thermostable O antigen and one thermolabile antigen which Oeding has designated as Ld antigen. From the results of absorption tests the gravis types are reported to contain only Ld antigen or perhaps only small amounts of O antigen. The intermedius types studied contained both Ld and O antigens in about the same amounts, and mitis strains also contain about the same amounts of Ld and O antigens. The Ld antigen is concluded to be the specific antigen of the diphtheria bacillus. The O antigen is thought to be a group

antigen responsible for the cross reactions between gravis, mitis, and intermedius and the more significant inter-reactions within the mitis and intermedius groups.

The immunological studies on the cellular constituents of C. diphtheriae are considered as another phase of investigation. Two workers Wong and T'Ung accomplished a considerable amount in investigating various chemical fractions extracted from C. diphtheriae.

1. Studies on the polysaccharide fraction:

In their first experiments Wong and T'Ung (1938) prepared antisera with heat killed whole organisms, using 2 strains of each type tested. Precipitation and absorption tests demonstrated that the polysaccharides were shared by all the types studied. Subsequently these authors (1939) serologically confirmed the group specificity of the polysaccharides and also found them weakly antigenic in rabbits.

The same authors (1939) then extended their survey of this fraction in C. diphtheriae. Precipitin antibodies were prepared against one strain from each of 5 distinct serological types. The precipitin reaction and absorption tests demonstrated 2 kinds of polysaccharides designated A and B. Polysaccharide A was present in 4 of the 5 types studied and included 3 mitis and 1 intermedius strains. Polysaccharide B reacted with its homologous antiserum only and was contained in a gravis strain.

2. Studies on the lipid fraction:

In serological reactions the lipid fraction was concluded by Wong and T'Ung (1939) to be group specific and non antigenic.

In a parallel investigation Hoyle (1942), by cross absorption experiments in complement fixation tests, demonstrated 2 lipoid antigens in C. diphtheriae. These included a specific antigen d present in large amount in mitis and in small amount in gravis and intermedius; and a non-specific antigen G present in large amount in gravis and intermedius and in small amount in mitis. The G antigen is also present in C. hofmanni. The author uses the term "lipoid antigen" to indicate an alcohol soluble haptene. It is possible that specificity is due to polysaccharide or other groups.

3. Studies on the protein fraction:

Wong and T'Ung (1939) observed the acid soluble protein to be inert serologically and antigenically. The alkali soluble protein reacted with homologous immune serum and was weakly antigenic in rabbits. This fraction is also heat-labile, being destroyed by heating to 56°C for 30 minutes.

This work was summarized by Wong (1940) who concluded that the protein is composed of at least a heat-labile type specific antigen and a heat-stable common antigen shared by all diphtheria organisms. The specific antigen is present in larger quantity and masks the reaction of the other. The common antigen may be demonstrated by inactivating the protein with heat.

A chemical analysis of the cell wall of C. diphtheriae (PW8) was carried out by Holdsworth (1952). A protein-carbohydrate complex was isolated and assumed to be the material of the cell wall. The protein portion was found to contain glucosamine and a high proportion of diamino pimelic acid. The oligosaccharide was composed of 2 molecules of D-galactose, one of D-mannose and three of D-arabinose.

Cummins (1954) employing a different approach, also ventured to discover something of the nature of the antigens of C. diphtheriae. In these experiments intact cells and cell wall material obtained by disintegrating whole cells were used. In agglutination tests suspensions of cell wall material were capable of absorbing specific antibodies from sera against intact cells, and could also induce the formation of specific antibodies in rabbits.

From the results of the experiments the author concludes the presence of 2 antigens in the cell wall of C. diphtheriae. The first is a superficial, specific antigen responsible for agglutination in intact bacteria and relatively heat-labile. The second antigen is a deeper group antigen and thought to be polysaccharide in nature. This latter antigen is heat-stable, susceptible to periodate action and can only be demonstrated in intact bacteria by absorption. The group antigen was found in one strain of C. ovis. The antigen could not be demonstrated in the other bacteria tested including, C. hofmanni, C. xerosis, and C. renale.

2. Serological Investigations Pertaining to Other Corynebacteria:

The agglutination reactions of corynebacteria isolated from lesions of various animals were studied by Merchant (1935). A close serological relationship was demonstrated between C. renalis and C. pseudotuberculosis. This relationship was also found to be reciprocal. A culture of C. equi used for comparison was agglutinated by its own antiserum only. Also included in this study were 5 strains of C. pyogenes which were found to be homogeneous in their agglutination reactions. Antisera prepared against 2 of the strains agglutinated all the strains studied.

In other attempts to classify C. pyogenes according to its antigenic behaviour Brown and Orcutt (1920) found no marked differences between 12 strains tested by precipitation reactions. Another worker, Minett (1922) reported that 4 strains were agglutinated to approximately the same titer by one serum. A similar experience with 10 strains was recorded by Jowett (1925).

Lovell (1937) also performed immunological studies on C. pyogenes. In his investigations the results of direct agglutination tests showed that the strains could be placed in 2 different groups, one of which was apparently a sub-type of the other. While some sera agglutinated all strains tested to approximately the same titer, sera produced from other strains agglutinated some suspensions to low titers or not at all.

In an extensive monograph dealing with the animal corynebacteria Parnas and co-workers (1952) also included some immunological studies on C. pyogenes. The study included 6 types of C. pyogenes: bovis, ovis, suis, equi, leporis, and humanum. The work was carried out using whole cells in agglutination reactions and also included precipitin tests with a haptene extracted by Lancefield's method. The authors concluded an antigenic heterogeneity among this group of corynebacteria. A very close relationship was observed between C. pyogenes var. humanum and C. pyogenes var. bovis. Cross reactions were also noted between C. pyogenes var. bovis and C. pyogenes var. ovis.

Ryff and Browne (1954) used 10 strains of C. pyogenes to prepare antisera against formalin killed and heated cultures. Cross agglutination and agglutinin absorption tests performed with these 10 strains plus an additional 47 strains indicated a consistent relationship within the species.

Bruner and co-workers (1939) attempted a serological classification of C. equi. Antisera were prepared against 7 strains of C. equi using formalin killed suspensions of the organisms. Agglutination tests demonstrated that the strains of C. equi were highly type specific. Extraction with acid at 100°C showed specific antigens by the complement fixation reaction. While all strains of C. equi apparently contain comparable amounts of species specific antigens, not all antisera contain large amounts of species specific antibody. These species specific antigens were not apparent in other species of corynebacteria used for comparison. Fourteen of the 20 cultures studied were divisible into 2 main serological types.

The work completed by Parnas et. al. (1952) was for the most part concerned with C. equi. These authors recognize 3 antigenic components in this species.

1. The E antigen or species specific antigen which is present in all strains of C. equi and may be demonstrated by the complement fixation reaction.

2. A group specific antigen which takes part in the agglutination reaction of the intact cell. By virtue of this antigen C. equi is divisible into 6 groups. No cross agglutination occurs between a strain of 1 group and sera of other groups.

3. A type specific antigen which is liberated by boiling with N/20 HCl and can be demonstrated by the precipitin reaction. This antigen is responsible for separation into types within each group.

The authors tested 20 strains of C. equi isolated from various localities in Poland and causing pyobacillosis in foals. All 20 strains

displayed the same species specific reaction as well as the same group and type specific reactions. Therefore these strains were concluded to be identical in their antigenic constitution with regard to their species, group, and type specific antigens. These results proved the previous assumption that only one type of C. equi was responsible for pyobacillosis of foals in Poland.

Three foreign strains of C. equi were also included in the investigation. Two strains exhibited the same group and type specific reactions and were considered identical, while the third strain formed a separate group.

The same authors discerned a common antigen between C. equi and a strain of C. hoagii. The presence of common antigens could not be demonstrated among the following bacteria by means of the complement fixation test: C. equi, C. pyogenes var. bovis, C. pyogenes var. suis, C. renale var. bovis, C. lipolyticum var. bovis, C. diphtheriae, and C. enzymicum.

Practically nothing is known concerning the antigenic characteristics of the corynebacteria associated with plants. The somatic antigens of 2 phytopathogens have been studied by Rosenthal and Cox (1953). Nine strains of C. michiganense and 2 of C. insidiosum were studied by agglutination and agglutinin absorption techniques. All strains of C. michiganense examined contained the somatic antigen designated B and 5 strains contained an additional antigen A. One strain of C. insidiosum also possessed antigens A and B and is antigenically identical with C. michiganense AB strains. Another strain of C. insidiosum included the specific antigen C in addition to antigen B.

Saxholm's diphtheria-like-corynebacteria are also of sufficient importance to warrant discussion. Saxholm (1951) reported the isolation

of diphtheria-like-corynebacteria (DLC) from cases of sore throat and from healthy contact carriers in Norway. His strains differed from C. diphtheriae morphologically and biochemically, but produced a toxin indistinguishable from diphtheria toxin.

Henriksen and Grelland (1952) studied the serological relationship of these organisms to one another and to the various types of C. diphtheriae isolated in Norway during the same period.

The 2 strains of C. ulcerans included in the testing and the DLC were alike in morphology and in cultural and biochemical properties. Serologically, all strains reacted to approximately the same titers with 2 DLC sera. The results of absorption tests indicate that all strains of DLC and C. ulcerans have the same agglutinogen. The results of agglutination tests with DLC sera and gravis, mitis, and intermedius strains indicate that none of the C. diphtheriae strains used in this study is closely related to the DLC-C. ulcerans group. In view of these results Saxholm's DLC and C. ulcerans may be considered identical.

The agglutination reactions of 6 species of anaerobic corynebacteria were examined by Linzenmeier (1954). The species included: C. anaerobium, C. avidum, C. parvum, C. granulosum, C. diphtheroides, and C. liquefaciens. Cultures which grew in coherent granules and autoagglutinable strains were not examined by direct agglutination but by absorption of agglutinins. The author also observed that suspensions heated to 56° and 100°C gave a higher agglutination titer than those not heated.

The species C. avidum was not divisible into serological groups and using a serum prepared against one strain, cross reactions were noted with strains of C. anaerobium, C. granulosum, C. liquefaciens, and C. parvum but not with C. diphtheroides. The agglutinogenic constitution of the

species C. avidum was concluded to be complex since after homologous and heterologous absorption the serum still agglutinates homologous and heterologous cultures except those of C. liquefaciens.

Antisera against C. anaerobium and C. liquefaciens also confirmed the existence of common agglutinogens between these species and other species of anaerobic corynebacteria except C. diphtheroides.

Antisera against C. parvum and C. granulosum partly confirmed the relationship between these species and the other anaerobic strains.

Serologically the species C. diphtheroides appeared to be almost unique and possessed a slight relationship only with the species C. avidum.

In summary, these studies demonstrated a reciprocal and close relationship between C. avidum and C. anaerobium on the one hand and C. parvum and C. liquefaciens on the other hand. All four species showed a slight relationship among themselves and with C. granulosum. In contrast, the species C. diphtheroides seemed to be quite distinct serologically.

3. Difficulties Encountered in Serological Investigations of Corynebacteria and Efforts to Overcome Them:

Freeman and Minzel (1952) have pointed out the lack of coordination in the preliminary grouping of cultures of C. diphtheriae, prior to serological study, according to the classic criteria of Anderson et. al. (1931). Freeman and Minzel cite several examples, such as the tendency on the part of a number of investigators to regard all starch fermenting strains of C. diphtheriae, regardless of their other properties, as belonging to the gravis group. Similarly, all non starch fermenting strains except intermedius are regarded by some as members of the mitis group. On the other hand, Frobisher (1938) has insisted that the terms gravis and mitis be used only when the strains so classified meet all the

original criteria of Anderson et. al. (1931). Still other investigators have employed the terms gravis and mitis without in any way describing the criteria they followed. Further confusion has been added to the problem of typing gravis strains by the use of the same Roman numerals for cultural and serological groups that are unrelated. Another difficulty in the way of a uniform classification is the employment of the terms "intermediate", "minimus", and "intermedius" to describe small colony strains of C. diphtheriae apparently belonging to the same cultural group. For grouping cultures of C. diphtheriae, prior to serological typing, Freeman and Minzel have proposed a classification based on fermentation reactions to clarify the typing problems of this species.

Although the literature is indicative of an extensive number of investigations concerning the serological typing of C. diphtheriae, nevertheless, the only commonly recognized gravis types are those of Robinson and Peeney (1936), which also comprise gravis types I to V of both Bynoe (1948) and Hewitt (1947). Perhaps of significance, in the failure of the many investigators in various parts of the world to correlate completely all their findings, is the diversity of technics recorded in obtaining suitable suspensions of all strains of C. diphtheriae.

Huang and Sia (1940) were among those confronted with this problem. A portion of their work was concerned entirely with attempts to obtain stable suspensions of C. diphtheriae for the agglutination reaction. These workers tried a variety of standard techniques which included: the use of glycerine as described by Eagleton and Baxter (1923), mechanical shaking as employed by Ewing (1933) and modified by Stewart (1938), and also Murray (1937), and the use of N/250 NaOH in washing a

Loefflers serum slope to obtain suitable suspensions as used by Robinson and Peeney (1936). These methods proved to be generally unsatisfactory as did the serum broth method developed by Knox (1937). Several other methods were attempted and involved, the use of the diffuse growth found in the supernatant following prolonged incubation, carrying out repeated transfers to avoid spontaneous agglutination, and growing the organism at room temperature. These methods also proved to be inconsistent.

The workers then turned their attention to the factors that govern the stability of bacterial suspensions, namely, the cohesive force between the bacteria and the repelling force - the electrokinetic potential between the surface of the bacteria and the diluent. So long as the electrokinetic potential difference is higher than the cohesive force of the bacteria, the suspension remains stable, and conversely, if the electrokinetic is less than the cohesive force the bacteria aggregate. The strains of C. diphtheriae examined in this study and exhibiting granular growth in broth were assumed to have a high cohesive force and a low electrokinetic potential difference. Those strains which grew diffusely but spontaneously agglutinate must have a higher electrokinetic potential difference than cohesive force but the difference between them must be very small since the addition of normal saline results in agglutination. This is explained by the fact that the potential difference decreases in the presence of low concentrations of electrolyte. It had been demonstrated that a high concentration of electrolyte tends to decrease the cohesive force. With this in mind Huang and Sia (1940) attempted to stabilize suspensions by adding concentrated solutions of electrolytes to decrease the cohesive force.

The electrolytes utilized were sodium chloride and magnesium chloride. More stable and less granular suspensions could be obtained with varying concentrations. However, very high concentrations such as M and 2M affected the titer in homologous systems. Difficulty was encountered in applying this technic to gravis strains. Normally the strains were grown in a meat infusion broth containing dextrose and serum, but the gravis strains were grown on Loeffler's serum slopes and suspended in N/4 NaCl. The workers also found M/2 $MgCl_2$ to give satisfactory results.

Minzel and Freeman (1950) also studied the problem of obtaining stable suspensions of C. diphtheriae. They compared the methods of recent investigators and included combinations of technics in some cases in an attempt to obtain satisfactory suspensions for typing purposes. Among the methods tried were those of Hewitt (1947), Bynoe and Helmer (1945), Huang and Sia (1940), Robinson and Peeney (1936), and Murray (1935). Concomitantly these investigators also utilized surface active agents including Tween 20, Tween 40, Tween 60, Tween 80, and Atlas G-2144. The most satisfactory results were obtained when Tween 80 was employed in the broth medium. The technique of Huang and Sia was also followed in the use of 1.5 per cent NaCl solution. In the table showing the comparisons of bacterial suspensions Minzel and Freeman indicate that several strains show the presence of large pseudo-agglutinated masses. There was no regular correlation between the strains displaying this phenomenon and the method used for preparing the suspension.

Another procedure for studying the agglutination reactions of those strains or species of corynebacteria which proved to be autoagglutinable or grew in coherent granules has been the technic of absorption of agglutinins as utilized by Linzenmeier (1954).

4. Application of Extracted Antigens in the Serological Analysis of Bacteria:

The problems encountered in the use of agglutination reactions to determine the antigenic relationships among the various species of corynebacteria are not unique for this group of organisms. The most widely adopted approach in such instances has resulted in attempts to free the desired antigens from the bacterial cell. While certain bacterial antigens like diphtheria toxin, streptolysin, and pneumococcal polysaccharides pass into the culture medium during growth or on autolysis and may be separated by chemical fractionation of the culture fluid, the usual procedure for obtaining antigens from microorganisms is by extraction of washed, intact or disintegrated cells with appropriate solvents. Some antigens can be extracted with water, saline, or neutral buffers, while others require acid or alkali or enzymatic digestion. Certain organic solvents have also been used. In some cases it is possible to extract the washed, intact organisms, but in others it is necessary first to disintegrate the cells mechanically by such methods as grinding, by alternate freezing and thawing, shaking with glass beads, or by exposure to sonic waves. In many instances it has been found advantageous to extract lipids with alcohol-ether, acetone, or chloroform, before applying aqueous procedures. In using any or all of these chemical and mechanical procedures, the possibility of degradation as a result of the treatment must be considered.

The streptococci perhaps represent the classic example wherein similar difficulties in serological investigations are encountered as found in the corynebacteria. The problem was solved, to a great extent, with the realization of active extracted. components. Within the streptococci

the general application of macroscopic agglutination tests was not possible due to the inability to obtain stable diffuse suspensions of the organisms. The original demonstration of the different serologically active polysaccharides resulted from the work of Lancefield (1928, 1933). A total of 13 groups have now been recognized, each with its peculiar polysaccharide antigen. The majority of strains isolated from pathological lesions in man share the same polysaccharide and are classified as group A. The polysaccharides themselves form an integral part of the bacterial cell, and for the group A strains form part of the cell wall. To obtain them in solution the organisms must be disrupted. To achieve this purpose, either Lancefield's (1928, 1933) acid extraction method, Fuller's (1938) formamide method, or Maxted's (1948) enzyme digestion method may be used. The presence of the polysaccharide can be demonstrated by a simple precipitin reaction using specially prepared high titered group antisera. The polysaccharides seem to play no part in the agglutination of streptococci.

The major groups may be further subdivided into types on the basis of other antigens either polysaccharide or protein in nature. The group A strains have been divided into at least 30 types on the basis of 2 protein antigens the M and T substances. Griffith (1934) distinguished these group A types by means of the slide agglutination reaction. Lancefield (1928, 1933) extracted type specific antigens from the cocci by hot acid, and identified them by the precipitin test. On the whole the results of Lancefield agreed with those of Griffith but some discrepancies occurred.

