

DETECTION OF ANTIBODIES TO CELLULAR
ANTIGENS OF STREPTOCOCCUS PYOGENES

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ABSTRACT

Group-specific polysaccharide was extracted from group A streptococci by various methods and after purification it was adsorbed on to polystyrene latex particles, which were used in an agglutination test to detect group-specific antibody in hyperimmune rabbit sera. The most pure and serologically specific extracts were obtained by extraction with formamide. Formamide-extracted polysaccharides of groups A-var, B, C, G and L were also adsorbed on to latex particles. Inter-group cross-agglutination reactions corresponded to known chemical relationships between group polysaccharides.

Latex agglutination tests were used to detect group A and A-var antibodies in human sera. Antibodies were found in 90% of patients suspected of suffering from streptococcal disease but in less than 40% of controls. In paired sera from patients with known streptococcal infections, initial antibody titres were high in most cases.

M-proteins from group A streptococci belonging to a number of types were extracted with hot acid and precipitated with 30% and with 60% saturated ammonium sulphate. The precipitates were designated P30 and P60 respectively. The supernatant (S60) was further

purified by precipitation at pH 5.5 and washing with absolute alcohol. Immunological tests showed that purified S60 fraction was more type-specific than P60 fraction. Absorption of rabbit hyperimmune M antisera with S60 fraction removed the specific bactericidal activity.

The purified S60 was used in latex agglutination tests to detect type-specific M antibodies in human sera. Sera from patients suspected of suffering from streptococcal infection and from control persons showed no significant difference in the number of serotypes for which antibody was present in high titre. Most of the paired sera from patients who had suffered from infection with group A streptococci of known serotype showed a significant rise in antibody titre to the infecting serotype. Rises in titre to heterologous serotypes also occurred frequently, but showed no constant pattern.

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PART ONE

INTRODUCTION: GROUP A STREPTOCOCCI

Streptococci are Gram-positive, catalase negative, spherical or ovoid cocci that are usually arranged in chains of varying lengths. The association of streptococci with human diseases such as erysipalis, puerperal sepsis and acute abscesses was demonstrated by Billroth, Pasteur, Ogston, Koch, Fehleisen and Rosenbach between the years 1874 and 1884 (for early references see Wilson & Miles, 1964). Later, streptococci pathogenic to animals were also isolated. The organisms pathogenic for man were called Strept. pyogenes by Rosenbach in 1884. However, this name is now used only for group A streptococci, which are the main cause of streptococcal disease in man.

The classification of streptococci is at present far from satisfactory. It may be made on the basis of (1) the presence of haemolysis on blood agar plates and its character, (2) biochemical characters, or (3) the group-specific polysaccharids. There are some correspondences between the subdivisions made by each of these methods, but there are also many discrepancies. In general, the streptococci fall into three main groups.

(1) The pyogenic cocci, most but by no means all of which

are β -haemolytic on blood agar (viz. the colonies are surrounded by a clear zone), and most of which possess a recognisable group-specific polysaccharide.

(2) A group of related species that have several common biochemical characters separating them from the pyogenic cocci and possess the group antigen D (or occasionally N). Most of them are found in animal faeces or in dairy produce (Deibel, 1964). Their action on blood is variable.

(3) The "viridans" streptococci, which produce alpha haemolysis (greening) on blood agar or are without any haemolytic activity. Group-specific polysaccharides have been found in relatively few of them and their classification is particularly difficult (Colman & Williams, 1965).

The group-specific antigens - the "C" polysaccharides of Lancefield (1933) - are each designated by a letter of the alphabet from A to T with the exception of I and J. At least one of them, the group D antigen, is not a true polysaccharide, but is a teichoic acid. Group A streptococci possess a carbohydrate group-specific antigen composed of rhamnose and N-acetylglucosamine.

I MORPHOLOGY AND STRUCTURE OF STREPTOCOCCUS PYOGENES

A. General

Like most streptococci, these organisms are usually spheres of varying size with an average diameter of 1 μ . They are arranged as pairs in chains, and at the time of division they elongate with the long axis perpendicular to the axis of the chain. The minimum number of cocci found in one chain is generally not less than eight, and longer chains are seen quite frequently. These chains result from the existence of intercellular bridges between individual cocci after cell division. Slade and Slamp (1956) showed that the chains could be disrupted into single cells and pairs by sonic oscillation for 1 minute. Excessively long chains can be produced by growing the organisms on broth to which has been added type-specific serum (Stollerman & Ekstedt, 1957). Growth in normal serum results in the formation of short chains. The mechanism of production of short chains was suggested by the results obtained by Ekstedt & Stollerman (1960 a & b) to be an enzymatic action, which is inhibited by the union of a surface antigen, e.g. M, R, with its homologous antibody.

Group A streptococci are non-motile. They are normally Gram-positive unless overdecolourised. However, unfavourable

conditions of growth lead to loss of this and other characteristics like shape and size which become highly irregular even within the same chain.