In the technic of Lancefield the bacteria are extracted by dilute HCl in a boiling water bath and both C substance and M antigens may be extracted

in this manner. Following extraction, cooling and centrifuging the supernatant is neutralized with NaOH to precipitate the nucleoproteins. After removal of the precipitated proteins the supernatant, which represents the crude extract, is tested against appropriate sera. The C and M substances can be separated by fractional precipitation with alcohol. The antisera are prepared against whole bacterial cells. For the production of grouping sera the strains of streptococci used should be as free as possible from type specific substances. Typing sera are produced with strains rich in type specific antigens and absorption of non type specific antibodies from the sera.

In brief, these have been the methods which have solved a difficult problem among the species of streptococci. Sharpe (1955) adopted the above method for the serological classification of the lactobacilli. A total of 412 strains was studied. Utilizing sera prepared against some of these strains and crude HCl cell extracts, it was possible to classify 312 (70 per cent) of these strains by precipitin tests into 6 groups and one sub-group. This classification was in agreement with one based on the physiological characteristics of the strains.

The antigenic structure of staphylococci has also been approached by means of chemical fractionation. Two polysaccharides have been isolated, Julianelle and Wieghard (1934, 1935). The first polysaccharide was isolated from pathogenic (A), and the second from non-pathogenic (B) strains. These polysaccharides are non-antigenic, type specific and react with rabbit immune sera produced against intact cells. In addition to the carbohydrates a complex protein substance has also been extracted. This protein is antigenic and responsible for species specificity of the staphylococci.

Within the Gram negative bacteria for example the coli, salmonella, and shigella groups, polysaccharides have been successfully extracted by such substances as: dilute acetic acid, trichloracetic acid, diethylene glycoll, phenol, and also glycine which was employed to disrupt the cells. It seems that for the isolation of carbohydrates trichloracetic acid may be satisfactorily used to extract any Gram negative species, however the efficiency of this solvent varies depending on the organism.

There is hardly a group or genus of bacteria within which experiments of this type have not been attempted. In many instances the results have not been as satisfactory as one might wish, nevertheless, experiments of this kind provide foundations for more fruitful endeavors.

5. Hemagglutination With Bacteria or Extracted Bacterial Components:

Bacteria or products extracted from the cell have also been demonstrated to play an active role in another phenomenon. The adsorption of components of bacteria onto erythrocytes, which are thus rendered agglutinable by antisera directed against these components or the whole cell, has been demonstrated by a number of investigators. The results of many workers suggested that, at least in some instances, a bacterial polysaccharide is involved in the adsorption process, and it has been generally assumed that the agglutination by immune sera is due to a reaction between specific antibody and the adsorbed polysaccharide. Purified protein preparations have not been shown to be similarly adsorbed by normal erythrocytes. Interest in protein fractions was again awakened when Boyden (1951) demonstrated the adsorption of proteins on erythrocytes treated with tannic acid and subsequent hemagglutination by antiprotein sera.

Similar experiments include those of Middlebrook and Dubos (1948) who demonstrated a specific hemagglutination reaction between sheep erythrocytes

treated with a component of a polysaccharide fraction of mammalian tubercle bacilli, and the sera of experimental animals or of tuberculous patients. Similarly, Alexander et. al. (1950) reported that erythrocytes treated with polysaccharides from P. tularensis are specifically agglutinated by the sera of persons recovered from tularemia or vaccinated against it, and by the serum of animals immunized with living cultures.

Neter and co-workers (1952) revealed that the hemagglutination and hemolysis tests can be used successfully for the demonstration of either antibodies active against E. coli O111 and 055 or the homologous antigens. The results of these workers indicated that it is possible with the aid of the indirect hemagglutination test to demonstrate antibodies against E. coli, serogroups O111 and 055, in human sera which fail to agglutinate the homologous bacterial suspensions. In recent work Neter et. al. (1956) showed that a suspension of red blood cells modified with several salmonella and shigella antigens is suitable as a polyvalent antigen for the detection of enterobacterial antibodies.

The same technic has been used by Meynell (1954) to study the antigens of the tubercle bacillus. The polysaccharide and protein antigens present in "purified protein derivative" (PPD) were separated by adsorbing the polysaccharides onto normal sheep red cells and the proteins onto red cells that had previously been tanned. These preparations were then tested against the appropriate antisera.

Many more examples of parallel studies may be cited. In many instances the eventual goal was to provide a useful and efficient method for diagnosis or confirmation applicable to clinical work.

Since the first demonstration of this method, at least the following species or types of bacteria have been demonstrated capable of modifying

red blood cells for agglutination or hemolysis by anti-bacterial antibodies:

Cocci:

Micrococcus pyogenes

Streptococcus pyogenes

Diplococcus pneumoniae

Neisseria meningitidis

Bacilli:

Hemophilus pertussis

Hemophilus influenzae

salmonellae

Salmonella typhosa

Shigella sonnei

Escherichia coli

Pasteurella tularense

Pasteurella pestis

Corynebacterium diphtheriae

Mycobacterium tuberculosis

6. Antigen-Antibody Reactions in Gels:

Specific precipitation of an antigen and antibody in gelified media has been used for bacteriological studies since at least 1932. Sia and Chung (1932) used a technic for the identification of pneumococcus types in which an opaque ring developed around colonies grown on layers of agar containing the homologous antiserum. At almost the same time Petrie (1932) made similar observations when he attempted the transformation of smooth into rough bacterial types by culturing them on media containing antiserum.

The extended use of a solid or semi-solid medium per se as the arena for the specific reaction of an antigen and its homologous antibody, or the analysis of mixtures of antigens and antibodies has resulted primarily from the investigations of two workers, J. Oudin and O. Ouchterlony.

Oudin Technic

The Oudin method involves the use of thin bore tubes, around 3 mm. internal diameter, which are filled one-half to two-thirds with a mixture of agar and immune serum. The antigen solution is then layered over the immune serum-agar mixture. As the antigen diffuses into the agar immune-serum layer a zone of precipitation is formed which moves down the tube as more antigen diffuses into the agar. The zone of precipitation has a sharp leading edge, while its upper part containing antigen-antibody complex is diffuse. The density of the precipitate is maximal at the leading edge. Precipitation occurs where the ratios of antigen and antibody are equivalent. Hence the process is that of precipitation, first at the interface, then dissolution in excess antigen and re-precipitation further down in the tube as the result of new equivalent ratios of antigen and antibody being formed, thus causing the zone to migrate. Therefore the more concentrated the antigen, the deeper the penetration of the precipitation zone.

The limiting conditions for the appearance of a zone in the gel may be stated as follows:

1.) The concentration of antibody must be sufficiently great for the zone of precipitation to be perceptible. Therefore, for each antibody there exists a concentration below which precipitation is not visible.

2.) The ratio of the initial concentration of antigen to the concentration of antibody must be distinctly greater than their equivalence ratio.

The minimum concentration of each antigen that can react to form a visible zone depends on the concentration of antibody and on the antigen-antibody ratio at equivalence. If the concentration of antigen is less than the minimum value required for equivalence, precipitation occurs in the antigen layer. If the initial concentration of antigen is equivalent to that of antibody, precipitation will occur only at the interface. Therefore it is advisable that the concentration be as high as possible. A high concentration does not introduce any complications.

When a single antigen-antibody system is present only one band is formed. In analyses of mixtures of antigens, multiple bands will usually be obtained. The number of bands can and frequently does correspond to the exact number of antigen-antibody systems present but fewer bands may appear for a number of reasons.

In a system which produces multiple zones there are several ways of identifying the components. For example, if one changes the concentration of only one antigen or of its antibody without affecting the concentration of the other reactants only one zone will be affected. Thus, if the concentration of one antigen is considerably increased, the zone of precipitation of that antigen and homologous antibody will appear considerably lower in the depth of the tube.

In another procedure let us assume a given solution M, containing several antigens, reacts with an anti-M serum, Am, to give several zones. One desires to know which of these zones are produced by antigens common to M and to another solution L suspected to contain one or more of them. In the procedure for identification two tubes are compared, one of which contains the antigens of L in higher concentration than the other. In practice one prepares a dilution of M in L and a similar dilution of M in any convenient diluent. Each dilution is allowed to react separately with anti-M

antobody, Am, in tubes. If comparison reveals that certain zones in M plus L show a higher penetration than in tube M, these zones are caused by antigens present in L.

The conclusions of Oudin were confirmed by Munoz and Becker (1950) using the method of supernatant analysis.

Bowen (1952) using Oudin's method, and Pope et. al. (1951), using a modification of the Oudin method examined the toxic filtrates from cultures of C. diphtheriae, and also purified toxins, toxoids, and antitoxins, and obtained multiple lines showing the presence of many antigens and antibodies.

Ouchterlony Technic

Ouchterlony's "double diffusion" method for the analysis of multiple antigen-antibody systems is basically the phenomenon of two reactants diffusing toward each other through a gel which contained neither reactant at the beginning of the experiment. Specific precipitation between the reacting components occurs at some point in the gel.

The common procedure is as follows: Molds are placed in Petri dishes and agar is added to a depth of 3 to 4 mm. The molds, which may be of any size, but are around 10 mm. x 10 mm., are then removed leaving wells. Two molds are placed parallel to each other with a third below and between them. The reactants are placed in these wells and allowed to diffuse toward each other. With this method various concentrations of the same antigen can be compared by reaction with homologous antibody, a known antigen can be compared with an unknown to determine whether they are related, and the fractionation or purification of substances such as toxins can be followed in a stepwise manner.

The appearance and formation of the specific pattern of the precipitation spectrum is best understood if the diffusion process is visualized in terms of migrating critical concentrations. Thus a precipitate is formed where the critical or equivalent concentrations of an antigen and its corresponding antibody meet. Neither antigen nor antibody can diffuse beyond the precipitation zone as they will immediately precipitate on meeting the other reactant. The precipitation streak will thus serve as a virtual barrier to the diffusion of the components precipitating in the reaction.

However, the appearance, in multiple antigen-antibody systems of a precipitate spectrum where each line corresponds to one pair of antigen and antibody, implies that the formation of a precipitate does not disturb the diffusion of other components or systems that are not involved in that particular reaction.

The agar plate test yields 3 different patterns of specific precipitation: Fusion of precipitate lines, complete intersection of the lines, or partial intersection of the lines.

Reaction of Identity: If the antigens or antibodies being compared form zones of precipitation which fuse completely, they are considered identical.

Reaction of Non-Identity: If the antigens or antibodies being compared form zones of precipitation which completely intersect, they are not identical nor related.

Reaction of Partial Identity: If the zones of precipitation formed partially intersect, with one line ending at the point of intersection and the other continuing to form a spur, the reactants are related or partially identical.

The Ouchterlony double diffusion technic was used by Halbert and co-workers (1955) in studying human streptococcal infections. From 0 to 5 bands were found with different human sera, from patients with various clinical diagnoses, in tests with streptolysin O concentrate. When several lines were seen one was usually quite intense, the others were moderate to very delicate. In tests with crude concentrates of the streptococcal culture supernates from 0 to 7 bands were seen. Patients with rheumatic fever showed many more bands than the non-rheumatic patients with both of the antigen preparations.

Bjorklund and Berengo (1954), also applying the double diffusion method, have studied the toxins from the filtrates of clostridia cultures. When toxin and antiserum, in optimal concentrations, were diffused towards each other each system gave rise to a quite typical precipitation pattern.

For example:

1. Cl. welchii: 7 different precipitation lines were presented.

2. Cl. histolyticum: The precipitate pattern was always represented by 2 very distinct and dense lines.

3. Cl. tetani: Here the precipitate pattern was found to be very complex and contained several ill defined lines some of them showing partial fusion. The number of lines was estimated from 8 to 12.

4. Cl. botulinum A: Nine lines were observed; one of them very distinct, another a little less prominent and the remaining ones all small and weak.

5. Cl. botulinum B: A total of 5 lines were formed, one of which was very distinct and 2 were rather prominent. The remainder were weak.

Since it has been the plan of the author to undertake a serological investigation of the corynebacteria, therefore, in this review the various

types of researches pertaining to the immunological characterization of the corynebacteria have been presented. The manifold problems encountered by other investigators and reported herein have also proved a barrier to the author and efforts were made to overcome them. The various extraction procedures, the hemagglutination test, and the Ouchterlony double diffusion method, described in this review have also played a part, to a varying extent, in our own experiments.

MATERIALS AND METHODS

MATERIALS AND METHODS

1. Strains Studied in the Investigations:

The following are the strains of corynebacteria studied and their source.

Species	Culture Number	Source
C. diphtheriae	14	Alexandra Hospital
C. diphtheriae (PW8)	174	University of Montreal
C. ovis (ovine)	120	NCTC 4680
C. hofmanni	134	NCTC 231
C. murium (Kutscheri)	135	NCTC 949
C. pyogenes (bovine)	136	NCTC 6448
C. renale	137	NCTC 7448
C. ulcerans	139	NCTC 7906
C. xerosis	140	NCTC 7238
C. xerosis	141	NCTC 8481
C. xerose	331	ATCC 373
C. hoagii	12	Royal Victoria Hosp. Clinical Lab.
C. bovis	132	NCTC 3224
C. bovis	320	ATCC 7715
C. equi	133	NCTC 1621
C. segmentosum	138	NCTC 934
C. "Q"	149	Dr. R.J. Avery
C. acnes	131	NCTC 737
C. enzymicum	288	ATCC 8156
C. hemolyticum	290	ATCC 9345
C. paurometabulum	281	ATCC 8368

Species	Culture Number	Source
C. fimi	282	ATCC 484
C. tumescens	285	ATCC 6947
C. michiganense	251	Dr. W.H. Burckholder Cornell University
C. poinsettiae	254	Dr. W.H. Burkholder Cornell University
C. insidiosum	255	Dr. W.H. Burkholder Cornell University
C. helvolum	194	Division of Bacteriology and Dairy Research Science Service, Ottawa
C. flaccumfaciens	195	Division of Bacteriology and Dairy Research Science Service, Ottawa
C. sepedonicum	196	Division of Bacteriology and Dairy Research Science Service, Ottawa

NCTC - National Culture Type Collection, London, England.

ATCC - American Type Culture Collection, Washington, D.C.

2. Maintenance of Cultures:

The various strains of corynebacteria were originally obtained in lyophilized form and thereafter maintained on Loeffler serum slopes and stored at 4°C. For additional cultivation of the strains the inoculum was obtained either from the growth on these stock slopes or from lyophilized culture. When required the stock slopes were sub-cultured every 10-12 weeks. In addition, each organism has been lyophilized in sufficient quantity to ensure a continued and standard supply of the original strains.

The strain of Streptomyces albus used for enzyme production was obtained from Dr. Maclyn McCarty of the Hospital of the Rockefeller

Institute for Medical Research. The strain was grown on 4 or 5 nutrient agar slopes for 5 days at 37°C. Due to the high degree of variability with respect to enzyme production on the same medium at different times it is important to avoid serial sub-culture of the strain used for production of the enzymes. Therefore, the growth from the above agar slopes was used to inoculate 30 agar slopes, and after incubation for 5 days at 37°C the slants were stored at 4°C to serve as the source of inoculum for subsequent experiments. The harvest from several slopes was also lyophilized.

3. Preparation and Standardization of Antigens:

Preparation of Antigens for Immunization.

The strains to be used as antigens for immunization were first grown in 10 ml. volumes of brain heart infusion broth containing 5 per cent sheep serum for 24 hours at 37°C. Transfers were then made to 100 ml. flasks of brain heart infusion broth containing Tween 80 in a final concentration of 0.7 per cent. Incubation was carried out at 37°C for 24 hours. Where it was required the time of incubation was increased to 30-36 hours or serum was included in the medium in a final concentration of 1.5 per cent. Following incubation the contents of each flask were transferred to a 200 ml. centrifuge bottle, centrifuged for 30-45 minutes (International Equipment Co. model), and the broth or supernatant discarded. The cells were then resuspended in 10-15 ml. of 1.5 per cent saline containing 0.4 per cent formalin, freshly added. The formalinized suspension was incubated at 37°C. for 24 hours. After standing the appropriate time the culture was tested for sterility by inoculating a loopful onto a blood agar plate and observing the plate for growth during incubation at 37°C for 40-48 hours. Meanwhile the cell suspension was stored in the refrigerator at 4°C. If the sterility test proved satisfactory the cells were washed 3 times using 1.5 per cent

sterile saline. The washing was accomplished by centrifuging the suspension at 3000 r.p.m. for 20 minutes (Servall angle centrifuge), removing the supernatant aseptically, and resuspending the cells in 10 ml. of sterile salt solution. When washed the cells were resuspended in 15-20 ml. of 1.5 per cent saline containing 0.4 per cent formalin and stored at 4°C as a stock suspension.

This scheme was slightly modified in preparing antigens of the soil species of corynebacteria. These strains were grown at room temperature or approximately 20°C for 3-5 days. The medium and subsequent treatment was the same as described above for the other corynebacteria.

In the last series of immunizations where booster doses were administered and several new sera were prepared this procedure was again modified. Instead of using formalin killed suspensions the cells were heated to 60°C for 1 hour, no Tween 80 was included in the medium, and 0.85 per cent saline was used in the place of 1.5 per cent saline.

Preparation of Antigens for the Agglutination Reaction.

The suspensions for the agglutination reactions were prepared in the following manner: The growth from a Loeffler's slope, maintained as a stock culture, was used to inoculate either, 10 ml. volumes of brain heart infusion broth containing 5 per cent sheep serum, or to seed directly 100 ml. volumes of broth containing 0.7 per cent Tween 80. Following incubation at 37°C for 24 hours, the growth was harvested by transferring the contents of each flask to a 200 ml. centrifuge bottle (International Equipment Co. model), and centrifuging for 30-45 minutes. The supernatant was decanted and the cells were resuspended in 20-25 ml. of 1.5 per cent saline. This preparation was maintained in the refrigerator as a stock suspension.

Again, depending on the strain, minor modifications were necessary such as: increase of time of incubation to 30-36 hours, incorporation of serum in the medium in a concentration of 1.5 per cent and, for the soil organisms, growth at room temperature or approximately 20°C for 3-5 days.

Standardization of Antigens.