B. Capsule

Capsules can be demonstrated in group A streptococci of very young cultures in serum broth during the logarithmic growth phase by India ink staining (Morison, 1940). During the stationary phase, capsular material is shed into the culture medium and can no longer be demonstrated on the cells. The capsular material is composed of the polysaccharide hyaluronic acid. Capsules of the same material are produced by members of group C. This capsular hyaluronic acid is more or less the same as the hyaluronic acid of animal tissue and is non-antigenic. Production of capsular hyaluronic acid stimulates the production by the streptococci of the enzyme hyaluronidase, which will be discussed fully later.

C. The cell wall

This is the tough rigid frame which gives the cell its characteristic shape. Cell walls may be obtained by the mechanical disruption of streptococcal cells in a Mickle's disintegrator (Salton & Horne, 1951) or by freeze-pressing (Edebo, 1961). The streptococcal cell wall consists of a mucopeptide backbone which provides rigidity

and support. On the surface are found the protein antigens viz. M, T and R. In between is the group-specific polysaccharide, which seems to be closely attached to the mucopeptide (Krause & McCarty, 1961). Apart from providing rigidity and shape the cell wall protects the protoplast under unfavourable conditions, e.g. against phagocytosis. However, it has no osmotic activity, which is a main function of the protoplast membrane. But the passage of extracellular products through the cell wall shows that it is not an absolute barrier. By virtue of the presence of M-protein, the cell wall provides an effective defence against phagocytosis. The various antigens in the cell wall will be discussed on pages 9-25.

II CULTURAL CHARACTERS OF STREPTOCOCCUS PYOGENES

A. Growth requirements

Group A streptococci grow best at a temperature of 37°C and are inhibited if the temperature exceeds 40°C. They are aerobic and facultatively anaerobic. They require a wide range of amino acids and vitamins particularly the B complex group of vitamins. Since they grow best in the presence of peptides, satisfactory synthetic media for their growth are difficult to achieve. Growth can be obtained in defined media, but the full range of antigens and extracellular products is not formed under these conditions. Streptococci metabolise glucose and give rise to lactic acid, which limits their growth; the use of buffer helps to overcome this difficulty.

B. Colonial morphology

Group A streptococci generally give rise to colonies not exceeding 1 mm in diameter on solid media. On blood agar they exhibit large zones of β -haemolysis surrounding the colonies. Colonies may have one of three different appearances: (a) Mucoid colonies are highly viscous due to the formation of abundant amounts of capsular hyaluronic acid. (b) Matt colonies appear as a result of drying or of the action of hyaluronidase on the capsule. They look

dry and irregular in surface. Both mucoid and matt colonies generally produce large amounts of M-proteins.

(c) Glossy colonies are regular in shape and their surface is shiny but they are smaller in size than the mucoid or matt colonies. They produce little or no M-protein.

Growth in broth gives a granular appearance, but this may be lost on repeated subculture, incubation at 22°C or changing the medium. Under such conditions, growth tends to be turbid and the chains are shorter.

III ANTIGENIC STRUCTURE OF STREPTOCOCCUS PYOGENES

The antigenic structure of group A streptococci is complex and as yet not completely understood. The antigens that have been most studied are of two classes, cellular antigens forming part or attached to the cell wall and extracellular antigens detectable in culture supernatants. The capsular hyaluronic acid is not antigenic.

A. The cellular antigens

These include the group-specific polysaccharide, various proteins including the M, T, R and other antigens, the cell wall mucopeptide and the glycerol teichoic acid.

1. The group-specific polysaccharide

The carbohydrate nature of this antigen was established by the work of Lancefield (1928 c, 1933). It forms two-thirds of the cell wall material and 6-10% of the dry weight of the whole cell (McCarty, 1952). The pure polysaccharide consists of about 60% L-rhamnose and 30% N-acetylglucosamine viz. an approximate ratio of 2:1 (Schmidt, 1952; McCarty, 1952; Salton, 1953; Krause, 1963).

In the cell or the cell wall the polysaccharide is masked by the surface proteins rendering it ineffective for agglutination reactions in group-

specific antisera. However, agglutination due to a group antigen was suspected by Griffith (1926) during his early attempts at the serological typing of streptococci. Later, after the group-specific polysaccharide had been recognized (Lancefield, 1933), he assumed that the cross-reactions amongst certain of his types were due to this antigen (Griffith, 1934) and could be absorbed out by heterologous streptococcal suspensions. Rosendal (1956) demonstrated the specific agglutination of streptococcal cells, which had been treated with proteolytic enzymes by their respective grouping sera. More recently, Cummins & Slade (1961) carried out agglutination reactions with cell walls treated with proteolytic enzymes. Nevertheless, the most commonly employed serological test for grouping of streptococci is a precipitin reaction in which the polysaccharide is in a crude extract obtained from the cells. There are various recognized methods of extraction: (1) Lancefield's acid-extraction method, in which the deposited cells are boiled in N/5 HCl (pH2) and neutralized with caustic soda; after centrifugation the supernatant is collected for use as antigen; (2) the hot formamide method (Fuller, 1938) in which the cells are heated in formamide at 160°C

and the soluble polysaccharide is separated from the residue by acid-alcohol and acetone fractionation; (3) treatment of the cells by the enzymes from the soil organism Streptomyces albus, which dissolve the cell wall (Maxted, 1948); (4) the use of a muralytic enzyme, group C phage-associated lysin, obtained by propagating the bacteriophage on group C streptococci and found to lyse the cell walls of streptococci of groups A, C, E, and under certain circumstances the cell walls of group H streptococci with the liberation of the group-specific polysaccharide (Maxted, 1957). Krause (1957) also reported similar results on groups A and C; (5) by autoclaving a suspension of cells (Rantz & Randall, 1955). The polysaccharide can be isolated from intact cells or from cell walls, but the latter are the most suitable starting material to obtain a pure polysaccharide.

The pure soluble polysaccharide is metabolically inert and non-toxic, and is rapidly excreted in urine when injected into animals (Schmidt, 1952). But, when it is injected intradermally in rabbits in the form of soluble cell wall fragments which also contain mucopeptide, nodular skin lesions develop (Schwab, Cromartie & Roberson, 1959).

Electrophoretic mobility of the polysaccharide is negligible. Its antigenic specificity is determined by β -N-acetylglucosaminide side chains on the rhamnose backbone of the molecule (Schmidt, 1952; McCarty, 1956, 1958). However, some strains of streptococci have been found to lose this specificity as a result of variation during passage through mice (Wilson, 1945). The work of McCarty & Lancefield (1955) and McCarty (1956) has shown that in fact this variation is due to the loss of N-acetylglucosamine moiety, thus exposing the rhamnose oligosaccharide which is the antigenic determinant of the variant strain. This variant polysaccharide (A-variant) reacts in antisera prepared against it, and also in antisera prepared against the parent polysaccharide but to a lesser degree. It has a higher ratio of rhamnose to glucosamine. The same authors showed that variation occurred during mouse passage in group A streptococci of several different serotypes. In addition, a strain was encountered - designated group A-intermediate - that possessed a polysaccharide intermediate in chemical and serological properties between group A and group A-variant polysaccharide viz. reacted equally in both antisera.

These variants so far have not been isolated from cases of natural infection in man, but they are established experimental mutants. However, accidental infections in laboratory workers have been reported (Lancefield, 1962).

2. Surface protein antigens

These constitute the outer layer of the cell wall, and are consequently referred to as the surface proteins. Sixteen different amino acids have been identified in these surface proteins (Salton, 1953; Tepper, Hayashi & Barkulis, 1960). There are three main classes of protein antigens namely the M-protein, the T antigen and the R antigen, and some minor antigens. The M and T antigens confer type-specificity on the organisms and have been extensively used for type classification of group A streptococci (Lancefield, 1940).

(a) The M-protein. The location of this type-specific antigen in the cell wall has been established in various ways. The first evidence was obtained by the removal of M-protein from the living streptococci by proteolytic enzymes without affecting their viability (Lancefield, 1943). Further studies by other workers (Salton,

1952; Barkulis & Jones, 1957; Cole & Hahn, 1962) confirmed the attachment of the M-proteins to cell walls. However, cell walls do not manufacture M-protein. This substance is an established product of protoplasts and L-forms (Sharp, Hijmans & Dienes, 1957; Gooder & Marted, 1958, 1961; Freimer, Krause & McCarty, 1959; Karakawa, Rotta & Krause, 1965). Moreover, Fox & Krampitz (1956) showed that the utilization by streptococci of amino acids in a growth medium for protein synthesis was dependent on the presence of purine and pyrimidine bases, vitamins and other growth substances.

The M-proteins although differing in antigenic specificity from type to type, share some common physical and chemical properties. Extraction of the antigen is commonly achieved by treatment with hot acid (Lancefield, 1928 a); boiling in acid does not impair its antigenic specificity. A more gentle method of extraction is treatment of cell walls with group C phage-associated lysin (Krause, 1957; Kantor & Cole, 1960). M-proteins are highly susceptible to proteolytic enzymes e.g. trypsin, pepsin. They are considered

to be alcohol soluble. Their isoelectric point has been defined as pH 5.3 (Lancefield & Perlman, 1952 a).

Studies on type 24 by Cayeux & Wahl (1964) showed that extraction by acid at 100°C fragmented the M-protein molecule into two parts each with different antigenic specificity in contrast to a single antigen obtained by acid extraction at 37°C. Wilson & Wiley (1963) demonstrated a type-specific fraction designated E in type 17 in close association with the M antigen; they suggested that this E antigen was a part of the M molecule which had been separated from it by acid hydrolysis and could be recognised only by immunoelectrophoresis. Fox & Wittner (1967) isolated a high molecular weight M antigen (Hi-M) of type 12 by extraction at alkaline pH at 37°C and a low molecular weight M antigen (Lo-M) by extraction in acid at 95°C. This Hi-M, which had more antigenic reactivity, when heated to 95°C at pH7 or 37°C at pH2, was partly converted to Lo-M. Moreover, Vosti & Johnson (1967) purified two fractions of low and high molecular weights from type 12 M-protein extracted by hot acid

and both fractions showed high antigenic activity. All these results signify one fact viz. that the M-protein can be split into at least two antigenic fractions.

Recent work of Fox & Wittner (1965) revealed a unique multimolecular structure of M-protein observed by gel electrophoresis in contrast to the homogeneity shown by analysis by ultracentrifugation and by immunodiffusion. The multimolecules of the M antigens of individual serotypes are identical in antigenic specificity but differ in molecular size.