During the preparation of the original series of antigens one organism was selected, strain 14 (C. diphtheriae), and a portion of the suspension was diluted to equal an opacity midway between tubes #1 and #2 of the Brown's series. The end point was determined by visual comparison: This dilution according to Brown's tables corresponded to approximately 500 x 10^6 organisms per ml., and when placed in the Evelyn Photoelectric Colorimeter yielded 54 per cent transmission of light with the #420 filter. This reading was then used as the standard of dilution for other suspensions, both for those used as antigens in immunization, and for those employed in the agglutination reaction.

The purified cell wall preparations were diluted to give a reading of 63 per cent transmission of light with the #420 filter in the Evelyn Photoelectric Colorimeter. This turbidity corresponds to a concentration of 375 x 10^6 organisms per ml.

Refer to the appendix for details in operating the Evelyn Photoelectric Colorimeter.

Preparation of Stock Suspensions for Extraction Experiments:

The strains to be extracted were first cultivated in 10 ml. volumes of brain heart infusion broth containing 5 per cent sheep serum. The inoculum consisted either of the growth washed from a stock Loeffler's slope or more usually of a lyophilized culture. Following incubation at

37°C for 24 hours the 10 ml. culture provided the inoculum for 100 ml. to 300 ml. volumes of brain heart infusion broth plus 1 per cent sheep serum. The 100 ml. flasks were incubated at 37°C for 24 hours.

In the earlier experiments the 100 ml. volumes were centrifuged following incubation and the cells washed twice with sterile 0.85 per cent saline. The cells were extracted immediately or resuspended in 5 ml. or 10 ml. saline and stored in the refrigerator at 4°C as a stock suspension.

In later experiments the 100 ml. culture was used to inoculate 6 liters of brain heart infusion broth, plus 1 per cent horse serum, contained in a 12 liter flask, and incubated with occasional shaking at 37°C for 24 hours. The cells were harvested in the de Laval separator, collected and washed twice with 0.85 per cent saline. The cells were then resuspended in 40 ml. to 50 ml. saline and stored in the refrigerator at 4°C.

The soil corynebacteria to be used for extraction were first grown from dried culture on 2 Loeffler slopes for 2 days at room temperature. The growth from the slopes was washed off with 3 ml. to 4 ml. 0.85 per cent saline and with a Pasteur pipette introduced into 5 Roux bottles, each containing 200 ml. of "Eugonagar". (For preparation of Eugonagar see appendix.) The Roux bottles were incubated at room temperature for 2 days. Then the growth was washed from the surface of the agar with 0.85 per cent saline, the washings combined and centrifuged. The cells were again washed with 100 ml. saline and resuspended in 50 ml. saline and stored at 4°C as a stock suspension.

4. Immunization of Animals:

For the production of antisera rabbits were injected intravenously, in the marginal ear vein, with bacterial suspensions in 1.5 per cent saline containing 0.4 per cent formalin. These suspensions were standardized to

500 x 10^6 organisms per ml. A series of increasing doses was administered at 72 hour intervals for a period of 3 weeks. The first injection was 0.25 ml., and subsequent inoculations were 0.5 ml., 1 ml., 2 ml., and so on, reaching a final injection of 6 ml. at the end of the 3 week period. Following a 5 day interval the injections were resumed. Three further inoculations were usually given, in volumes of 7, 8, and 9 ml. respectively, at 5 day intervals. The animals were bled by cardiac puncture on the sixth day following the last injection. The blood, approximately 50 ml. per animal, was allowed to clot at room temperature and then placed in the refrigerator overnight. The sera were collected the following day and stored in vials at 4°C. Merthiolate in a final concentration of 1/10,000 was added as a preservative. The time consumed from the initial dose to the final bleeding was approximately 6 weeks.

This procedure was modified slightly when the booster doses were administered and new sera were prepared near the end of the study. The amount of antigen injected did not exceed 3 ml., 5 or 6 injections were given for the booster sera, and 0.85 per cent saline was used with no formalin added since heated cultures were utilized.

5. Procedure for the Agglutination Reaction:

In this study the agglutination reactions were carried out by employing the usual procedure of doubling dilutions of antisera. A series of 10 dilutions of serum was used ranging from a 1/10 to 1/5120 dilution. The total volume in each tube was 1 ml. and consisted of 0.5 ml. of the diluted antiserum to which was added an equal amount of antigen, standardized to 500 x 10^6 organisms per ml. Appropriate antigen controls were included consisting of 0.5 ml. of 1.5 per cent saline and 0.5 ml. of the antigen

suspension. The tubes were placed in a water bath at 56°C for 6 hours. The degree of agglutination was recorded after 6 hours in the water bath at 56°C, and after standing overnight at room temperature. A system of plus marks was used to denote the degree of agglutination; one plus (+) to distinguish that amount barely visible to the naked eye, four plus (++++) to indicate complete agglutination. The intermediate degrees were measured by two plus (++) and three plus (+++). The titer of each serum is represented by the highest dilution showing complete agglutination (++++).

Essentially the same procedure was followed in agglutination reactions using the cell wall material. However, the suspensions were standardized to an opacity equal to 375 x 10^6 organisms per ml., and the tubes were left in the water bath at 56°C for 2 hours.

The last series of experiments in this investigation pertained to the extraction of antigens from the species studied. For each organism that was grown in bulk and extracted a portion of the stock culture was standardized and tested in an agglutination reaction against the homologous immune serum to determine the agglutinability of that particular batch of cells. The reactions were carried out using 0.85 per cent saline with incubation in a water bath at 40°C for 6 hours. The tests were re-examined after standing overnight at room temperature.

6. Preparation of Cell Wall Material:

The cell wall material used in these experiments was prepared following the technic of Cummins (1954) and is described below. Using the harvest from a stock Loeffler's slope as the inoculum, each strain was cultivated in 100 ml. volumes of brain heartinfusion broth containing 0.7 per cent Tween 80. Incubation was carried out at 37°C for 24 hours, and

then the content of each flask was transferred to a 200 ml. centrifuge bottle and centrifuged for 30-45 minutes (International Equipment Co. model). The supernatant was decanted and the cells of each 100 ml. flask resuspended in 4 ml. sterile distilled water. This suspension together with 6 gm. of glass Ballotini beads#12 was placed in a 50 or 100 ml. round bottom flask and shaken for 3 to 4 hours on a laboratory shaker. When disintegration was estimated to be above 90 per cent, as judged by stained smears, the supernatant was collected and the glass beads washed several times with 2 to 3 ml. volumes of distilled water. The combined washings were centrifuged at low speed, 2000 - 2500 r.p.m., Servall angle centrifuge, for 10 minutes to remove any remaining beads and unbroken bacteria. The supernatant was collected and centrifuged at high speed to throw down the cell wall material. This required 8000 - 10,000 r.p.m. for 10 minutes (Servall highspeed angle centrifuge). The final deposit was washed again in 10 ml. distilled water at high speed. The sediment represents the crude cell wall material.

Purification of the cell wall material was brought about by the action of Liquor Tryspin Compound (Allen and Hanbury Ltd.). The tryspin was first brought to pH 7 with 0.1N - NaOH using phenol red as the indicator and added to the crude cell wall suspension in a final concentration of 1/20. The suspending medium for the tryspin was 0.033M phosphate buffer pH 8. This solution was allowed to act on the cell wall material for 3 hours at 37°C. When the treatment with tryspin was complete the suspension was centrifuged at high speed, 8000 - 10,000 r.p.m. for 10 minutes. The deposit was washed with 10 ml. of distilled water at high speed. The supernatant was decanted and the deposit suspended in 5 to 10 ml. of 1.5 per cent saline, and stored at 4°C in the refrigerator. This preparation represents the purified cell wall material.

7. Extraction Procedures:

The work of several authors such as Cummins (1954) and Wong and T'Ung (1939) has indicated that, at least for C. diphtheriae, a somatic polysaccharide is responsible for group specificity in serological reactions.

The extraction procedures that follow represent an attempt to isolate polysaccharide material or perhaps an antigenic substance that would aid in the immunological characterization of the corynebacteria.

Fuller's Formamide Method.

The method of Fuller (1938) is used exclusively for the extraction of polysaccharides since the treatment with formamide destroys all the proteins.

The bacteria to be extracted were spun down and the supernatant discarded. Then 15 volumes of formamide were added to the cellular deposit, the contents thoroughly mixed, and heated for 20 minutes in an oil bath at 150°C. After cooling and centrifuging the supernatant was decanted and to it was added 2 1/2 volumes of acidified - absolute alcohol containing 5 per cent of 2N HCl. The material was allowed to stand in the cold at 4°C for several hours or overnight until maximal precipitation occurred. The precipitate was filtered off through Whatman filter paper #2 and re-extracted twice with small quantities of 70 per cent alcohol. The combined alcohol extracts were mixed with an equal volume of acetone and again precipitation allowed to take place in the cold at 4°C. Usually several hours were required. After centrifuging the acetone was decanted and the precipitate dissolved in a small quantity of water, 2 to 3 ml. The part that remained undissolved, largely nucleoprotein, was removed by centrifugation and the supernatant represented the polysaccharide extract ready for testing.

Lancefield's Method

The method of Lancefield (1928, 1933) was also used in attempting to extract bacterial antigens. This technic is described as liberating both protein and carbohydrate substances which may be separated by fractional precipitation with alcohol.

The bacterial sediment from 100 ml. of broth culture is collected after centrifugation and suspended in 1 ml. of N/10 HCl, made up in 0.85 per cent saline. A loopful of the suspension should give a reddish color when mixed with a drop of 0.01 per cent solution of thymol blue. If necessary enough HCl should be added to adjust the reaction to a pH between 2.0 and 2.4. The mixture is then transferred to a centrifuge tube and heated in a boiling water bath with occasional shaking for 10 minutes, then cooled and centrifuged. The supernatant is decanted into a second centrifuge tube and a few drops of 0.01 per cent phenol red are added which will give a faint yellow color to the solution. Then N/5 NaOH is added slowly, drop by drop, to the extract. The correct reaction, pH 7 is reached when the first pink color appears. If too alkaline, the extract is neutralized with N/20 HCl. In neutralizing a precipitate is formed which is centrifuged. The clear supernatant, representing the crude extract, is then tested against the appropriate sera.

When this method was followed as the general procedure for obtaining extractions in the last series of experiments the procedure was slightly modified. Portions of the stock cultures, representing the harvest from 6 liters of broth, were centrifuged to provide a packed cell volume of 0.4 to 0.7 ml. Usually about 10 ml. of the stock suspension was sufficient. To this sediment was added 1 to 2 ml. of N/10 HCl, and heating in the boiling water bath was carried out for 15 to 20 minutes. In the neutralization 1N NaOH

was added until the first trace of cloudiness appeared, usually 1 or 2 drops, and then N/10 NaOH was added until pH 6 to 6.5 was attained as determined by pH indicator papers. Then the precipitate was centrifuged and the supernatant used for testing.

Preparation of Streptomyces albus Filtrate for Disruption of Cells.

The medium for the production of S. albus filtrate was prepared according to the method of McCarty (1952). (See appendix for preparation.) Using Roux bottles this medium is dispensed in 100 ml. per bottle. If larger bottles are used the medium is distributed in the same proportion, i.e., 100 ml. of medium per 1000 ml. volume of container. The spores from 1 stock agar slant, in 5 ml. distilled water, are used to inoculate 3 Roux bottles. This method provides a relatively shallow layer of medium. The bottles are incubated at 37°C.

Growth on this medium is rapid and a continuous sheet of surface growth, well covered with a white sporulated surface, is obtained in 48 to 72 hours, The maximum yield of enzymes is achieved at this time, and it is important not to continue incubation beyond this point since the activity of the filtrates decreases after reaching the maximum.

The amber culture medium was removed by decanting the fluid from the sheet of surface growth through a filter. The pooled culture fluid was brought to 0.7 per cent saturation with $(NH_4)_2SO_4$ by the addition of 472 gm. of solid salt per liter of fluid. This precipitate is resuspended in M/15 phosphate buffer pH 8, about 10 ml. for each liter of original culture, and stored in the refrigerator at 4°C with a few drops of chloroform as a preservative.

Glycine Disruption of Cells.

An attempt was made to follow the procedure of Hays and Stanley (1950) in using glycine to disrupt the bacterial cells with the concommitant liberation of polysaccharide material.

The strain of corynebacteria was grown in 100 ml. volumes of brain heart infusion broth. After incubation at 37°C for 24 hours the cells were collected, washed twice with 0.85 per cent saline, and resuspended in saline to form a heavy suspension. Sufficient of a 3M glycine solution at pH 8 and at 37°C was then added to the thick bacterial suspension to give a final glycine concentration of 1M. The mixture was well shaken and incubated in a water bath at 40°C for 24 hours.

When lysis occurs the suspension is then dialysed against de-ionized distilled water to remove the glycine. Two changes of water are made over a period of 48 hours. The material is centrifuged and the deposit discarded. Five volumes of ethanol, containing a trace of sodium acetate, are added to the supernatant and the resulting precipitate extracted twice with distilled water. Material precipitated by 5 volumes of ethanol is again obtained, dissolved in distilled water and centrifuged. The deposit is discarded. The aqueous solution is extracted with a chloroform-amyl alcohol mixture, 0.25 volume of chloroform + 0.1 volume of amyl alcohol to remove protein material, then reprecipitated with 5 volumes of alcohol and the precipitate either resuspended in distilled water for immediate testing or lyophilized.

Acetic Acid Method.

Acetic acid was also used in an attempt to obtain polysaccharide material. The organisms were grown in brain heart infusion broth, either in 100 ml. or 6 liter volumes and incubated at 37°C for 24 hours. The

cells were collected by centrifugation and washed twice with 0.85 per cent saline. Glacial acetic acid was added to a heavy stock suspension to effect a final concentration of 1 per cent. The suspension was heated in a boiling water bath for 90 minutes. After cooling the material was centrifuged and 5 volumes of absolute alcohol were added to the supernatant, and precipitation allowed to take place at 4°C. The precipitate was taken up in a small amount of saline, 1 to 2 ml., after centrifuging and the insoluble material removed. The clear extract was then tested.

8. Preparation and Procedure for Hemagglutination Reactions:

Some experimentation was also carried out using the hemagglutination reaction, a procedure, which was hoped would reveal in a quantitative degree the presence of both protein and carbohydrate antigens in crude extracts. Both the method of using tannic-acid treated cells as followed by Stavitsky (1953) for the detection of proteins, and the Keogh method for demonstrating carbohydrates as adopted by Meynell (1954) were tried.

The red blood cells used in both of the following methods were obtained from whole sheep blood preserved with Alsever's solution, 1.2 volumes to 1 volume of blood, and stored at 4°C. (See appendix for preparation of Alsever solution).

Keogh Method: (Non-Tannic Acid Treated Cells)

Sensitization: Portions of the stock suspension of sheep red blood cells in Alsever's solution were centrifuged, the cells washed 3 times with 10 ml. volumes of physiological saline, and finally diluted in saline to obtain a 2.5 per cent suspension. This required the addition of 40 ml. saline to 1 ml. of packed red blood cells. Using this diluted red blood cell suspension, 10 ml. amounts were centrifuged to retrieve packed cells in 0.25 ml. volumes. To the packed cells was added 4.5 ml. of buffered saline, pH 7.2, or 0.5 ml. of the various dilutions of extracts to be adsorbed and tested.

Following incubation of the suspension at 37°C for 2 hours, the cells were washed 3 times with 10 ml. volumes of saline. Finally 15 ml. of saline was added to each tube of packed cells to form a 1.7 per cent suspension of sensitized sheep red blood cells.

Absorption of Normal Hemolysins from the Test Sera: Five ml. of the 2.5 per cent suspension of sheep red blood cells were centrifuged. To the packed cells was added 0.1 ml. of the immune serum and 0.9 ml. of saline. The materials were mixed well and incubated for 10 minutes at room temperature. After centrifuging the supernatant was used as the 1/10 dilution of antibody for the test.

Titrations: Doubling dilutions of the test sera in saline were made in Kahn tubes. Each tube contained 0.4 ml. diluted serum and received 0.05 ml. of the 1.7 per cent suspension of sensitized cells. The racks were shaken and incubated at 37°C for 3 hours. The tests were read after this 3 hour period and again after standing overnight at room temperature. Agglutination of the red blood cells was read in the same manner as bacterial agglutination using the same system of plusses to denote the degree of agglutination. The agglutination was detected by gently resuspending the red cells and observing the degree of clumping.

Controls: 1. Sensitized cells in the diluent.

2. Normal sheep cells in the highest concentration of test serum.

Stavitsky Method: (Tannic Acid Treated Cells)

The sheep red blood cells in Alsever solution were washed 3 times with saline, and 1 ml. of packed cells was then diluted with about 40 ml. of buffered saline, pH 7.2. Preparation of Tannic acid cells: one ml. of sheep cells diluted as above plus 1 ml. of 1/20,000 dilution of tannic acid were incubated in a water bath at 37°C for 10 minutes. The cells were then

centrifuged gently and washed with 1 ml. of buffered saline, pH 7.2, and resuspended in 1 ml. of saline. These tannic acid cells were kept at 4°C for not more than 18 hours before use.

Sensitization of tannic acid cells: Four ml. of buffered saline, pH 6.4, plus 0.5 ml. of bacterial extract, and 1 ml. tannic acid cells were mixed in this order and kept at room temperature for 10 minutes. The cells were then centrifuged, washed once with 2 ml. of 1/100 normal rabbit serum in saline, and resuspended in 1 ml. of 1/100 normal rabbit serum.

Titrations: Doubling dilutions of the test antiserum in 0.5 ml. amounts were prepared in Kahn tubes using 1/100 normal rabbit serum in saline as the diluent. To each tube was added 0.05 ml. of tannic acid extract-sensitized cells. The tubes were shaken and kept at room temperature and the reactions were read after 3 and 12 hours.

Controls: 1. Sensitized cells in the diluent.

2. Normal sheep cells in the highest concentration of test serum.

Reading of Reaction: The titer is expressed as the highest dilution of an antiserum which gives a definitely positive reading. The reaction was read and graded according to the pattern formed by the agglutinated cells on the bottom of the tube. The various grades are as follows:

++++ Compact granular agglutination

+++ Smooth mat on bottom of tube, with folded edges

++ Smooth mat on bottom of tube, edges somewhat ragged

\+ Narrow ring of red around edge of smooth mat

\- Discrete red button in the center of the bottom of the tube.