The M-proteins are non-toxic. This fact was elicited during investigations of the antigenic properties of this substance in mice and rabbits (Stamp & Hendry, 1937; Hirst & Lancefield, 1939; Schmidt, 1957; Kantor & Cole, 1960). Kaplan (1958) showed that M-proteins injected in mice were deposited mainly in the endocardium of the heart, the glomerular tufts of the kidneys, the alveolar walls of the lungs and also in the reticulo-endothelial cells of the spleen, liver and lymph glands. These findings were confirmed by Miller (1961). All of these experiments have

established both antigenicity and non-toxicity in experimental animals. In man, administration of the M-protein showed no evidence of toxicity (Schmidt, 1960; Potter, Stollerman & Siegel, 1962) but induced type-specific antibody formation in non-immune subjects and gave a secondary response in patients known to have had experience with homologous types. However, Lawrence (1952) reported that intradermal injection of M-protein caused skin reactions in 36% of 293 adult patients presumably as a result of sensitization to streptococcal products during previous infections. The use of adjuvants with M-protein considerably enhanced their antigenicity; mineral oil adjuvant was used by Hayashi & Walsh (1961) in immunising rabbits with either M-protein or isolated cell walls. Fox (1964) and Fox & Wittner (1966) tested several adjuvants including Bordetella pertussis vaccine, sodium alginate, polyethylene glycol, dextran, mineral oil, peanut oil and aluminium hydroxide but only Bord. pertussis, mineral oil, peanut oil and aluminium hydroxide enhanced M-protein antibody response.

M-proteins are believed to be a major

virulence-factor in group A streptococci. This is clearly demonstrated by the fact that M-proteins counteract phagocytosis which, on the other hand, is enhanced by the presence of antibodies against these antigens. This is the basis for the bactericidal test described by Todd (1927 a and b) and modified by Kuttner and Lenert (1944). The association of M-protein with virulence is well documented by Lancefield (1962). Virulence may be lost with the loss of M-protein e.g. by repeated subculture in artificial media. Virulence is, to some extent, host-specific viz. virulent strains isolated from human patients are avirulent for mice; but strains rendered mouse virulent by passage through these animals still retain their virulence to man. This suggests that, although M-protein is a major virulence factor, other factors also play some role in virulence e.g. capsule (Foley & Wood, 1959). Finally, strains isolated from convalescent patients suffering from streptococcal infections may exhibit only small amounts of M-protein (Rothbard & Watson, 1948).

The antibodies to M antigens are protective

against their respective serotypes viz. they induce type-specific immunity (Wannamaker, Denny, Perry, Siegel & Rammelkamp, 1953; Denny, Perry & Wannamaker, 1957). The protective properties of these antibodies were demonstrated in experimental animals by many workers both actively and passively (Stamp & Hendry, 1937; Hirst & Lancefield, 1939; Lancefield & Perlman, 1952 a; Kantor & Cole, 1960; Hayashi & Walsh, 1961, etc.). Sera of patients suffering from group A streptococcal infections equally protected mice against experimental infections and exhibited bacteriocidal activity (Todd, 1927 b; Lyons & Ward, 1935; Kuttner & Lenert, 1944; Rothbard, 1945; Rothbard, Watson, Swift & Wilson, 1948; Lancefield, 1958, 1959; Maxted, 1956; Stoyanova, 1966). The long-chaining test has also been used to measure streptococcal type-specific immunity (Stollerman & Ekstedt, 1957; Stollerman, Siegel & Johnson, 1959; Dunlap & Harvey, 1967). Non-biological methods for the detection of the type-specific antibodies are also available by the application of the haemagglutination test (Holmes, 1958; Quinn & Lowry, 1963, 1967; Vosti & Rantz,

1964; Fox, 1964; Nishimura, Kumagai & Kobayashi, 1966). The latex agglutination test has also been employed (Molina & Saletti, 1961; Bray, Bass, Dodge & Travis, 1966; Brighton, 1967). Lian & Pierce (1961) used a bentonite flocculation test for the same purpose. (For a full discussion of these methods see the section on the diagnosis of streptococcal diseases).

In serological typing of group A streptococci, M-proteins are identified by a precipitin test with crude acid extracts (Lancefield, 1928 a & b) and absorbed typing sera (M antisera). This is carried out in capillary tubes (Swift, Wilson & Lancefield, 1943). Accurate differentiation and identification of the various M-type antigens is carried out by the indirect bactericidal test (Maxted, 1956; Lancefield, 1957, 1958).

(b) The T antigens. These are protein in nature and are type-specific. Unlike the M-proteins they are susceptible to hot acid but resist trypsin digestion and to a lesser extent pepsin digestion. Their extraction is, therefore, conveniently achieved by treatment of the cells with proteolytic enzymes at low temperatures

(Lancefield & Dole, 1946; Pakula, 1951); they are insoluble in alcohol and their isoelectric point has been defined as pH 4.9 (Lancefield & Perlman, 1952 a). They are strongly antigenic and in the rabbit induce agglutinating antibodies which, however, do not protect mice against infection with homologous streptococci. T antigens extracted with proteolytic enzymes precipitate with their homologous antisera (Lancefield & Dole, 1946; Pakula, 1951; McLean, 1953). They are also active in complement fixation and in inhibition of the corresponding T-agglutination reactions.

The serological classification of group A streptococci introduced by Griffith (1934) was established by means of slide-agglutination of intact cells. This reaction depends on the presence of the T antigen and hence it is now universal practice to use trypsinized cells for T-typing. By this method Griffith identified 30 types. At the present time over 50 types are recognised, but some of them (types 7, 16, 20, 21 and 45) have now been identified as members of groups C and G.

(c) The R antigens. So far these protein

antigens have been identified in a restricted number of types. The R antigen of type 28 is also found in types 2 and 48 (Lancefield, 1957) as well as in some strains of groups B, C and G (Marted, 1949; Lancefield & Perlman, 1952 b). The R antigen of type 3 has not been found in any other type (Lancefield, 1958). These antigens react in both precipitation and agglutination reactions but their antibodies are not protective. They resist tryptic digestion but not peptic digestion. They are more resistant to heat and acid than the T antigens and hence may be found in acid extracts.

(d) Other surface antigens. The B antigen (Hamblly, 1958) is another protein similar to the M-protein in that it is extractable with N/5 HCl at 100°C. It is also susceptible to tryptic and peptic digestion, but is lost during mouse passage. It is found in various types, but it is not type-specific. Its antibody is not bactericidal and its role in mouse protection is not known.

The F antigen (Wilson & Wiley, 1963) is an antigen found in some strains of serotype 17 but its antibody does not enhance bactericidal activity

of blood (see page 47).

3. The mucopeptide

This forms the rigid framework of the cell wall and is composed of alternating units of N-acetylglucosamine and N-acetylmuramic acid linked to a short peptide chain consisting of the amino acids alanine, glutamic acid and lysine in an approximate ratio of 3:1:1 together with smaller amounts of glycine (Krause & McCarty, 1961). The mucopeptide can be extracted either chemically by the action of enzymes such as Streptomyces albus enzymes, group C phage-associated lysin and egg lysozyme, or physically by sonic disintegration of the cell walls. Hot formamide (Fuller, 1938) removes all the group-specific polysaccharide from cells or cell walls and leaves behind the mucopeptide layer, which can then be solubilized by enzyme treatment (Krause & McCarty, 1961). Mucopeptides from various bacteria are antigenic and immuno-chemically related (Abdulla & Schwab, 1965 a & b; Karakawa, Lackland & Krause, 1966b). The specific antigenic moiety of mucopeptide in group A streptococci was attributed by Karakawa & Krause (1966) as a result of inhibition tests to the peptide part of the molecule and also in certain instances to the hexosamine

polymer. They concluded that immunization with mucopeptide would result in the appearance of antibodies directed against either antigenic determinant.

The mucopeptide-polysaccharide complex obtained as cellular fragments by sonic disintegration of cell walls induces a nodular skin reaction of a chronic nature when injected intradermally into rabbits (Schwab, Cromartie & Roberson, 1959; Schwab & Cromartie, 1960; Schwab, Gooder & Maxted, 1962). Cromartie & Craddock (1966) have also demonstrated cardiac lesions resembling those of rheumatic fever in mice after injection of this material. Abdulla & Schwab (1966) showed that injection of the mucopeptide without the polysaccharide produces an acute dermonecrotic reaction after 4 hr, but that the chronic nodular lesion which followed injection of cell wall fragments did not appear for 7 days. Cell wall antigens can be detected in the tissues long after their parent cells have disappeared, suggesting that they are released during disintegration of the streptococci in phagocytes (Ohanian & Schwab, 1967). Moreover, the intravenous injection of mucopeptide produces a pyrogenic response in rabbits together with a localized Schwartzman reaction (Rotta, 1967a).

4. Teichoic acids

These antigenic substances are not attached to the cell wall, but occur on the surface of intact cells. They are polyglycerophosphates which can be extracted by hot acid or formamide, trypsinization of the cells or extraction at pH 9 at room temperature (McCarty, 1959). McCarty (1959) demonstrated them in most haemolytic streptococci, in some of which (groups D and N) they form the group-specific antigen; also he demonstrated them in staphylococci and aerobic sporing Gram-positive bacteria but not in pneumococci, clostridia, corynebacteria or Gram-negative bacteria. A polyglycerophosphate (PGP) antigen in streptococci was also described by Stewart (1961). The biological significance of teichoic acids is not yet defined, and so far they play no role in the practical aspects of the diagnosis of streptococcal diseases.

B. The extracellular antigens

These are substances found in the supernatant broth of the culture, but the demonstration of their respective antibodies in human serum indicates that they are also produced by streptococci in vivo. It is possible that some of them play a part in the pathogenicity of the organisms either by contributing to tissue invasion or

by causing intoxication of the patient. In the following passages only those of diagnostic and epidemiologic importance will be reviewed in any detail.

1. The soluble haemolysins

These substances present in the culture supernate are lethal to experimental animals when injected intravenously as well as being haemolytic. The animal shows haemoglobinuria and intravascular haemolysis on post-mortem examination. These haemolysins are responsible for the β -haemolysis and consist of two distinct substances known as streptolysin O and streptolysin S (Todd, 1934, 1938, 1939).