The normal rabbit serum and test sera were absorbed as in the Keogh method.

9. Capillary Tube Method for Testing Extracts:

The crude extracts were tested against homologous and heterologous sera in capillary tubes. The tubes are about 7 cm. in length and correspond in size to No. 18 gauge wire, approximately 1 mm. external diameter. In carrying out the tests 2 parallel rows of Kahn tubes are set up. Each tube in the first set receives 2 drops of the heterologous sera to be tested and is labelled. One drop of the extract is dispensed into each tube of the parallel series. The capillary tubes are first wiped with a piece of moistened tissue paper, then one end is dipped into a test serum and the tube filled about one-third. This portion of the tube containing serum is next placed in the corresponding Kahn tube containing extract and carefully, to avoid mixing, filled another one-third, then wiped clean and both ends are sealed in the Bunsen flame. Before sealing the liquid is allowed to flow toward the center of the tube to insure sufficient space at both ends for sealing. Then one end of the tube is imbedded in plasticene so that the extract islayered over the serum. A control tube of serum plus saline is also included for each test.

The reactions are examined after 1 hour, 4 to 5 hours, and also after standing overnight. A positive reaction is denoted by the appearance of a cloudy zone or ring at the interface of serum and extract or more usually in the upper or lower half of the column of liquid. The degrees of reaction are read as follows:

+++ Very strong reaction occurring immediately on addition of the reagents, the zone of reaction is milky in appearance and precipitates to the bottom of the tube quite rapidly.

++ Strong reaction similar to above although not as intense, may precipitate after 5 to 6 hours or overnight.

\+ A light but definite cloudiness requiring 1 to 2 hours to reach a maximum, and may or may not precipitate on standing.

± A very light zone barely visible to the naked eye when the test is compared to the control, requires several hours to develop and does not precipitate.

10. Agar Plate Precipitin Test:

Medium: Purified, good quality agar (Difco) was prepared to a final concentration of 1.5 per cent with the addition of NaCl to 0.85 per cent and merthiolate to 1/10,000.

Method: 5 to 6 ml. of melted agar was poured into Petri plates and allowed to solidify forming a base. Round stainless steel molds, 8 mm. external diameter, were placed on the agar base, 1 in the center of the plate and 3 molds around the plate. The peripheral molds were situated so that they were 20 mm. from each other and a distance of either 10 mm. or 20 mm. from the center mold. Then an additional 20 ml. of melted agar was poured over the plate and allowed to set. Finally the molds were removed leaving wells, 8 mm. in diameter.

The basins were filled with the reagents using Pasteur pipettes, for example, antiserum in the center well and different dilutions of the extract in the peripheral wells. The plates were then sealed with adhesive plaster and kept at room temperature. Beginning from 2 to 3 days later the plates were examined for lines of precipitation between the wells and observed for 6 to 8 weeks. The basins were refilled consecutively for 2 or 3 days or as required.

EXPERIMENTAL RESULTS

EXPERIMENTAL RESULTS

I. Preparation of Antisera:

Prior to undertaking the experiments antisera were produced in rabbits against the following 14 species. Preceding each strain is the identification number as used in this laboratory.

12. C. hoagii
131. C. acnes
132. C. bovis
133. C. equi
138. C. segmentosum
149. C. "Q"
281. C. paurometabulum
288. C. enzymicum
290. C. hemolyticum
251. C. michiganense
254. C. poinsettiae
255. C. insidiosum
282. C. fimi
285. C. tumescens

In addition antisera were already available from a previous study, Yurack (1954) against the following strains.

14. C. diphtheriae
120. C. ovis
134. C. hofmanni
135. C. murium (Kutscheri)
136. C. pyogenes
137. C. renale
139. C. ulcerans
140. C. xerosis
174. C. diphtheriae (P.W. 8)
194. C. helvolum
195. C. flaccumfaciens
196. C. sepedonicum

Relatively late in this work booster doses were administered to the original animals with the following species.

120. C. ovis
132. C. bovis
136. C. pyogenes
174. C. diphtheriae (P.W. 8)

Where these sera are used in tests they are indicated by the letter B following the strain number.

At the same time additional sera were produced in new animals against the following species.

135. C. murium

139. C. ulcerans

251. C. michiganense

When much higher titered sera were produced after the booster doses it was intended to inject all of the remaining animals, however the time required for this scheme would not have permitted the completion of all the extraction experiments in the late phase of the investigation. Therefore the animal inoculations were discontinued.

II. Attempts to Obtain Stable Antigen Suspensions for the Agglutination Reaction:

When the first rabbit immune sera were collected, endeavors were made to determine the agglutinating titers of these sera against the homologous strains. Five strains were selected and antigens prepared for agglutination reactions. The tests were set up and when readings were made after the prescribed period of incubation it was impossible to determine the degree of specific agglutination in 4 of the 5 reactions due to autoagglutination and the formation of large thready mucoid clumps.

Similar difficulties were encountered in an earlier phase of the work, Yurack (1954) and attempts were made to prevent autoagglutination. The methods used included the use of lyophilized cultures as a means of maintaining stable antigens for agglutination reactions, the effects of various concentrations of saline on the stability of antigen suspensions, the use of Tween 80 in trials to overcome thread-like agglutination and a comparison of the agglutinability of unheated and autoclaved suspensions. Although

each of these technics showed an improvement in some instances, a method which consistently stabilized antigen suspensions for the agglutination reaction was not realized.

It was hoped that direct agglutination reactions could still be used in this study and further attempts were made to obtain stable antigens. The following experiments were carried out with this end in view.

Experiment 1

In this experiment several variations of the suspending medium for the washed cells were assayed for their effect on the stability of the suspension for the agglutination reaction. The diluent for the serum dilutions was also altered with respect to NaCl concentration.

Therefore 2 strains were selected, 132 C. bovis and 12 C. hoagii, which had displayed autoagglutination to an extent which prevented reading of the direct homologous agglutination tests. The strains were grown in 100 ml. volumes of brain heart infusion broth and the cells collected by centrifugation, washed and standardized for agglutination reactions following the procedure outlined in Materials and Methods section 2. The suspending medium for the washed cells included Subtosan, a plasma substitute, phosphate buffer pH7.0 and 0.5 per cent phenol in physiological saline. Then homologous agglutination reactions were carried out beginning with a 1/20 dilution of antibody. The results are presented in sections A-F of Table 1. The numbers in this as in subsequent tables correspond to the same degree of agglutination as would plus signs.

TABLE 1

A.

Antigen Suspension in 10 per cent Subtosan in 1.5 per cent Saline.

Antiserum Dilutions in 1.5 per cent Saline.

Strains	Reciprocal of Antiserum Dilutions									
	40	80	160	320	640	1280	2560	5120	10240	C
132.	1	1	1	2	2	2	2	2	2	4
12.	2	3	4	4	4	4	4	4	4	3

B.

Antigen Suspension in 10 per cent Subtosan in 0.85 per cent Saline.

Antiserum Dilutions in 0.85 per cent Saline.

Strains	Reciprocal of Antiserum Dilutions									
	40	80	160	320	640	1280	2560	5120	10240	C
132.	1	1	1	2	2	2	2	2	2	4
12.	2	3	4	4	4	4	4	4	4	3

C.

Antigen Suspension in Phosphate Buffer pH 7.0

Antiserum Dilutions in 1.5 per cent Saline

Strains	Reciprocal of Antiserum Dilutions									
	40	80	160	320	640	1280	2560	5120	10240	C
132.	1	1	1	2	2	2	2	2	2	4
12.	2	3	4	4	4	4	4	4	4	3

TABLE 1 (continued)

D.

Antigen Suspension in 0.5 per cent Phenol in 0.85 per cent Saline

Antiserum Dilutions in Distilled Water

Strains	Reciprocals of Antiserum Dilutions									
	40	80	160	320	640	1280	2560	5120	10240	C
132.	1	2	2	2	2	2	2	2	4	2
12.	2	3	4	4	4	4	4	3	3	2

E.

Antigen Suspension in 0.85 per cent Saline

Antiserum Dilutions in Undiluted Subtosan

Strains	Reciprocal of Antiserum Dilutions									
	40	80	160	320	640	1280	2560	5120	10240	C
132.	2	2	1	1	1	1	1	1	1	3
12.	2	3	4	4	4	4	4	4	4	3

F.

Antigen Suspension in 0.85 per cent Saline

Antibody Dilutions in Distilled Water

Reactions Carried Out at Room Temperature

Strains	Reciprocal of Antiserum Dilutions									
	40	80	160	320	640	1280	2560	5120	10240	C
132.	4	4	4	4	4	4	4	4	2	2
12.	4	4	4	4	4	4	4	4	3	3

In the tests using strain 132 C. bovis all the tubes containing serum displayed very fine floccules with the cells adhering to the bottom of the tube. However the antigen control showed large fluffy floccules. The titrations using subtosan as the suspending medium for the antigen suspension also indicated a definite inhibition for agglutination.

The form of the agglutinated cells with strain 12 C. hoagii varied with both small and large floccules evident in all of the tubes. This was also the case in the antigen controls. Subtosan did not seem to inhibit the agglutination of this strain.

The variations in technic described here did not solve the problem connected with the use of whole cells as a standard procedure in agglutination reactions.

Experiment 2

In this experiment the stability of an antigen suspension over a range of pH values was tested in phosphate buffer. Strain 132 C. bovis was grown in 100 ml. volumes of brain heart infusion broth containing 0.7 per cent Tween 80 and 1.5 per cent sheep serum. After centrifuging the cells were collected and washed in distilled water and then resuspended in distilled water. This stock suspension was standardized and then aliquots centrifuged. The cellular deposit was resuspended to its original volume in phosphate buffer. The buffer pH ranges included: pH 5.4, 5.8, 6.0, 6.2, 6.4, 6.6, 6.8, 7.0 and also 1.5 per cent saline. These suspensions were allowed to remain overnight in the ice box at $4^{\circ}C$. When examined the following day they were slightly granular (+). All of the buffered suspensions over the range of pH values displayed a uniform degree of autoagglutination.

The suspensions in phosphate buffer were thoroughly mixed by pipette and antigen controls were set up as would normally be used in agglutination reactions; they consisted of 0.5 ml. of 1.5 per cent saline and 0.5 ml. of the standardized buffered suspensions. The tubes were incubated in a water bath at 42°C for 2 hours and then left overnight at 37°C. All of the suspensions showed a considerable degree of autoagglutination when examined the following day. There seemed to be no difference in the degree of autoagglutination within the phosphate buffer range pH 5.4 to 7.0 and including the suspension in 1.5 per cent saline.

Therefore, it could be concluded that the preparation of antigen suspensions in phosphate buffer over a broad pH range did not effect the desired stability of the suspension.

Experiment 3

An observation prompted another experiment to determine the stability and agglutinability of suspensions prepared from cells grown on blood agar plates. Strain 132 C. bovis was subcultured from a stock Loeffler's slope onto a nutrient and blood agar plate. Examination of the plates following incubation at 37°C for 24 hours showed that the colonies on the nutrient agar plate were uniformly rough while the growth on the blood agar plate was smoother and of a semi-matte appearance. Hence the following experiment.

Four strains; 132 C. bovis, 12 C. hoagii, 281 C. paurometabulum and 133 C. equi, which had been subcultured on Loeffler's slopes every 2-3 days for 2 weeks, were inoculated onto blood-agar plates from the stock slope. The growth from these plates was then used to seed six blood-agar plates for each strain tested. Three of the plates contained rabbit blood and three contained sheep blood. The plates were incubated

at 37°C for 24 hours and then the growth was removed by washing with 1.5 per cent saline containing 0.7 per cent Tween 80. The cells were centrifuged and the saline plus Tween 80 replaced by 1.5 per cent saline. The suspensions were standardized for the agglutination reaction and tested against the homologous antisera beginning with a 1:20 dilution. The results are presented in Table 2. The serum dilutions are expressed as reciprocals. In the table S and R indicate suspensions obtained from plates containing sheep or rabbit blood respectively.

TABLE 2

Homologous Agglutination Reactions

Antigen Suspensions from Blood-Agar Plates

		Reciprocal of Serum Dilutions									
Strain		40	80	160	320	640	1280	2560	5120	10240	C
132	S	4	4	4	4	4	4	4	3	3	2
132	R	4	4	4	4	4	4	3	3	3	2
132^{x}	S	4	4	4	4	4	4	4	3	2	2
12	S	4	4	4	2	1	-	-	-	-	-
12	R	4	4	4	1	-	-	-	-	-	-
12^{x}	S	4	4	4	4	2	1	-	-	-	-
281	S	4	4	4	4	4	4	4	4	4	4
281	R	4	4	4	4	4	4	4	4	4	4
281^{x}	S	4	4	4	4	4	4	4	4	4	4
133	S	-	-	-	-	-	-	-	-	-	-
133	R	-	-	-	-	-	-	-	-	-	-

S denotes antigen from plate with sheep blood.

R denotes antigen from plate with rabbit blood.

x indicates second animal used for immunization.

Experiment 4

An almost identical experiment was carried out with 5 species of soil corynebacteria: 251 C. michiganense, 254 C. poinsettiae, 255 C. insidiosum, 282 C. fimi, and 285 C. tumescens.

Antigen suspensions were prepared, as previously described, from blood-agar plates containing sheep blood. Homologous agglutination reactions were then carried out with halving dilutions of antiserum from 1:20. The results are recorded in Table 3.

TABLE 3

Homologous Agglutination Reactions

Antigen Suspensions from Agar Plates Containing Sheep Blood

Strain	Reciprocals of Serum Dilutions									
	40	80	160	320	640	1280	2560	5120	10240	C
251	4	1	-	-	-	-	-	-	-	-
254	Stringy Mucoid Clumps									
255	4	4	4	3	1	-	-	-	-	-
282	3	3	2	1	-	-	-	-	-	-
285	4	4	4	4	4	3	3	2	1	1

Experiment 5.

In this experiment some of the suspensions prepared from blood agar plates and used in Experiments 3 and 4 were heated in flowing steam at 100°C. for 30 minutes to test the effect of this treatment on homologous agglutinability. The suspensions of strains 12, 133, 251, 254, 255, and 282 were so treated and then used in homologous agglutination titrations with halving dilutions of antiserum from 1:20. These results are tabulated in Table 4.

TABLE 4

Homologous Agglutination Reactions

Antigen Suspensions from Blood-Agar Plates but Heated at 100°C. for 30 Minutes

Strain	Reciprocal of Serum Dilutions									
	40	80	160	320	640	1280	2560	5120	10240	C
12	4	4	4	4	4	4	2	-	-	-
133	4	1	-	-	-	-	-	-	-	-
251	4	4	2	1	-	-	-	-	-	-
254	4	4	4	4	3	2	1	-	-	-
255	4	4	4	4	2	1	-	-	-	-
282	Cells settled and adhered to tube. Not possible to read agglutination.									

The results of these tests indicate that the stability of antigen suspensions for the agglutination reaction, from cells grown on blood-agar plates is somewhat improved. This is evident with strain 132 C. bovis and in particular with regard to strain 12 C. hoagii when the tests and controls as reported in Table 2 are compared with those of Table 1. The soil corynebacteria also produced satisfactory suspensions with one exception strain 254 C. poinsettiae.

The agglutinability of strain 12 C. hoagii is considerably reduced if grown on blood plates. However heating of the suspension restores the original titer as observed when the results of Tables 1 and 4 are compared with the reactions shown in Table 2.

The soil species of corynebacteria generally exhibit quite low agglutination titers. The agglutinability of these species is somewhat altered by heating and it may be true that specificity is also altered in the process. It was previously observed Yurack (1954), that autoclaved cultures of corynebacteria generally show an altered agglutinability not only with respect to the homologous reaction but also in heterologous reactions.

It is interesting to note that strain 133 C. equi doesn't agglutinate with homologous serum even when a heated suspension is used. Strain 251 C. insidiosum also shows a very low agglutination titer.

From the titers observed in these experiments it does not seem feasible to use the direct agglutination reaction in serological studies of corynebacteria.

III. Experiments with Cell Wall Material:

In earlier experiments Yurack (1954), attempts were made to duplicate the work of Cummins (1954) who demonstrated a type specific protein and group specific polysaccharide in C. diphtheriae using cell walls in agglutination reactions. Under the particular conditions in this laboratory the experiments were not successful. However the method and results as described by Cummins appeared quite straight forward and seemed ideal for the intended serological survey of the corynebacteria.

The following experiments were carried out to re-appraise his technic under the conditions existing in this laboratory.

Experiment 1

In this first attempt to duplicate the work of Cummins, strain 174 C. diphtheriae was selected. The organism was grown in 100 ml. volumes of brain heart infusion broth and incubated at 37°C. for 24 hours. The cells were collected by centrifugation, washed in physiological saline and then resuspended in saline. The suspension was transferred to a round bottom flask and shaken with glass Ballotini beads for several hours. When disintegrat ion was maximal, as judged by stained smear the liquid material was decanted and unbroken cells and glass beads removed by centrifugation at low speed. The crude cell wall substance was deposited by centrifuging at high speed, and washed in physiological saline, and then resuspended in saline. This crude cell wall suspension was standardized in the photo-electric colorimeter to a reading of 65 per cent transmission of light. The standardized crude cell wall suspension was set up in a homologous agglutination reaction with halving dilutions of serum from 1:10 to 1:5120. No reaction was observed.

Experiment 2.

The procedure outlined in Experiment 1 was repeated for a second strain of C. diphtheriae. Crude cell wall material was prepared from strain 14, standardized and titrated in a homologous agglutination reaction with halving dilutions of serum from 1:20 to 1:10,240. No reaction was observed.

Experiment 3

Continuing with this technic a crude cell wall suspension was again prepared from strain 14, standardized, and then tested with halving dilutions of homologous serum from 1:20 to 1:5120 in an agglutination reaction. Complete agglutination was noted in the first tube only. No other evidence of agglutination was visible.

The remainder of the crude cell wall suspension was then purified by treatment with tryspin in a final dilution of 1:20. Following the action of tryspin on the crude cell wall material for 3 hours at 37°C, centrifugation of the mixture was carried out at high speed. The deposited cell walls were washed with physiological saline and then resuspended in saline and standardized. This purified preparation was then set up in a homologous agglutination reaction with halving dilutions of serum from 1:20 to 1:5120. No trace of agglutination was evident.

Experiment 4.

Still attempting to obtain cell wall preparations appropriate for the agglutination reaction, a third trial was made with strain 14 C. diphtheriae. Crude cell wall material was obtained in the usual manner, standardized and then tested with halving dilutions, from 1:20 to 1:5120, of the homologous serum. Complete agglutination was evident only in the first tube which represents a 1:40 dilution of serum.

Experiment 5.

Another attempt was made following the same procedure, but this time with a different species, 134 C. hofmanni. A crude cell wall suspension was prepared as previously explained and purified with tryspin used in a final dilution of 1:20. The purified suspension was then set up in a homologous agglutination reaction with halving dilutions from 1:20 to 1:5120 of serum. Complete agglutination of the cell walls was again observed only in the first tube in the series, a 1:40 final dilution of serum.

Therefore this technic, although it conformed as closely as possible to that described by Cummins (1954), as applied in these experiments did not meet expectations and was not judged satisfactory for serological analysis.

IV. Extraction Experiments Using Fuller's Formamide Method:

Since the attempts to obtain stable antigen suspensions of whole cells, and satisfactory preparations of cell walls for the direct agglutination reaction failed, it seemed logical to direct future experiments toward the extraction of antigenic fractions from the cells. Cummins (1954), and Wong and T'Ung (1939), demonstrated that for C. diphtheriae at least, a polysaccharide is responsible for group specificity in serological reactions. It was hoped that a similar fraction having the same property could be extracted from other members of the genus corynebacterium which would aid in the serological grouping of the species studied.

The first technic to be tried was that of Fuller (1938), employing formamide for the extraction. This method is used only for the extraction of polysaccharide material because formamide destroys all proteins in the process.

Experiment 1

The first species to be extracted using the formamide method, with the intention of obtaining a polysaccharide fraction active in serological reactions, was a soil corynebacterium, 255 C. insidiosum. The strain was first grown in 100 ml volumes of brain heart infusion broth for 24 hours at 37°C. Then following centrifugation, 15 volumes of formamide were added to the cellular deposit and extraction carried out in an oil bath at 150°C. for 20 minutes. After cooling and centrifuging the material was precipitated in the cold at 4°C. from the decanted supernatant by the addition of 5 volumes of acidified alcohol. The resulting precipitate was collected by filtration through #2 Whatman paper and re-extracted several times with small quantities of 70 per cent alcohol. The alcohol extracts were combined and material again precipitated in the cold by the addition of

an equal volume of acetone. The precipitate was spun down, and after decanting the supernatant, was dissolved in a small quantity of distilled water. A portion remained insoluble and was removed by centrifugation. The clear supernatant, representing the polysaccharide fraction, was then ready for testing. The extract was then tested with homologous immune serum by the capillary tube method as outlined under Materials and Methods section 9. The extract from strain 255 was tested with a 1:10 dilution of serum in the following dilutions: 1:10, 1:20, 1:40, 1:60, 1:80, 1:100. All of the tests were negative.

Experiment 2

Continuing with this method a formamide extract was obtained from another species 137 C. renale, following the procedure outlined in Experiment 1. The extract was tested in capillary tubes with a 1:10 dilution of homologous serum. The dilutions of extract included : 1:10, 1:50, 1:100, 1:200 and then halving dilutions to 1:12,800. All of the tests were negative.

Experiment 3

Still experimenting with the formamide method polysaccharide material for serological testing was prepared from a third species 14 C. diphtheriae. The extract was prepared in the usual manner but was re-precipitated several times with the alcohol mixture and then finally precipitated with acetone. This purified and final acetone precipitate was very small in amount but completely soluble in water. Dilutions of the extract, 1:10, 1:50, 1:100, 1:200 and then halving to 1:12,800, were tested with a 1:10 dilution of the homologous immune serum by the capillary tube method. All of the tests were negative.

The extracts used in this series of experiments tested positive for carbohydrate by the Molisch reaction.

As the investigation continued and more experience was gained it was realized that the failure to obtain results with the formamide extract might have been due to improper testing of the reagents. Under usual conditions the maximal results in capillary tubes are obtained when extract and antiserum are used undiluted, or at least when the serum is used undiluted. Therefore considering the dilutions of serum and extracts used in testing for antigenic substances in these experiments, no definite conclusions can be drawn.

V. Experiments Using Lancefield's Extracts in Hemagglutination Tests.

Since the method of Fuller failed to extract antigenic materials from the species of corynebacteria tested, it was necessary to test other extraction procedures. The difficulties encountered in serological studies with corynebacteria run parallel to a similar situation among the streptococci. An example is the difficulty in both genera in obtaining suitable cell suspensions for direct agglutination tests. Some of the methods which were adopted in extracting antigenic fractions from streptococci were also tried in these experiments. Therefore efforts were now directed toward preparing Lancefield's extracts from corynebacteria.

One of the obvious conclusions that can be made from the previous experiments using whole cells in agglutination reactions is the generally low antibody titers exhibited by sera prepared against species of corynebacteria. It seems self-evident that a more sensitive quantitative test would be an invaluable tool for the serological characterization of species of corynebacteria. Such a technic is the hemagglutination test. Since Lancefield's method, at least in the streptococci, liberated both protein

and carbohydrate fractions in the Lancefield extracts by using the appropriate procedures. For this purpose the method of Stavitsky (1952) using tannic acid treated red blood cells to adsorb proteins and the Keogh technic, as adopted by Meynell (1954), using non-tannic acid treated cells to detect carbohydrate were utilized.

Experiment 1

For this experiment six species were chosen:

120 *C. ovis*	140 *C. xerosis*
132 *C. bovis*	254 *C. poinsettiae*
139 *C. ulcerans*	255. *C. insidiosum*

The strains were cultivated in 100 ml. volumes of brain heart infusion broth. The cells were collected by centrifugation and extracted with 1 ml. N/10 HCl in a boiling water bath. After cooling, the material was centrifuged and the supernatant was removed and neutralized with N/10 NaOH which resulted in the precipitation of nucleoprotein. This insoluble protein was removed by centrifuging and the clear supernatant was used for testing.

Before proceeding with the hemagglutination tests the crude extracts from the 6 species were first tested in capillary tubes against homologous antiserum to determine whether sufficient active antigenic material to produce a reaction by this method had been liberated. Only two strains, 132 *C. bovis* and 140 *C. xerosis* gave strong positive reactions.

The extracts from the 6 species were then used in an attempt to sensitize sheep red blood cells for the hemagglutination test. The Keogh method for carbohydrate adsorption and the Stavitsky method for protein adsorption were both used. For sensitization by both procedures 0.5 ml. of undiluted extract was used. The sensitization procedures and the titrations using halving dilutions of serum were performed as outlined under Materials and Methods section 8. The results are found in Table 5. In the table the numbers correspond to the same degree of agglutination as plusses.

TABLE 5

Hemagglutination Tests with Crude Lancefield's Extracts

Keogh Method

Non-tannic Acid Treated Red Blood Cells

Extracts from Strains	Reciprocal of Serum Dilutions 10	20	40	80	160	320	C_1	C_2
132	1	-	-	-	-	-	-	-
140	1	-	-	-	-	-	-	-
120	4	3	2	1	-	-	-	-
139	-	-	-	-	-	-	-	-
254	1	-	-	-	-	-	-	-
255	-	-	-	-	-	-	-	-

Stavitsky Method

Tannic Acid Treated Red Blood Cells

Extracts from Strains	Reciprocal of Serum Dilutions 10	20	40	80	160	320	C_1	C_2
132	-	-	-	-	-	-	-	-
140	-	-	-	-	-	-	-	-
120	4	3	3	2	2	2	-	-
139	-	-	-	-	-	-	-	-
254	3	2	2	1	1	1	-	-
255	3	2	2	1	1	-	-	-

Controls; Keogh and Stavitsky; C_1 - Sensitized cells plus the diluent
C_2 - Normal sheep cells in highest concentration of test serum.

From the table one may observe that the extracts from strains 132 and 140 which gave a positive reaction with homologous serum in capillary tubes did not react in the hemagglutination test with either method. The extracts from 2 strains, 254 and 255, which were negative with homologous serum in the capillary tube test, showed considerable titers in the hemagglutination test using the Stavitsky method. A positive titer in the hemagglutination test using both procedures was obtained with the extract from strain 120 although this extract also failed to react with homologous serum in the capillary tube test.

Experiment 2

Another crude Lancefield extract was prepared from strain 132 C. bovis following the same procedure used in Experiment 1. The extract was tested with homologous serum by capillary tube and found to be strongly positive. In the previous experiment where 0.5 ml. of undiluted extract was used for adsorption onto red blood cells in the hemagglutination test, no reaction was visible by either the Keogh or Stavitsky method. In this experiment 0.5 ml. of varying dilutions of extract were used for sensitization of the red blood cells. Antibody was prepared in halving dilutions from 1/20 to 1/10,240 for both methods.

Tests:

Stavitsky Method: Tannic acid treated red blood cells:

The extract from strain 132 was tested in the following dilutions: 1/10, 1/20, 1/50, 1/100, 1/500, 1/1000.

Result: All tests with all dilutions of the extract were negative.

Keogh Method: Non-tannic acid treated red blood cells:

The extract from strain 132 was tested in the following dilutions:

1/10, 1/50, 1/100, 1/500, 1/1000, 1/5000, 1/10,000.

Result: All tests with all dilutions of the extract were negative.

These tests indicate that, under the conditions described here, material liberated in the Lancefield extraction process, although active with homologous serum in capillary test tubes, is not suitable for the hemagglutination reaction. Some reactions were obtained, notably with strains 254, 255 and 120, using the Stavitsky Method and also by strain 120 using the Keogh Method, but generally the reactions were not uniform nor were the titershigher than many observed in the direct whole cell agglutination reaction. Therefore the hemagglutination procedure is not applicable for all strains of corynebacteria and does not serve the purpose for serological grouping of the corynebacteria.

VI. Experiments with Glycine to Disrupt Bacterial Cells for the Extraction of Polysaccharide Material

Although the previous experiments to obtain uniformly active polysaccharide fractions from corynebacteria were unsuccessful it was, nevertheless, hoped that a method would be found that could be adapted to this group of organisms.

Hayes and Stanley (1950) reported satisfactory results in obtaining polysaccharides from E. coli using glycine to disrupt the cells with the subsequent liberation of the desired fraction. Consequently an attempt was made to apply this method to corynebacteria.

Experiment 1

In this experiment strain 132 C. bovis was selected because extraction of serologically active material from this organism by Lancefield's Method had previously been demonstrated. The strain was grown in 100 ml.

volumes of brain heart infusion broth and incubated at 37°C for 24 hours. The cells were collected by centrifugation, washed in physiological saline and resuspended in saline to obtain a heavy bacterial suspension. Then 3M glycine was added to the suspension in an amount to yield a final concentration of 1 M. The suspension plus glycine was mixed well and incubated at 40°C for 24 hours. Examination of the cells by stained smear after this period of incubation, showed no evidence of lysis. Incubation of the mixture for a further 24 hours still evidenced no hint of lysis.

Experiment 2

This experiment was undertaken to prove, or disprove, the liberation of material from strain 132 following treatment with 1 M glycine. The procedure detailed in Experiment 1 was repeated using this strain. Although incubation of the cell suspension with 1 M glycine produced no evident lysis the extraction procedure was continued. The suspension was then dialysed against distilled water to remove the glycine. Then the material was centrifuged and the deposit discarded. Five volumes of alcohol were added to the supernatant and the material was allowed to stand overnight in the cold at 4°C. No trace of precipitate formed confirming the observation that lysis of the cells did not occur.

Experiment 3

The purpose of this experiment was to determine the effect of heating of the cell suspension with regard to glycine sensitivity. Strain 132 C. bovis and strain 140 C. diphtheriae were grown in brain heart infusion broth and washed suspensions of cells prepared as in Experiment 1. The suspensions of these strains were then heated to 60°C for 1 hour. One ml. of 3 M glycine pH 8 was added to 2 ml. of the heated heavy bacterial

suspension, final glycine concentration 1 M, and the mixture was well shaken and incubated at 40°C for 24 hours. As judged by stained smear, no evidence of lysis was observed at 24 and 48 hours.

Experiment 4

In this experiment the pH of the suspending medium for glycine, phosphate buffer, was tested over a range of values for the effect on glycine activity. Therefore small quantities of 3 M glycine were prepared in phosphate buffer at pH values ranging from pH 7.0 to pH 8.2. One ml. volumes of unheated stock suspensions of strains 132 and 140 were dispensed in test tubes. For both strains each tube of cell suspension received 0.5 ml. of 3 M glycine in phosphate buffer at pH 7.0, 7.2, 7.4, 7.6, 7.8, 8.0, and 8.2. Following incubation of these mixtures at 40°C for 24 hours, no indication of lysis was observed.

The results of these experiments warrant the conclusion that under the conditions described above, glycine does not lyse the tested species of corynebacteria.

VII. Experiments to Lyse Corynebacteria by Extracellular Enzymes of Streptomyces albus to Obtain Polysaccharides:

Recently McCarty (1952) has reported that filtrates of Streptomyces albus literally dissolve suspensions of intact Streptococci as well as cell wall material. The filtrate acts upon the protein-carbohydrate complex of the cell wall, separating the protein and carbohydrate components. The method was used by McCarty to obtain the C. carbohydrate from streptococcal cell walls. This technic, if applicable, seemed ideal for obtaining carbohydrate from corynebacteria, and experiments to test the efficacy of this method were drafted.

A strain of S. albus was received from Dr. McCarty and grown on the surface of a specially prepared medium (See appendix for preparation) in Roux bottles. Following incubation for 48 to 72 hours, the culture medium was poured off and filtered, then concentrated 100-fold by precipitation with ammonium sulfate. The precipitate is reconstituted in M/15 phosphate buffer pH 8, about 10 ml. for each liter of original culture, and represents the enzymatic filtrate.

Experiment 1

It was first necessary to determine whether the prepared filtrate would be effective in bringing about lysis of corynebacteria. For this purpose strain 14 C. diphtheriae was grown in 100 ml. volumes of brain heart infusion broth for 24 hours at 37°C. The cells were then collected by centrifugation and washed with physiological saline. From the cell deposit a heavy suspension was prepared. To a 5 ml. portion of this heavy cell suspension was added 0.5 ml. of concentrated S. albus filtrate and the mixture incubated at 37°C for 24 hours. Prior to incubation 0.1 ml. of the suspension was removed, added to 5.9 ml. saline and tested in the Evelyn Photoelectric Colorimeter for per cent transmission of light. Using the No. 420 filter a reading of 62 per cent transmission of light was obtained. Following incubation a 0.1 ml. aliquot was again removed from the suspension, added to 5.9 ml. saline and tested in the photoelectric colorimeter as before. A reading of 76 per cent transmission of light was obtained.

It was therefore concluded that a certain amount of lysis of the cells had occurred.

Experiment 2

In this experiment a polysaccharide fraction was obtained from strain

14 C. diphtheriae using the filtrate from S. albus to liberate the material, and tested for activity with homologous serum by the hemagglutination reaction.

Strain 14 was grown in 100 ml. volumes of brain heart infusion broth and incubated at 37°C for 24 hours. The cells were collected by centrifugation, washed with physiological saline and then resuspended in saline so that a heavy suspension resulted. Sufficient S. albus filtrate was added to the heavy cell suspension to effect a final concentration of 1:5 and the material was incubated at 37°C for 24 hours. Following incubation the cells were removed by centrifugation and the decanted supernatant treated with 5 volumes of absolute alcohol and precipitation allowed to take place in the cold at 4°C. The precipitate was spun down and following removal of the supernatant, dissolved in 1.5 ml. of distilled water. Insoluble material was removed by centrifuging and the clear supernatant, the polysaccharide fraction, was used for testing.

This extract was tested in the hemagglutination reaction following the procedure of Keogh using non-tannic acid treated sheep red blood cells. For sensitization, 0.5 ml. amounts of the following dilutions of extract were used: 1/50, 1/100, 1/1000, 1/5000, 1/10,000. The titrations were carried out using the antiserum in halving dilutions from 1/10.

All tests with all dilutions were negative.

Experiment 3

In this experiment polysaccharide fractions for testing by the hemagglutination reactions, were obtained from strains 14 C. diphtheriae and 132 C. bovis using the S. albus filtrate. However the crude extract was further purified before testing by treatment with a chloroform-amyl alcohol mixture to remove any remaining proteins.

Strains 14 and 132 were seeded into 300 ml. amounts of brain heart infusion broth and incubated at 37°C for 24 hours. The cells of both strains were collected by centrifugation, washed with physiological saline, then suspended in 5 ml. saline. To this heavy suspension was added 1 ml. of S. albus filtrate and the mixture incubated at 37°C for 24 hours. Following incubation the cells were centrifuged and discarded and the supernatant treated with 5 volumes of absolute alcohol and precipitation allowed to take place in the cold at 4°C. The precipitate that formed was spun down and, following removal of the supernatant, dissolved in 1.5 ml. of distilled water. Insoluble material was removed by centrifuging and the clear supernatant is the polysaccharide extract used for testing in the previous experiments.

In this experiment the extract was then further purified by shaking with a chloroform-amyl alcohol mixture. The chloroform absorbs proteins and settles out as a layer below the aqueous extract. The alcohol merely prevents frothing. The removed protein appears as a "skin" between the chloroform and extract layers. The aqueous extract layer is pipetted off the underlying chloroform protein layer and shaken with a fresh chloroform-alcohol mixture. The process is repeated until the interface between the aqueous extract and chloroform layers no longer shows a protein skin, about 8 to 10 treatments. This protein free extract is now used for testing.

These de-proteinized extracts from strains 132 and 14 were tested in the hemagglutination reaction following the procedure of Keogh using non-tannic acid treated sheep red blood cells. For sensitization 0.1 ml., 0.2 ml., and 0.5 ml. of the undiluted extracts were used. The titrations were carried out using the antiserum in halving dilutions from 1/10. The results are presented in Table 6. The number corresponds to the degree of agglutination represented by one plus (+).