(a) Streptolysin O. This lysin is oxygen-labile and thus rendered inactive and loses its haemolytic power on exposure to oxygen at room temperature. It can be reactivated by reduction with 0.1% sodium hydrosulphite. It is stable in the cold if protected from atmospheric oxygen. However, inactivation at 55°C is irreversible. It is produced by strains of groups A, C (human) and G streptococci, and when isolated it retains its antigenicity. The fact that it is susceptible to digestion by trypsin indicates its protein nature (Bernheimer, 1948). Streptolysin O forms

β -haemolysis more rapidly than streptolysin S, but only when the culture is grown anaerobically or in the depth of the medium. Strains of group A streptococci that fail to produce streptolysin O may occasionally be encountered (Herbert & Todd, 1944).

Streptolysin O combines with its antitoxin in both reduced and oxidised form. After infection, antibodies to it are formed; they can be measured by determining the amount of lysin neutralized by the serum (see page 43).

Streptolysin O has been incriminated by Todd (1942) in toxicity to leucocytes. Its lethal effect on intravenous injection might be due to its cardiotoxic action (Bernheimer & Catoni, 1945; Kellner, Bernheimer, Carlson & Freeman, 1956; Halbert, Bircher & Dahle, 1961).

(b) Streptolysin S. This is a lipoprotein and is oxygen stable, but very sensitive to acid and heat and may be stored satisfactorily only at -73°C . It is responsible for the haemolysis produced by colonies growing on the surface of blood agar. Its production is stimulated by ribonucleic acids (Bernheimer & Rodbart, 1948),

and it can be reasonably purified from the broth by fractionation with methanol under controlled conditions of temperature, pH and ionic strength (Cinader & Pillemer, 1950). Unlike the O lysin, the S lysin is produced by all Lancefield groups of streptococci and shows strict group-specificity. It is antigenic only when present in the organism but loses antigenicity when released in the free state. Weissmann, Keiser & Bernheimer (1963) reported that both streptolysins cause the release of lysosomal enzymes by the disruption of lysosomes of mammalian cells. It has been incriminated as a cause of renal tubular lesions during experiments on mice (Tan & Kaplan, 1962). However, streptolysin S plays no part in the serological tests used for the diagnosis of streptococcal infection.

2. The erythrogenic toxin

This toxin is produced by many strains of group A streptococci and by some strains of groups C and G streptococci. It is responsible for the skin rash in scarlet fever. The intradermal injection of the anti-toxin in patients with this disease leads to blanching of the erythematous rash (Schultz-Charlton reaction). On the other hand, the intradermal injection of the

toxin-containing filtrate in a dilution of 1:100 gives rise to an erythematous and oedematous reaction at the site of injection within 6-24 hr covering an area of more than 10 mm in diameter in non-immune persons (a positive Dick test). Immune subjects give a negative Dick test viz. no reaction at the site of injection, because the toxin is neutralized by circulating antitoxin.

Strains of group A streptococci which are not active producers of erythrogenic toxin can be induced to produce it by infecting them with temperate phages from toxin-producing strains (Zabriskie, 1964). About 80% of group A strains produce a serologically identical erythrogenic toxin, while the remaining 20% may produce one of two other serologically different toxins (Coffey, 1938).

3. Hyaluronidase

Streptococcal hyaluronidase differs from testicular hyaluronidase in that it breaks down hyaluronate to trisaccharides and not to disaccharides, and that it does not attack chondroitin sulphate. In addition to group A streptococci, it is also produced by certain members of groups B, C and G. The enzymes of the latter two groups are serologically distinct from

that of group A. The simultaneous production of this enzyme and its substrate hyaluronic acid by the same strain seems at first sight to be inconsistent. However, the production of capsules is prevented by the addition of hyaluronidase to the culture medium (McClellan, 1941). The enzyme appears late in the logarithmic growth phase (Pike, 1948) in contradistinction to the capsular material which appears during the early logarithmic phase and then decreases during the stationary phase. Furthermore, Hill, James & Maxted (1963) produced evidence that hyaluronic acid was shed into the medium at the beginning of the stationary phase and then depolymerised by hyaluronidase very slowly. The addition of hyaluronic acid to the culture medium stimulates the production of hyaluronidase (McClellan, 1941; Rogers, 1945).

Hyaluronidase is antigenic and stimulates antibody formation following streptococcal infection. This antibody specifically inhibits the action of hyaluronidase on hyaluronic acid. The antihyaluronidase must be distinguished from an acute phase non-specific inhibitor found in patients sera during the acute infection. This non-specific inhibitor acts in the presence of magnesium and is inactivated by heating the serum

to 56°C for 30 min.

4. Fibrinolysin (streptokinase)

This enzyme was first described by Tillett & Garner (1933). It is produced by almost all group A streptococci and some members of groups C and G, and occasionally by a few strains in groups B and F. There is wide variation in the amount of enzyme produced by different strains. The enzyme resists heating at 100°C for 50 minutes; it activates plasminogen, the precursor of the serum protease, which dissolves fibrin clot (Milstone, 1941; Christensen, 1945). This action may be inhibited by natural inhibitors in the plasma. In assaying the enzyme, this difficulty is overcome by heating the serum at 56°C for 45 minutes. Its assay is accomplished by adding a constant amount of a mixture of thrombin and fibrinogen to increasing dilutions of the enzyme and incubating at 37°C for 1 hour. The end-point is the highest dilution that gives lysis of the clot (Report, 1947).