TABLE 6

Hemagglutination Tests with Polysaccharide Extracts from Strains 14 and 132

Volume of Extract used for Adsorption	Reciprocal of Serum Dilutions													
	20	40	80	160	320	640	1280	2560	5120	10,240	20,480	40,960	C_1	C_2
Strain 132														
0.1 ml.	-	-	-	-	-	-	-	-	-	1	1	1	-	-
0.2 ml.	-	-	-	-	-	-	-	-	-	-	1	1	-	-
0.5 ml.	-	-	-	-	-	-	-	-	-	-	-	1	-	-
Strain 14														
0.1 ml.	-	-	-	-	-	-	-	-	-	-	1	1	-	-
0.2 ml.	-	-	-	-	-	-	-	-	-	-	-	1	-	-
0.5 ml.	-	-	-	-	-	-	-	-	-	-	-	-	-	-

These tests show a slight positive reaction in the very high serum dilutions indicating that only traces of carbohydrate material were present in the extracts for adsorption.

Although the method of using enzymatic filtrates of S. albus to bring about lysis of bacterial cells was not altogether unsuccessful, nevertheless the procedure followed here did not prove very efficient. Therefore this technic is not recommended as a general procedure for the serological analysis of corynebacteria.

McCarty points out that the maximum production of enzymes is very variable even with the same strain on the same medium. It may well be that the preliminary manipulation with the culture of S. albus, such as growth on

McGill nutrient agar and failure to accurately determine sporulation microscopically may have influenced considerably the yield of active enzymes.

VIII. Extraction Experiments with Acetic Acid to Obtain Polysaccharides:

In a perusal of the literature dealing with extraction methods as applied to the bacterial cell, the use of dilute acetic acid is frequently described as having been employed to extract polysaccharides from both Gram positive and Gram negative species of bacteria. Therefore it was decided to conduct a series of experiments to analyse this method as a procedure to obtain active polysaccharide material, when applied to corynebacteria.

Experiment 1

This experiment was designed to test the effectiveness of acetic acid as a vehicle for the extraction of serologically active material. The remainder of a stock suspension of strain 132 C. bovis amounting to 4 ml. was diluted to 10 ml. To this suspension was added 0.2 ml. glacial acetic acid. After mixing well the suspension was heated in a boiling water bath for 90 minutes. Then the material was cooled and centrifuged and the deposit discarded. The decanted supernatant was treated with 5 volumes of absolute alcohol and the mixture left in the ice box overnight at 4°C. The precipitate was spun down and, following removal of the supernatant, was dissolved in 1 ml. of distilled water. The insoluble portion was removed by centrifugation and the clear supernatant tested against the homologous immune serum. In the capillary tube test the extract from strain 132 and homologous serum, when tested undiluted, gave an immediate and strong positive reaction. This experiment demonstrated that acetic acid could liberate material active serologically at least from strain 132.

Experiment 2

It was now necessary to prepare active extract from strain 132 in an amount sufficient for testing against all the heterologous sera at hand to determine the usefulness of the extract for serological grouping purposes.

Accordingly, a lyophilized culture of strain 132 C. bovis was used to inoculate 10 ml. of brain heart infusion broth. Following incubation at 37°C for 24 hours the 10 ml. culture was used as the inoculum for 100 ml. of brain heart infusion broth and this 100 ml. flask, in turn, used to seed 12 liters of broth. Following incubation at 37°C for 24 hours the cells were harvested by centrifugation in the DeLaval continuous flow centrifuge. The cells were washed with physiological saline and resuspended in 50 ml. distilled water. Then 0.8 ml. glacial acetic acid was added to the suspension and extraction carried out in a boiling water bath for 90 minutes. Following cooling and centrifuging the supernatant was decanted and treated with 5 volumes of alcohol. Insoluble material was allowed to precipitate overnight in the cold at 4°C. The centrifugation was carried out, the supernatant removed and the precipitate dissolved in 3 ml. distilled water. Insoluble material was removed by centrifugation and the clear supernatant used for testing. This extract from strain 132 produced a strong reaction with homologous immune serum when the reagents were tested undiluted in capillary tubes.

The extract from strain 132 was now tested in capillary tubes with all the heterologous sera to determine what relationships, if any, exist between this strain and other species of corynebacteria. In the tests the extract and sera are used undiluted. The results may be found in Table 7 A and B.

TABLE 7A

Acetic Acid Extracts Tested With

Homologous and Heterologous Sera

Sera	Acetic Acid Extracts from Strains		
	132	320	140
12	-	-	-
14	2	1	-
120	-	-	-
131	1:-	-	2:1
132	3:3	2:2	-
133	-	-	---
134	2:1	-	-
135	-	-	-
136	-	-	-
137	-	-	-
138	-	-	1:-
139	-	-	-
140	2	2	3

2 results for the same extract-serum combination indicate that 2 animals were immunized.

NT - Not Tested.

TABLE 7B

Acetic Acid Extracts Tested With

Homologous and Heterologous Sera

Sera	Acetic Acid Extracts from Strains		
	132	320	140
149	-	-	-
174	1	1	-
281	2:-	2:-	-
288	-	-	-
290	1:1	2:-	1:-
194	-	-	-
195	-	-	-
196	-	-	-
251	2	2	1
254	-	-	-
255	-	-	-
282	-	-	-
285	-	-	-
Lanc. D.	NT	2	2

Experiment 3

An extract was prepared from a second strain of C. bovis 320 to test the reactions with serum against strain 132 C. bovis and to compare the results of heterologous reactions from both strains.

The strain 320 was grown in a 6 liter volume starting from 10 ml. and 100 ml. inocula. The method as outlined in Experiment 2 was followed, using acetic acid to obtain the crude extract with alcohol precipitation of the supernatant following removal of the cells. The precipitate was dissolved in a small amount of distilled water, 2-3 ml., and the soluble portion used for testing. The extract gave a strong reaction when tested with serum 132 in capillary tubes. The extract from strain 320 was then tested with the other heterologous sera. The results are included in Table 7 A and B for comparison with those of the extract from strain 132.

From the table it is evident that the acetic acid extracts of strains 132 and 320 both show reactions with sera against strains 14 C. diphtheriae, 140 C. xerosis, 281 C. paurometabulum, 290 C. hemolyticum, 251 C. michiganense, and a weak reaction with 174 C. diphtheriae. The extract from strain 132 also reacts with both sera against strain 134 C. hofmanni and with one serum against strain 131 C. acnes. In addition the extract from strain 320 reacts strongly when tested with Lancefield's group D serum. The extract from strain 132 was not tested with Lancefield's serum.

Experiment 4.

It was also decided to test the extract from strain 132 with homologous serum by the agar plate diffusion process, as outlined under Materials and Methods section 10, to determine if more than one antigen was responsible for the cross reactions observed. One line was observed between the wells containing extract and serum undiluted.

Experiment 5.

Experiments were now continued using acetic acid as the medium of extraction to determine if successful results could also be obtained from other species. Consequently strain 140 C. xerosis was grown in 12 liters of brain heart infusion broth from lyophilized culture via the intermediate volumes of 10 ml. and 100 ml. cultures. The cells were collected, washed with physiological saline and extracted with acetic acid as previously described. Following extraction the cells were removed by centrifugation and the supernatant treated with 5 volumes of alcohol. The resulting precipitate was spun down and dissolved in 5 ml. of distilled water following removal of the supernatant. Insoluble material was removed and the clear supernatant used for testing. The extract from strain 140 when tested with homologous serum in capillary tubes resulted in a strong positive reaction (++). In keeping with the purpose to discover serological relationships among the species of corynebacteria studied, the extract from strain 140 was tested by the capillary tube method with the heterologous sera and also with Lancefield's grouping sera A, B, C, D, E, F, G, H, and K. A strong positive reaction was observed with the extract from strain 140 and Lancefield's group D serum. Thereafter all active extracts were also tested with Lancefield's group D serum. The results of the other heterologous tests using the extract from strain 140 may also be found in table 7, A and B. The extract and the sera in the tests were used undiluted.

The results of the tests indicate a strong reaction between the extract from strain 140 and the sera against strain 131 C. acnes with Lancefield's group D serum. Weaker reactions were evident with sera against strains 138 C. segmentosum, 290 C. hemolyticum, 194 C. helvolum and 251 C. michiganense.

Experiment 6

It was decided to prepare an extract from a second strain of C. xerosis in order to compare the heterologous reactions of both strains. Therefore a second strain of C. xerosis 331 was inoculated from dried culture into 12 liters of brain heart infusion broth in the usual stepwise manner through 10 ml. and 100 ml. cultures. The same procedure of acetic acid extraction and alcohol precipitation was used to prepare an extract for testing. When the extract from strain 331 was tested with the serum against strain 140 a strong and definite reaction was obtained but of a streaky and granular nature rather than a homogeneous cloudy area.

The extract was then tested against the heterologous immune sera. When the first set of tests was completed a reaction of the same intensity was observed in every tube. It was apparent that a non-specific antigen was responsible for the reactions and that the extract was unsatisfactory for further testing.

However an attempt was made to remove the non-specific substance in the extract from strain 331.

Several precipitations with alcohol were tried but non-specific reactions with heterologous sera were still obtained. Then the extract, in 1.0 ml. amounts was layered over 0.3 ml. amounts of heterologous sera in Kahn tubes in an attempt to absorb the non-specific material from the extract. When precipitation at the interface was maximal the upper clear layer of extract was removed and layered over another 0.3 ml. volume of heterologous serum. This process was repeated 7 times. Following this treatment the extract from strain 331 still produced a positive reaction with serum against strain 140 in capillary tubes. Tests with heterologous sera also displayed a positive reaction though to a lesser degree than observed before absorption.

Considering the amount of material expended and the results obtained this procedure was not considered practical.

Experiment 7.

Another attempt was made to prepare an active extract from a second strain of C. xerosis. Consequently strain 141 was seeded into 12 liters of brain heart infusion broth from lyophilized culture as previously outlined. The cells were collected by centrifugation in the De Laval continuous flow centrifuge, washed and a heavy suspension prepared in 50 ml. saline. In this experiment it was intended to compare the efficiency of acetic acid and hydrochloric acid as extractives.

Therefore the heavy suspension of strain 141 was divided into two equal portions. The first portion was extracted with dilute acetic acid in the usual manner. The cells were removed from the crude extract and the supernatant treated with 5 volumes of alcohol. The resulting precipitate was dissolved in 5 ml. of distilled water.

The second portion of the suspension of strain 141 was centrifuged and 30 ml. of N/10 HCl was added to the cellular deposit. The suspension was then extracted in a boiling water bath for 20 minutes. After cooling and centrifuging the supernatant was removed and neutralized with N/10 NaOH. Insoluble proteins precipitated and were removed by centrifugation. The supernatant was then treated with 5 volumes of alcohol and the material allowed to precipitate in the cold at 4°C. The precipitate was spun down, and, following removal of the supernatant, was dissolved in 5 ml. of distilled water. The insoluble portion was removed by centrifugation.

Both the acetic acid extract and the hydrochloric acid extract from strain 141 were tested with serum against strain 141 in capillary tubes. The reactants were used undiluted. No reaction was observed.

However an observation was made concerning the efficiency of the HCl and HAC extraction methods. When the preliminary extracts were treated with alcohol from 2 to 3 times more material was precipitated from the crude HCL extract than was obtained from the HAC extract.

Experiment 8

The procedure followed in Experiment 7 of dividing the cell suspension and extracting the equal portions by acetic acid and hydrochloric acid respectively was repeated. This time a soil species 251 C. michiganense was selected. The strain was started from lyophilized culture and was grown in 5 Roux bottles containing "Eugonagar" (See appendix for preparation). The suspension was divided equally and the 2 portions extracted with HCL and HAC respectively. The cells were removed from the crude extracts, and treated with 5 volumes of alcohol. The precipitates that formed were spun down, and following removal of the supernatant, dissolved in 5 ml. of distilled water. Insoluble material was removed by centrifugation. The HCL and HAC extracts from strain 251 were then tested against homologous immune serum in capillary tubes using both reagents undiluted. No reaction was observed.

It was again evident that the HCL method was by far the more efficient extraction technic with regard to quantity of material extracted.

Experiment 9

Another attempt was made to obtain an active extract using acetic acid. In this experiment species 134 C. hofmanni was chosen. The strain was grown in 12 liters of brain heart infusion broth, from lyophilized culture, in the same manner as explained in previous experiments. The cells were collected by centrifugation and extracted with dilute acetic acid. Following extraction the cells were discarded after centrifugation and the supernatant treated with 5 volumes of alcohol. The material that precipitated was

centrifuged and the supernatant discarded. The precipitate was dissolved in 5 ml. of distilled water and the insoluble material removed by centrifugation. The extract from strain 134 tested negative with homologous serum in capillary tubes using both reagents undiluted.

Another observation was the disappearance of activity within 5-7 days from the active extracts obtained from strains 132, 320 and 140.

Although with the acetic acid technic the objective was attained in several experiments with strains 132, 320 and 140, the activity of these extracts soon disappeared and the results with other species were completely negative. Therefore it was concluded that this method was too inconsistent to be used as a general procedure and the instability of the material obtained in the process a definite disadvantage from the point of view of testing.

IX. Experiments with Hydrochloric Acid to Obtain Antigenic Fractions from Corynebacteriaceae:

The observation in Experiments 7 and 8 of the preceding section demonstrated that hydrochloric acid is a much more efficient extractive than is acetic acid. It may be recalled that in Section V of Experimental Results, 6 strains of corynebacteria were extracted employing Lancefield's hydrochloric acid method with the intention of using these extracts in the hemagglutination test. Preliminary testing of these extracts with homologous serum by the capillary tube method showed that the extracts from strains 132 and 140 were highly active. These reflections prompted a return to the use of hydrochloric acid as the extracting medium.

It was intended to re-assay this technic for the production of active antigenic fractions to be used in capillary tube tests with homologous and heterologous sera. It was hoped that, providing the method was generally applicable, the reactions of extracts and heterologous sera would furnish a pattern that could be applied in grouping the species of corynebacteria studied.

Experiment 1.

The first species to be extracted in the series of experiments was strain 255 C. insidiosum. A lyophilized culture was used to inoculate 2 Loeffler slopes which were incubated at room temperature for 2 days. The Loeffler slopes were then used to seed 5 Roux bottles containing "Eugonagar" which were also incubated for 2 days at room temperature. Following incubation the cells were washed from the surface of the agar with physiological saline, centrifuged and then the cellular deposit extracted with 30 ml. N/5 HCL in a boiling water bath for 20 minutes. After cooling and centrifuging the supernatant was removed and neutralized with N NaOH and N/5 NaOH. On

neutralization nucleoprotein precipitated and was removed by centrifugation. The supernatant was then treated with 6 volumes of alcohol and the material allowed to precipitate in the cold at 4°C. The precipitate was spun down and, following removal of the supernatant, dissolved in 6 to 8 ml. saline. Insoluble material was removed by centrifugation and the clear supernatant used for testing.

When the extract from strain 255 and homologous serum, both reactants undiluted, were tested in capillary tubes no reaction was evident. Then the extract was diluted 1/10, 1/50, 1/100 and 1/200 and tested by the same procedure with undiluted serum. All of the extract dilutions reacted with homologous serum to the same degree, the maximum intensity being obtained with a 1/100 dilution of extract.

On the following day the extract was retested with homologous serum in capillary tubes, prior to being used in heterologous reactions and it was discovered that the serological activity had virtually disappeared rendering the extract unsatisfactory for further testing.

Experiment 2.

Continuing with this procedure another strain was selected for extraction in an attempt to liberate a serologically active and stable fraction.

Therefore strain 290, C. hemolyticum, was grown in 12 liters of brain heart infusion broth from lyophilized culture. The cells were collected by centrifugation and extracted with 30 ml. of N/5 HCL in a boiling water bath for 30 minutes. After cooling and centrifuging the supernatant was decanted and neutralized with N NaOH and N/5 NaOH and the insoluble proteins removed by centrifugation. The supernatant was then treated with 6 volumes of alcohol and material allowed to precipitate in the cold. The precipitate was spun down, and following removal of the supernatant, dissolved in 7 to 8 ml. of distilled water. The insoluble portion was removed by centrifugation.

The extract from strain 290 was then tested in capillary tubes with homologous serum using the following dilutions of extract: Undiluted, 1/10, 1/50, 1/100, and 1/200. A very heavy floccular precipitate was obtained with the undiluted and 1/10 dilutions of extract. A less intense positive reaction was observed with the 1/50 and 1/100 dilutions. In addition a second homogeneous and light zone was visible with undiluted serum and extract dilutions 1/10, 1/50, and 1/100. This zone appeared in the lower portion of the tubes.

On testing the extract from strain 290 with heterologous sera a non-specific heavy floccular reaction was evident in all tubes. Further tests were therefore discontinued. It may be recalled from the preceding section that the acetic acid extract of strain 290 also exhibited the same type of non-specific reactions with heterologous sera. It is also interesting to speculate that the second zone visible in the homologous reaction with strain 290 may be due to specific antigen.

Experiment 3.

Following the same procedure used in the previous experiments a hydrochloric acid extract from a third species 136 C. pyogenes was prepared. The strain was grown in 12 liters of brain heart infusion broth and the cells collected and extracted with HCL. Material was again obtained by alcohol precipitation of the supernatant with the aqueous soluble portion used for testing.

The extract, when tested in capillary tubes with serum against strain 136, both reactants undiluted, showed a good reaction (+). When the extract was tested with homologous booster serum 136 B a more intense reaction was visualized (++).

The following day the extract was retested with homologous serum and again the activity had almost completely disappeared. No further tests were

carried out.

The extracts described above as being prepared by extraction with HCL with subsequent precipitation by alcohol were tested for carbohydrate by the Molisch reaction. The extracts produced a strong and immediate positive reaction indicating the presence of carbohydrate reactive groups.

Experiment 4.

Although the foregoing experiments were not completely successful, nevertheless they were encouraging, in that serologically active material was obtained. It is interesting to ponder on the effect that the alcohol treatment may have produced with regard to the serological activity of the extracted components. With this in mind the extraction procedure was modified omitting the alcohol precipitation and experimenting with the crude hydrochloric acid extract.

Consequently strain 136 C. pyogenes was grown in 6 liters of brain heart infusion broth. The cells were collected by centrifugation in the De Laval, washed with saline and then resuspended in 50 ml. saline. The 10 ml. portions or more of this stock suspension were centrifuged so that the packed cell volume was from 5 to 8 ml. The cellular deposit was then extracted with 1 to 1.5 ml. of N/10 HCL by heating in a boiling water bath for 15 to 20 minutes. After cooling and centrifuging the supernatant was decanted and brought to pH 6.2-6.6 using N NaOH and N/5 NaOH. The nucleoproteins that precipitated were removed by centrifugation. The supernatant represents the crude extract ready for testing.