The role of fibrinolysin in the pathogenesis of streptococcal infection is not known, but it seems to attack the fibrin barriers that help to localise the lesion. It is antigenic, and in convalescent

sera antibodies against it are detectable. Its antibody level in serum can be estimated by determining the highest dilution of serum that inhibits lysis of a clot by a standard dose of the enzyme.

5. Streptococcal proteinase

The studies of Elliot (1945) showed that this enzyme is best produced by group A streptococci grown in broth of pH 5.5-6.5. M-proteins are destroyed by it. However, the production of the enzyme is suppressed by mouse passage of the streptococcus. In general, little proteinase is produced between pH 7.0-8.0, which is the optimal pH for the growth of the organisms. Oxidation inactivates proteinase; it is most active at pH 8.0 and is destroyed by heating at 70°C for 30 minutes. Both proteinase and its precursor have been isolated in a crystalline form (Elliot, 1950). Crystalline precursor can be activated by treatment with trypsin in low concentrations or by the addition of reducing agents e.g. thioglycollic acid, cysteine hydro-chloride. In addition to M-proteins, proteinase also destroys fibrin, casein, gelatin, fibrinolysin and many other proteins, thus resembling papain and other related enzymes (Elliot, 1950). Like other proteolytic enzymes, proteinase causes tissue damage

in experimental animals (Kellner & Robertson, 1954), but whether it causes similar changes in natural infections is not known.

The enzyme is antigenic, and antibodies to it may be detected in the sera of patients by a haemagglutination test (Ogburn, Harris & Harris, 1958). The precursor possesses two antigenic components, one of which is identical with that of the proteinase (Elliot, 1950).

6. Deoxyribonuclease

This enzyme is also known as streptodornase. It attacks deoxyribonucleic acid and is produced by a wide range of group A strains (Tillet, Sherry & Christensen, 1948; McCarty, 1948). It is also produced by members of several other groups, notably groups C and G. Wannamaker (1958, 1962) reported four serologically different deoxyribonucleases designated A, B, C and D. They are inactivated by versene as well as by their respective specific antibodies. Activation of the enzymatic activity is effected by magnesium. The most commonly encountered enzyme of these four is deoxyribonuclease B, a fact which is consistent with the wide distribution of the corresponding antibody in the sera of patients. For this reason, it is

a useful substitute of ASO determinations for diagnostic purposes (Wannamaker & Ayoub, 1960). In streptococcal infections there is no evidence that it attacks the nucleic acids of the tissue cells, but it seems to attack nucleoproteins in the pus at the site of infection.

7. Diphosphopyridine nucleotidase (DPNase)

This enzyme attacks Coenzyme DPN liberating nicotinamide, and hence its other name nicotinamide adenine nucleotidase (NADase). Evidence of its presence in streptococcal filtrates and its activity on DPN was provided by the work of Carlson, Kellner & Bernheimer (1956, 1957). The enzyme is highly toxic for leucocytes when they ingest streptococci, and evidence for this leucotoxicity by DPNase was obtained by Wilson (1957) and by Bernheimer, Lazarides & Wilson (1957). DPNase is antigenic, and its activity is inhibited by convalescent serum. Discrepancies may be encountered in the detection of DPNase antibody due to the presence in the culture filtrates of an inactive form of the enzyme or a zymogen which combines with this antibody (Ayoub & Wannamaker, 1963). It is produced by streptococci of group A and of several other groups, including groups C and G.

In group A, its production seems to be associated with certain M-types (Lazarides & Bernheimer, 1957).

8. Amylase

This enzyme attacks starch, glycogen and amylopectin, and the presence of these substances in the medium enhances its production (Crowley, 1950, 1954). On the other hand, a starch-like carbohydrate may be formed by the same strains of streptococci under different conditions of culture (Crowley, 1955; Crowley & Jevons, 1955). Nothing is known about its antigenicity and immunising power.

9. Esterase

This enzyme attacks β -naphthyl acetate (Stock, Uriel & Grabar, 1961).

10. Lipoproteinase

This antigen is detected by serum opacity reactions (Krumwiede, 1954; Gooder, 1961). Another group A streptococcal factor responsible for opacity in aged sera has been described by Hill & Wannamaker (1968).

IV ANTIGENIC RELATIONS OF GROUP A TO OTHER GROUPS OF HAEMOLYTIC STREPTOCOCCI

The most striking of these relationships is in the chemical composition of the cell walls and group-specific polysaccharides of groups A, B, C, E and G, all of which contain L-rhamnose as a main monosaccharide (Cummins & Harris, 1956; Slade & Slamp, 1962; Krause, 1963). These similarities in chemical composition are responsible for cross-reactions between the group-specific polysaccharides of some of these groups viz. groups A and A-variant on one hand (McCarty & Lancefield, 1955; McCarty, 1956) and C and C-variant on the other hand (Krause & McCarty, 1962; Araujo & Krause, 1963; Krause, 1963). Group G was found to contain in addition to rhamnose, galactose and N-acetyl-galactosamine (Krause, 1963) but its antigenic determinant is believed to be the L-rhamnose monosaccharide unit (Curtis & Krause, 1964). Serological cross-reactions between groups A, C and G were reported by Cummins & Slade (1961).