The crude extract was tested in capillary tubes with homologous serum against strain 136 and booster serum 136 B. More intense reactions were obtained with this crude extract than were exhibited by the alcohol

precipitated fraction in Experiment 3. Also the serological activity of the extract did not diminish with time.

The extract was then tested by the capillary tube method with the heterologous sera available.

Moreover a portion of the stock suspension for extraction was removed, and standardized for the agglutination reaction. The standardized suspension was then used to titer sera 136 and 136 B.

In addition an agar diffusion test plate was also set up with the extract from strain 136 and homologous serum 136 B and observed for the formation of lines.

Subsequent Experiments

The general procedures as outlined above in Experiment 4 for strain 136 were carried out for 1 strain of all of the species, except one, against which an antiserum had been produced. One strain 131 C. acnes failed to grow from lyophilized culture. The results of these experiments will be presented in Tables 8 to 10. Briefly summarized, the tables present the data obtained in the following experiments for each strain.

1. Homologous macroscopic agglutination reaction
2. Agar plate diffusion test of the serologically active HCl extracts with homologous serum
3. Capillary tube tests of the HCl extract with homologous and heterologous immune sera. In Table 10, wherein these results are presented, the numbers correspond to the same degree of reaction as plus signs.

TABLE 8

Homologous Macroscopic Agglutination Reactions

Strain	Serum	Reciprocal of Serum Dilutions									
		20	40	80	160	320	640	1280	2560	5120	C
12	12	4	4	4	4	4	4	-	-	-	-
	*12	4	4	4	4	4	-	-	-	-	-
14	14	4	4	4	4	4	4	2	1	-	-
132	132B	4	4	4	4	4	4	4	4	4	-
	*132B	4	4	4	4	4	4	4	4	4	-
133	133	4	1	-	-	-	-	-	-	-	-
	*133	4	4	1	-	-	-	-	-	-	-
134	134	2	2	2	2	3	4	4	4	4	1
136	136	4	4	4	4	4	4	3	2	1	-
	136B	4	4	4	4	4	4	4	4	4	-
137	137	-	1	2	4	4	4	2	1	-	-
140	140	1	3	4	4	4	4	4	2	2	-
149	149	-	1	4	4	4	4	2	1	1	-
	*149	2	3	4	4	4	3	2	1	-	-
281	281	1	-	-	-	-	-	-	-	-	-
	*281	1	-	-	-	-	-	-	-	-	-
288	288	3	4	4	4	4	4	4	4	4	-
	*288	3	4	4	4	4	4	3	-	-	-
290	290	2	3	3	4	4	4	4	3	2	-
	*290	2	3	4	4	4	4	3	2	1	-
194	194	4	4	4	4	4	2	2	1	-	-
195	195	4	4	4	1	-	-	-	-	-	-
196	196	4	4	4	4	4	1	--	-	-	-
251	251	3	1	-	-	-	-	-	-	-	-
254	254	1	4	4	4	4	4	-	-	-	-
255	255	4	4	4	4	1	-	-	-	-	-
282	282	-	-	-	-	-	-	-	-	-	-
285	285	4	4	4	4	4	4	1	-	-	-

* - Indicates a second animal used for immunization

B - Indicates booster serum

TABLE 9

Agar Plate Diffusion Tests

Positive Results of HCl Extracts and Homologous Serum

Extracts From Strains	Lines Observed
12	1 weak diffuse line
14	1 very strong and 1 strong sharply defined line, and 1 very defined diffuse line
120	2 very strong sharply defined lines and at least 1 strong and 1 weak diffuse line
132	2 very strong and sharply defined lines, and 2 very strong but diffuse lines
139	1 weak diffuse line
140	1 strong and 1 weak diffuse line
149	1 weak diffuse line
174	1 very strong and 1 strong sharply defined line, and 1 strong and 1 weak diffuse line
288	1 very strong sharply defined line, 1 very strong diffuse line, and 1 weak well defined line
194	2 strong and well defined lines
195	1 strong and well defined line
251	1 weak diffuse line
254	1 very strong well defined line
255	1 weak diffuse line

TABLE 10

Results of Tests of Crude HCl Extracts with Homologous and Heterologous Sera

Strains	Sera 12	14	120	B 120	131	132	B 132	133	134	135	N 135	136	B 136	137	138	139	N 139	140	149	174	B 174	281	288	290	194	195	196	251	N 251	254	255	Sera 282	285	L.D.
12	3:3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	-	2	-	-	-	-	2:-	2:1	-	-	2	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
120	-	-	2	4	3:2	2:-	3:2	-	-	-	-	-	3	-	-	-	-	-	-	-	-	-	2:-	1:-	-	-	2	-	-	-	3	2	2
131	Not Tested																																	
132	-	-	-	-	1:1	3:3	4:4	-	-	-	-	1	2	-	-	-	-	2	-	1	-	1:1	2:1	1:1	-	-	-	-	-	-	-	-	-	2
133	-	-	-	-	-	-	1:-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
134	-	-	-	-	-	-	2:1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
135	-	-	-	1	-	-	-	-	-	2	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-
136	-	-	-	2	2:1	-	-	-	-	-	NT	22	3	-	-	-	NT	-	-	-	-	2:1	-	-	-	-	-	NT	-	-	-	2	-
137	-	-	-	-	-	-	3:1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
138	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2:2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
139	-	-	-	2	-	-	-	-	-	-	1	-	1	-	-	2	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
140	-	-	-	2	2:1	2:2	2:2	-	-	-	-	-	2	-	-	-	-	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
149	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3:2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
174	-	2	-	-	-	-	1:1	-	-	-	-	-	1	-	1:-	-	-	-	-	2	3	-	-	-	-	-	-	-	-	-	-	-	-
281	-	-	-	1	-	-	2:1	-	-	-	2	-	2	-	1:-	1	-	2	-	-	1	2:1	2:2	-	-	-	1:1	1	-	-	-	-	-	-*
288	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3:1	-	-	-	-	-	-	-	-	-	-	-
290	Non Specific Reactions																							2:2										
194	-	-	-	-	3:3	-	1:-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3/2	-	-	-	-	-	-	-	-	-*
195	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-
196	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
251	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	2	-	2	-	-	-*
254	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-
255	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	2	-	3	-	-	-*
282	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
285	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

* Refer to text for explanation

Where 2 results appear under one serum, a second animal was used for immunization

Table 8 includes only the agglutination reactions of those strains exhibiting stable antigen suspensions.

Table 9 includes only the results of those agar plate diffusion tests where definite lines were observed.

Explanation for strains in Table 10 indicated by a * sign:

Strain 281:- The homologous reaction of the HCl extract from strain 281 was not very strong and appeared in the extract layer of the capillary tube. The reaction was immediate and took the form of a fluffy mass which soon precipitated. The same type of reaction occurred with many of the heterologous sera and this extract and is recorded in the table. However, the cross reactions of extract 281 with the sera of strains 196 and 288 displayed a definite homogeneous zone in the center of the tube which was still visible after the tests stood at room temperature overnight. In addition, the test with serum 196 also showed the rapidly developing fluffy reaction in the extract layer of the tube which soon precipitated.

Strain 194:- In the homologous capillary tube reaction of the HCl extract from strain 194, 2 definite zones of varying intensity were observed, and the reaction is recorded in the table as 3/2.

Strains 251 and 255:- In the homologous capillary tube tests of the HCl extracts of these strains the maximum reaction was obtained when the extracts were used either in a 1/10 or 1/50 dilution.

From the results of the heterologous tests with the crude HCl extracts no obvious groups are evident. However, a number of reciprocal reactions were observed and these are recorded in Table 11.

TABLE 11

Reciprocal Reactions of Crude HCl Extracts

With Heterologous Sera

Sera	Extracts from Strains							
	120	132	136	140	174	281	251	255
120B	H	-	2	-	-	-	-	-
132B	-	H	-	2	1	2	-	-
136B	3	-	H	-	-	2	-	-
140	-	2	-	H	-	-	-	-
174B	-	1	-	-	H	-	-	-
281	-	1	2	-	-	H	-	-
251N	-	-	-	-	-	-	H	2
255	-	-	-	-	-	-	2	H

H - Indicates homologous reaction

Numbers refer to degree of reaction as recorded in Table 10

A breakdown of the results of Table 10 from the point of view of extract activity, demonstrating the number of extracts showing no activity, those active with homologous serum only, and those taking part in increasing numbers of cross reactions, is presented in Table 12.

TABLE 12

Distribution of Activity of Crude HCl Extracts

	Number	Per Cent of Total
Extracts Showing No Activity	3	12
Extracts Active with Homologous Serum Only	6	24
Extracts Showing Non-Specific Activity	1	4
Extracts Showing: 1 Cross Reaction	5	20
2 Cross Reactions	2	8
3 Cross Reactions	1	4
4 Cross Reactions	3	12
5 Cross Reactions	0	0
6 Cross Reactions	1	4
7 Cross Reactions	0	0
8 Cross Reactions	0	0
9 Cross Reactions	1	4
10 Cross Reactions	1	4
11 Cross Reactions	1	4
	25	100

X. Preliminary Experiment with Paper Chromatography:

The completion, as far as was possible, of the tests with crude hydrochloric acid extracts in the preceding section demonstrated that active serological components could be liberated from most species of corynebacteria. Generally it was observed that the presence of numerous lines in the homologous agar plate diffusion tests also indicated several cross reactions of the particular crude HCl extract with heterologous sera. However, the nature of the reactive substances remains completely unknown.

Therefore, some preliminary attempts were made, using paper chromatography, to determine the presence of protein and polysaccharide fractions in the crude hydrochloric acid extracts, their relative numbers and perhaps also relative concentrations. Since paper chromatography has not been reduced to a routine procedure for the separation of either proteins or polysaccharides, the following "runs" at best represent only preliminary experiments.

Materials and Precedure Followed:

Paper: Whatman No. 1 chromatographic paper, measuring 18 1/2 x 22 1/2 inches, was used in all tests.

Specimen: Approximately 0.06 ml. of specimen was applied on a line 3 cm. long and 7.6 cm. from the top edge of the paper. Ten specimens were applied per sheet with a distance of 1.5 cm. between them. The papers were then suspended from polyethylene troughs housed in a large wooden cabinet that was coated inside with wax. The atmosphere of the cabinet was saturated for several hours before use with the solvent to be added in the process.

Solvent:

Polysaccharide:- The solvent used for separating the polysaccharide components was a mixture of N-butanol:ethanol:water 10:1:2 V/V respectively following the technic of White and Secor (1953).

Protein:- Several preliminary runs were carried out using an acetone: water mixture in X/X 1:1, 3:1 V/V and also 25 per cent acetone in water as described by Simonetti (1953). The best separation of proteins was achieved with acetone:water in 3:1 V/V, as recommended by Reid (1950), therefore this mixture was used for the separation of proteins.

The papers containing the specimens were suspended in the cabinets and 100 ml. of the appropriate solvent was added to the troughs and allowed to run down the paper for 8 hours. The papers were then removed and hung to dry.

Fixing and Staining:

Polysaccharide:- After the chromatograms had dried they were fixed by immersion in a mixture of 20 ml. of neutral formalin and 80 ml. of absolute ethanol for 15 minutes. Following this treatment the papers were again dried. When dry the papers were stained by spraying with mucicarmine following the technic of Hamerman (1955). After the chromatograms had been sprayed, they were repeatedly rinsed in distilled water containing 5 per cent glacial acetic acid until the background color was largely removed; then they were washed in distilled water and hung to dry. The polysaccharide bands were revealed as bright pink on a very light pink or colorless background.

Protein:- The dried chromatograms were fixed in a drying oven at 100 C for 5 minutes. Following fixation the papers were stained following the method of Durrum (1951) by dipping them for 30 minutes in a solution of 0.1 per cent bromphenol blue in absolute alcohol saturated with $HgCl_2$, then rinsed in water until all the bakcground color was removed and finally hung up to dry. The protein bands were revealed as bright blue areas on a colorless background.

The Rf value of each band was calculated as follows:

$$Rf = \frac{\text{Distance in cm. moved by the band}}{\text{Distance in cm. moved by the advancing front of liquid}}$$

The Rf values from different extracts which approximated each other numerically were assumed to be due to a similar component and were grouped in this manner. Thus, from the results of the protein chromatography experiments, a total of 8 groups were observed while 5 groups were delineated from the results of the polysaccharide runs.

The intensity of the bands was also graded visually according to the degree of color using 4 to indicate the maximum observed with all others relative to this maximum.

The results of this experiment are recorded in Tables 13 and 14. The Rf values of the bands observed are presented as well as the relative intensity of the band.

The results of the chromatography experiments demonstrate a multiplicity of protein and polysaccharide components in the crude HCl extracts. Generally the most intense bands were visible at the origin with virtually every extract tested exhibiting both a protein and a polysaccharide component at this point. The only exception was noted with strain 288 which exhibited no detectable protein, even at the point of application of the specimen.

In general the procedure for polysaccharide components yielded sharp distinct bands, while the proteins were separated into distinct areas. There was considerable tailing in some protein bands with higher Rf values indicating that sharp separation was not achieved by the method used.

TABLE 13

Results of Paper Chromatography for Proteins

Showing Rf Values and Intensity of Bands

Extracts from Strains	Bands A Rf I	B Rf I	C Rf I	D Rf I	E Rf I	F Rf I	G Rf I	H Rf I
12	0:1	-	-	-	-	.61:1	-	.97:1
14	0:2	.06:1	-	-	-	.68:1	.90:1	.97:1
120	0:3	.08:2	.15:3	.23:1	.58:1	-	.87:2	.93:1
132	0:2	.08:1	-	-	.56:1	-	.87:1	.94:1
133	0:2	.06:1	-	-	-	.60:1	.89:1	.93:1
136	0:2	.08:1	-	-	-	.61:1	.86:1	-
137	0:3	-	-	-	.59:1	-	.90:2	.94:1
138	0:1	.08:1	-	-	-	-	-	.94:1
140	0:2	.08:1	-	-	.58:1	-	.90:1	.97:1
149	0:1	.08:1	-	-	-	.62:1	.87:1	.99:1
174	0:2	.06:1	-	-	-	.66:1	.90:1	.99:1
288	-	-	-	-	-	-	-	-
194	0:2	.08:1	-	-	-	-	-	.92:1
195	0:2	.08:1	-	-	-	.63:1	-	-
196	0:1	.05:1	-	-	.59:1	-	-	.92:1
251	0:1	.05:1	-	-	-	.62:1	-	-
254	0:1	-	-	-	-	.63:1	.89:2	.93:1
255	0:1	.04:1	-	-	.58:1	-	.84:1	-
282	0:2	-	-	-	.59:1	-	.86:1	.92:1
285	0:1	-	-	-	.59:1	-	-	.93:1

TABLE 14

Results of Paper Chromatography for Carbohydrates

Showing Rf Values and Intensity of Bands

Extracts from Strains	Bands A Rf I	B Rf I	C Rf I	D Rf I	E Rf I
12	0:3	-	-	-	-
14	0:3	-	-	-	.13:1
120	0:4	.01:1	.04:1	-	.13:1
132	0:3	-	.05:1	-	.13:1
133	0:4	-	-	-	-
136	0:3	-	.04:1	-	-
137	0:3	-	.04:1	-	-
138	0:3	-	.05:1	.08:1	.13:1
140	0:3	-	.05:1	-	.13:1
149	0:3	-	.05:2	-	-
174	0:3	-	-	-	.13:1
281	0:3	-	.04:2	.10:1	.13:1
288	0:4	-	.04:1	-	-
194	0:3	-	-	-	.13:1
195	0:3	-	.04:1	-	-
196	0:3	-	.04:1	-	-
251	0:2	-	-	-	.13:1
254	0:3	.01:1	.04:2	-	-
255	0:2	-	-	-	.13:1
282	0:3	-	.04:1	-	-
285	0:3	-	-	-	-

DISCUSSION OF RESULTS

DISCUSSION OF RESULTS

In the present investigation of the serological relationships of the corynebacteria, antisera were produced in rabbits against 14 species of corynebacteria. Immune sera against another 12 species were already on hand from a previous work (Yurack 1954) thus making available for study immune sera against 26 species of corynebacteria comprising most of the usually accepted human, animal, and plant strains.

With the collection of antisera, the homologous agglutination titers of 4 out of 5 strains of the first group tested were impossible to determine due to autoagglutination and the formation of large stringy masses of cells. When similar difficulties were encountered in an earlier phase of the work of Yurack (1954) attempts were made to overcome them. The methods used included the use of lyophilized cultures as a means of maintaining stable antigens for agglutination reactions, the effects of various concentrations of saline on the stability of antigen suspensions, the use of the detergent Tween 80 to overcome thread-like agglutination, and a comparison of agglutinability of unheated and autoclaved suspensions. Although each of these technics showed an improvement in some instances, a method which consistently stabilized antigen suspensions for the agglutination reaction was not realized.

Further attempts to obtain stable antigens for the direct agglutination reaction using Subtosan, a plasma substitute, phosphate buffer pH 7.0 and 0.5 per cent phenol in physiological saline respectively as diluents for the antigen were not satisfactory. Subtosan was found to inhibit the agglutination of one of the strains tested.

Phosphate buffer, tested over a pH range from 5.4 to 7.0 did not enhance the stability of the tested strain of corynebacterium. The same degree of

autoagglutination was observed with all the aliquots of the suspension throughout the buffered pH values tested.

Antigen suspensions for agglutination reactions prepared from cells grown on blood agar plates show some improvement. The agglutinability of one of the strains tested, 12 C. hoagii was considerably reduced when the antigen suspension was prepared in this manner. However, heating of the suspension restored the original titer.

The antigen suspensions of the plant species of corynebacteria, prepared from cells grown on blood agar plates, were quite satisfactory for agglutination reactions. However quite low titers were observed. The agglutinability of these species is altered by heating and it is possible that specificity is also altered by this treatment. It was previously observed by Yurack (1954) that autoclaved cultures of corynebacteria generally show an altered agglutinability in heterologous reactions as well as in homologous tests. It was also notable that strain 133 C. equi did not agglutinate with homologous serum even when a heated suspension was tested.

When the many low titers observed and the degree of autoagglutination are considered, it seems that under the conditions of these experiments the direct agglutination reaction is not satisfactory as a general procedure in serological studies on corynebacteria.

The conclusion stated above is at least partially contradicted if one examines Table 8 which contains the homologous agglutination titers of the strains to be used in extraction experiments. In the extraction experiments stable antigen suspensions were the rule, with the exception of some 5 species, including those species which showed extensive autoagglutination in the experimental section devoted to attempts to obtain suitable suspensions for the agglutination reaction.

In the earlier experiments antigen suspensions for the agglutination reaction were prepared from the harvest of 100 ml. flasks of broth previously inoculated via 10 ml. broth and lyophilized culture. In the later extraction experiments the antigen suspension was obtained from a stock culture prepared from the harvest of 6 liters of broth inoculated in a stepwise manner from 100 ml. broth via 10. broth and lyophilized culture. Although, in the 50 ml. stock culture prepared from 6 liters of growth, it was difficult to completely resuspend all of the cells the large clumps soon settled, leaving a heavy yet homogeneous and stable suspension for agglutination reactions in the upper half of the container. It would seem that the stepwise increase of inoculum for seeding the 6 liter culture tended to produce a more smooth phase of growth and that the culture in bulk quantities allowed for the absolute number of smooth cells to be much greater than in the smaller 100 ml. volumes of broth.

The next series of experiments were performed in an attempt to duplicate the work of Cummins (1954), in preparing cell wall suspensions suitable for use in direct agglutination reactions. With the crude and tryspin purified cell wall preparations of 3 species, including 2 strains of C. diphtheriae, no significant reactions were obtained in homologous agglutination tests with halving dilutions of serum. The technic used in these experiments conformed as closely as possible to that described by Cummins. The only obvious difference lies in the apparatus used for disintegration of the bacterial cells. Cummins reports the use of the Mickle disintegrator requiring shaking with glass beads for only 1 hour, whereas, in this study an ordinary laboratory shaker was used necessitating a 3-5 hour period of shaking with glass beads for maximal cell destruction. It is felt that the structural continuity of the cell walls necessary for detectable macroscopic agglutination was damaged to a degree thereby rendering invalid the agglutin - ation method as a means of testing.

The next logical procedure seemed to be directed toward the extraction of antigenic fractions from whole cells, since the attempts to obtain stable antigen suspensions of whole cells and satisfactory preparations of cell wall material for the direct agglutination reaction failed.

Polysaccharide fractions were obtained from 3 species of corynebacteria including 2 strains of C. diphtheriae, using Fuller's (1938) formamide method. Dilutions of the extracts from 1/100 to 1/12,800 were tested with a 1/10 dilution of homologous serum by the capillary tube method. All of the tests with all extracts were negative. However the extracts reacted positive for carbohydrate by the Molisch reaction.

As more experience with extraction procedures was acquired it was realized that the failures with this technic might have been due to improper testing of the reagents. Under usual conditions the maximum results in capillary tubes are obtained when the extract and serum are used undiluted, the most important condition being the use of undiluted immune serum. Therefore, a consideration of the dilutions of serum and extract used for testing in this series of experiments does not warrant any definite conclusions.

Crude Lancefield's hydrochloric acid extracts when tested quantitatively for protein and carbohydrate fractions by the Stavitsky and Keogh hemagglutination technics respectively, also did not meet expectations. Six species were extracted and in a preliminary testing for serological activity by the capillary tube method only the extracts from strain 132 C. bovis, and 140 C. xerosis showed a positive reaction with homologous serum. However these 2 active extracts produced no titer in the hemagglutination tests for either protein or carbohydrate. This seems all the more unusual if one considers that in later experiments crude hydrochloric acid extracts of

these strains exhibited more than 1 line in the agar plate diffusion test, indicating the presence of several antigens. The crude hydrochloric acid extracts from 3 strains showed positive titers with the hemagglutination test by the Stavitsky method for detecting proteins. One of these extracts also exhibited a titer in the Keogh test indicating adsorption of polysaccharide material.

Material liberated in the hydrochloric acid extraction process, although active with homologous serum in capillary tube tests, does not seem to be suitable for the hemagglutination reaction under the conditions of these experiments. The reactions that were obtained were not uniform nor were the titers as high as those observed in the whole cell direct agglutination test.

Glycine in phosphate buffer, over a pH range from 7.0 to 8.2, employed in attempts to disrupt the bacterial cells in order to obtain a polysaccharide fraction, failed to lyse the tested species of corynebacteria to a degree that could be detected.

The trials with extra cellular enzymes from the culture filtrates of Streptomyces albus as used by McCarty (1952) to produce bacterial lysis of species of corynebacteria, with the subsequent isolation of polysaccharide material for testing by the hemagglutination reaction, were not satisfactory. Polysaccharide fractions from 2 strains 132, C. bovis and 140, C. xerosis, obtained by this method and tested by the Keogh hemagglutination method showed slight positive reactions in the very high serum dilutions, around 1/20,000 indicating that only traces of carbohydrate material were in the extracts available for adsorption.

McCarty (1952) emphasizes that maximum enzyme production is very variable even on the same medium with the same strain. It is also probable

that the preliminary manipulations with the strain of S. albus, such as growth on McGill nitrient agar and failure in the ability to determine sporulation microscopically may have influenced considerably the yield of active enzymes.

The use of alcohol precipitated fractions from acetic acid extracts in capillary tube tests with homologous and heterologous sera was satisfactory only for 2 strains of C. bovis and 1 strain of C. xerosis. The extracts obtained from the other species tested were inactive or non-specific. However even the serologically active extracts lost their activity within a short period of time, 3-5 days. When aliquots of a stock suspension were extracted by hydrochloric acid and acetic acid respectively and alcohol precipitated fractions obtained from both crude extracts, it was observed that the hydrochloric acid extraction method liberated from 2 to 3 times more material than acetic acid. It was therefore concluded that hydrochloric acid is a more efficient extractive for corynebacteria.

Alcohol precipitated fractions from hydrochloric acid extracts of the species tested were found to be active by the capillary tube method with homologous serum, but the activity virtually disappeared overnight. It is conceivable that alcohol fractionation was detrimental to the serological activity of the liberated components.

A return to the crude hydrochloric acid extracts, with some minor modifications, produced the best results in the capillary tube tests with homologous and heterologous sera. Out of the 25 species extracted, 6 of the crude hydrochloric acid extracts did not react with homologous serum, while 1 extract exhibited non-specific reactions. However, three of the extracts inactive with homologous serum, did show a varying degree of

activity with serum 132B. Another group of 6 extracts reacted with homologous serum only. The remaining extracts exhibited some cross reactions, with some strong reciprocal reactions also observed.

The agglutination titers of those extracts showing no homologous activity generally were no lower than many titers exhibited by strains producing active extracts. It is possible that the immune serum of these inactive strains did not contain sufficient antibodies against the fractions liberated to yield a visible result by the capillary tube method. It may also be possible that the extraction process destroyed groupings necessary to produce the activity tested for in these experiments.

The extracts of those strains against which booster sera were available showed a more intense degree of reaction with the booster serum than that observed with the lower titer serum in homologous tests by capillary tube. Therefore, in these cases at least, a relationship exists between the degree of homologous reaction of the extract and the agglutinating titer of the serum. In addition, many of the cross reactions observed in heterologous tests with the booster sera were not evident with the lower titer serum against that strain. This situation is apparent particularly with sera 132 and 132B. It is probable that in such a potent serum antibodies against minor and more general antigens are present to a much higher degree and are responsible for many of the cross reactions observed, when similar antigens are present, even in small amount, in heterologous extracts.

In the agar plate diffusion tests the number of lines obtained varied from 1 to 4, indicating the minimum number of active antigens extracted. The results of these tests also show, to an extent, the complexity of antigenic composition of many species as well as the efficiency of the extractive.

Both the thin sharply defined lines, and the diffuse type of line were evident, and reputed to be due to protein and carbohydrate substances respectively, by such workers as Oudin (1952) and Ouchterlony (1953). In most instances there was a correlation between the number of cross reactions exhibited by a particular extract, and the number of lines produced in the agar plate diffusion test, as well as in the number of protein and carbohydrate bands observed in the chromatography experiments.

Unfortunately no obvious groups are discernable from the results of the heterologous tests of the crude hydrochloric acid extracts by the capillary tube method. Generally, the extracts from the plant species of corynebacteria do not react with other sera nor were cross reactions generally observed within this group of organisms. However, the hydrochloric acid extract from one species, 194 C. helvolum, produced an immediate and strong reaction with both sera against strain 131, C. acnes. A slight reaction was also observed overnight with serum 132B, C. bovis. The extract from strain 194 was the only member of the plant group of organisms showing 2 distinct zones in the homologous capillary tube test and also 2 lines in the agar plate diffusion test.

Also within the plant group of organisms the extracts and sera of 2 species, 251 C. insidiosum, and 255 C. michiganense, exhibited strong reciprocal reactions by the capillary tube method. These results confirm the work of Rosenthal and Cox (1954) who also found these species to be closely related serologically, and that some strains of both species were indistinguishable serologically. In view of these findings, and also considering the fact that the extracts from strains 251 and 255 did not react with any other sera, it would seem that the designation of these strains as

2 separate species is unwarranted, and that one strain should be described as a variant of the other.

In the heterologous capillary tube tests of the extracts of the species of the human and animal groups, numerous cross reactions were noted. In addition more reactions were evident with these extracts and sera against strains of the plant group of organisms than was the reverse. The reciprocal reactions that were observed have been presented in Table 11. It was interesting to note that the reactions of the 2 strains of C. diphtheriae, 14 and 174 were not reciprocal. The extract from strain 174 did react with the serum against strain 14 but the reverse was not true.

It is also noteworthy that the hydrochloric acid extracts from 3 strains, 120 C. ovis, 132 C. bovis, and 140 C. xerosis reacted strongly in capillary tube tests with Lancefield's group D serum.

The results of the chromatography experiment indicate a multiplicity of protein and carbohydrate components in the crude hydrochloric acid extracts. In most instances more protein bands were observed per strain than carbohydrate bands. The extracts tested, with only one exception, showed an intense protein and carbohydrate band at the point of application of the specimen. It is probable that these immobile fractions are part of a large protein-carbohydrate complex.

With one strain, 288 C. enzymicum, no detectable protein was observed even at the line of application of the specimen. Only 2 carbohydrate bands were visible in the chromatography run, but the extract from strain 288 with homologous serum in the agar plate diffusion test produced 3 lines, 2 of which were strong and diffuse and 1 weak but well defined. The strong reaction observed with this extract and homologous serum in capillary tubes is therefore most likely due to polysaccharide.

In view of the difficulties encountered in this laboratory in obtaining whole cell suspensions of species of corynebacteria for the agglutination reaction, and the similar difficulties encountered by other workers such as Eagleton and Baxter (1923), Robinson and Peeney (1936), Huang and Sia, and Minzel and Freeman (1950), and with no consistent solution to the problem, it seems that the use of whole cells in direct agglutination reactions is not feasible in attempting a serological analysis of the corynebacteria as a group. Some workers, such as Cummins (1954) and Parnas (1952), have also reported that with some species at least, group antigens play no part in whole cell agglutination tests. Therefore, these observations indicate that any exhaustive survey of the members of the genus corynebacterium, with regard to a serological grouping, should be directed toward the extraction of antigenic fractions from the whole cells.

In this investigation the most promising results were obtained using crude hydrochloric acid extracts of the cells, tested by the capillary tube method with immune sera against intact organisms. From the results obtained no obvious and clear cut groups were defined although some observations were made. In general, the extracts from the plant species of corynebacteria do not react either with sera against organisms of the human and animal groups nor with the sera against strains within the plant group. Most of the cross reactions, including reciprocal reactions, were observed among the commonly recognized members of the human and animal groups of corynebacteria.

It is the opinion of the author that the most promising method of obtaining species specific antigens from corynebacteria for serological grouping is the technic used by Lancefield and others in solving a similar problem among the β-hemolytic Streptococci.

SUMMARY

VII. Polysaccharide fractions prepared by bacterial lysis with extracellular enzymes from the culture filtrates of Streptomyces albus were not satisfactory in hemagglutination tests.

VIII. The results obtained in capillary tube tests with alcohol precipitated fractions from acetic acid extracts were too limited for this method to be used as a general procedure in the serological analysis of the corynebacteria.

IX. The activity in capillary tube tests of alcohol precipitated fractions from hydrochloric acid extracts virtually disappeared overnight. Crude hydrochloric acid extracts, from the species of corynebacteria tested, produced the most consistent results in capillary tube tests with homologous and heterologous sera.

X. As expected, the results of the chromatography experiment indicate a multiplicity of protein and carbohydrate components in the crude hydrochloric acid extracts of the species of corynebacteria tested.

It is felt that the complexity of antigenic structure, which resulted in the overlapping reactions observed when the hydrochloric acid extracts were employed as antigens, will tend to complicate any serological analysis, with emphasis on grouping, of the members of the genus corynebacterium.

CLAIM TO ORIGINALITY OR CONTRIBUTION TO KNOWLEDGE

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1. The methods assayed in this investigation, although not original, have been applied, for the first time, in a serological analysis of the corynebacteria as a group; particularly with regard to the extraction of antigenic fractions from the cells and the procedures followed in testing these extracts.

2. These investigations represent the first attempt at a comprehensive survey of all of the representative organisms within the genus corynebacterium.

3. Of the various technics tried the most consistent results were obtained with crude hydrochloric acid extracts, as developed by Lancefield, and tested by the capillary tube method. In this study the basic extraction procedure was modified, in that 0.4 to 0.8 ml. of packed cells were extracted with 1 to 2 ml. of N/10 HCl and neutralization carried only to pH 6.2-6.5 rather than to neutrality.

4. In addition, something of the number and nature of the extracted fractions has been determined by agar plate diffusion tests and chromatography experiments.

APPENDIX

APPENDIX

MEDIA:

1. Brain Heart Infusion Broth (Difco) was the medium employed in growing strains for the various preparations.

2. Loeffler's Serum Medium (Coagulated):

The ingredients are 3 parts serum, sterilized by filtration through a Seitz EK film, and one part of sterile 1 per cent Dextrose Broth. Using sterile precautions, fill into screw cap tubes, about 7 ml. per tube. Slope in the autoclave and heat long enough to solidify the serum without making it opaque (75°C for 1 hour). Then autoclave at 120°C for 20 minutes for sterility.

3. "Eugonagar" Preparation:

Formula per liter of Distilled Water:

Trypticase	15.0 gm.
Phytone	5.0 gm.
Sodium Chloride	4.0 gm.
Sodium Sulfite	0.2 gm.
l - Cystine	0.2 gm.
Dextrose	5.0 gm.
Agar	15.0 gm.

Final pH 7.0

4. Medium for the Production of Streptomyces albus Filtrate:

Salt Mixture per Liter:

NaCl	5.0 gm.
K_2HPO_4	2.0 gm.
$MgSO_4.7H_2O$	1.0 gm.

$CaCl_2$ ..... 0.04 gm.

$FeSO_4.7H_2O$ ..... 0.02 gm.

$ZnSO_4.7H_2O$ ..... 0.01 gm.

To this salt mixture is added:

Bacto-Casamino Acids,

purified, Difco ..... 0.5 per cent

Glucose ..... 0.5 per cent

BUFFER SOLUTIONS:

1. Phosphate Buffer for Tryspin Suspension:

Potassium phosphate buffer 0.033M was the suspending medium for tryspin in the cell wall experiments. A ratio of 95 ml. of K_2HPO_4 to 5 ml. of KH_2PO_4 results in a 0.033M buffer of pH 8.0.

2. Buffered Saline for Hemagglutination Experiments:

a). Buffered saline pH 7.2: This solution was prepared by mixing 100 ml. of physiological saline and 100 ml. of a buffer consisting of 23.9 ml. of 0.15M KH_2PO_4 and 76.0 ml. of 0.15M Na_2HPO_4.

b). Buffered saline pH 6.4: This solution was prepared by mixing 100 ml. of saline and 100 ml. of a buffer composed of 32.2 ml. of 0.15M Na_2HPO_4 and 67.7 ml. of 0.15M KH_2PO_4.

3. Phosphate Buffer for Various Antigen Suspensions:

Where phosphate buffer is indicated in the Experiments of Section II of Experimental Results as the suspending medium for an antigen suspension, Soerensen's 0.15M phosphate buffer, in appropriate dilutions, was used.

ALSEVER'S SOLUTION:

Alsever's solution was employed for preserving sheep red blood cells for use in hemagglutination reactions. The solution is prepared as follows.

Dextrose ..... 2.05 gm.

Sodium Citrate ..... 0.8 gm.

Sodium Chloride ..... 0.42 gm.

Distilled Water ..... 100 ml.

The pH is adjusted to 6.1 with 10 per cent citric acid and autoclaved at 120°C for 15 minutes. One volume of blood is added to 1.2 volumes of Alsever solution.

SURFACE ACTIVE AGENT:

The surface active agent used in this investigation, Tween 80, is a nonionic partial ester, polyoxyethylene sorbitan monooleate. Fresh solutions were prepared at a maximum of 30 days. Tween 80 was diluted 7 ml. per 100 ml. of distilled water to give a 7 per cent solution, and autoclaved. Ten ml. of this solution was added to 90 ml. of brain heart infusion broth to produce the desired concentration of 0.7 per cent of the surface active agent in the final preparation.

METHOD OF OPERATING THE EVELYN PHOTOELECTRIC COLORIMETER:

The correct use of the Evelyn Photoelectric Colorimeter is described as follows: The correct filter, (#420), was placed in the light beam of the instrument. Next an Evelyn tube containing the blank, 6 ml. of 1.5 per cent saline, was put into the tube holder. The galvanometer lamp was then turned on by means of the galvanometer lamp switch, and the galvanometer adjusted to zero by moving the coarse zero adjuster on top of the galvanometer and the glass scale. Then the colorimeter lamp was turned on and the galvanometer adjusted to 100 by means of the coarse and fine adjustments on the instrument. The tubes containing 6 ml. of the sample were now placed in the instrument and the percent transmission was read directly from the galvanometer scale.

MOLISCH REACTION:

To 5 ml. of the test solution, in a test tube, add 2 drops of Molisch reagent, a 5 per cent solution of α-naphthol in alcohol. After mixing thoroughly the tube is inclined and about 3 ml. of concentrated sulfuric acid (H_2SO_4) is allowed to flow down the side of the tube thus forming a layer of acid beneath the test solution. If carbohydrate is present a reddish violet zone appears at the interface between the 2 liquids.

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