As has been mentioned several times in the section on the extracellular products of group A streptococci, several of these products are also produced by strains of groups C and G and sometimes by members of other groups.

The streptolysin O, the erythrogenic toxin, hyaluronidase, streptokinase, deoxyribonuclease, are all produced by some strains in groups C and G and in some cases by group B streptococci. Hyaluronic acid capsules are also produced by group C streptococci.

Maxted (1949) reported the presence of type 28 R antigen in some strains of groups B, C and G. Recently, Maxted & Potter (1967) described three group G strains which had an M antigen indistinguishable from that of group A type 12 streptococci. Moreover, the type-specific antigens of group C are proteins but their relations with the M-proteins of group A streptococci have not yet been fully investigated.

The relationships between haemolytic streptococci may prove to be important in immunity against infection with these organisms. They may also be of great help in understanding the pathogenesis of the non-suppurative sequelae of streptococcal infection.

V CROSS-REACTIONS BETWEEN STREPTOCOCCAL ANTIGENS AND TISSUES

The occurrence of rheumatic fever and glomerulonephritis as sequelae to streptococcal infection is well established, but the pathogenesis and the actual mechanism of causation of these diseases is not yet known. In the efforts to find a satisfactory explanation, the role of auto-immunity and hypersensitivity induced by the infecting streptococci has been investigated by many workers. This involved a detailed study of the relation of streptococcal antigens to mammalian tissue, particularly the heart, kidney and skeletal tissue. Kaplan & Meyeserian (1962) demonstrated a cross-reaction between group A streptococci and heart tissue of a rheumatic patient by means of complement fixation tests and immunofluorescence. This was followed by the description of a cross-reactive antigen in the cell walls of group A streptococci. It was shown to be closely associated with the M-protein (Kaplan, 1963, 1964). Further confirmation came by the work of Kaplan & Suchy (1964) who showed that this cross-reactivity was not only given by streptococcal antisera but also occurred with antisera prepared primarily against mammalian heart tissue. Furthermore, Kaplan & Svec (1964)

detected in patients with suppurative and non-suppurative streptococcal diseases a streptococcal antibody which cross-reacted with human heart tissue. They found this antibody in 24% of patients with uncomplicated streptococcal infections but in the majority of those suffering from acute rheumatic fever, rheumatic heart disease or acute glomerulonephritis. The streptococcal antigens cross-reacted with mammalian antigens that were found mainly in the sarcolemma of heart and skeletal muscle and in the smooth muscles of blood vessels. Kaplan (1964) suggested the probable existence of more than one cross-reactive antigen in the streptococcal cell. This was confirmed by Lyampert, Vvedenskaya & Danilova (1966) who described four such antigens and suggested that the immunizing action of cross-reactive antigens depended on the presence or absence of immunological tolerance to them in the subject immunized.

However, progress in this field of study is rapid and more workers are involved. Zabriskie & Freimer (1966) studied another streptococcal antigen that showed a cross-reaction with the sarcolemma of muscle and gave good evidence that it was located in the protoplast membranes of all streptococci of group A but not of other groups. A further approach was made by Nakhla & Glynn (1967) who demonstrated another antigen in most of the strains of group A streptococci they studied, and one strain of group

G streptococci, and pointed out that it was distinct from the M-protein and probably a complex of protein, polysaccharide and lipid. They also isolated from human saliva a similar substance which gave the same cross-reaction with cardiac and voluntary muscle. Finally, Goldstein, Halpern & Robert (1967) reported the occurrence of a cross-reaction between the group-specific polysaccharide of group A streptococci and the glycoproteins of the heart valves.

To conclude, Kaplan's observations have been fully confirmed, but it seems to be too early to assume with certainty any major role played by these cross-reactive antigens in the pathogenesis of the sequelae of streptococcal infection.

VI LABORATORY METHODS FOR THE INVESTIGATION OF STREPTOCOCCAL DISEASES

The laboratory methods used for the diagnosis of the individual cases and for epidemiological investigations of infection with group A streptococci fall into three groups viz. the isolation of the organism, the detection of its type and the detection of an antibody response in the patient. Although the isolation and identification of group A streptococci plays an important part in the diagnosis of infection in the individual case, it is often not possible to be sure that the patient from whom the organism is isolated is suffering from clinical streptococcal disease unless there is a rise in serum antibody. Neglect to observe these precautions may lead to a gross overestimate of the incidence of primary streptococcal disease and to an underestimate of the frequency with which non-suppurative sequelae follow it. Typing methods are of little importance in the investigation of the individual patient, but are essential in most epidemiological studies.

A. Isolation and identification of group A streptococci

A good account of modern methods for the isolation and identification of group A streptococci is given by Williams (1958); since the present work is not connected

with this subject, they will not be discussed further.

B. Type identification of group A streptococci

As mentioned in a previous section, group A streptococci possess two main type-specific antigens viz, the M-protein and T antigen. Griffith (1934) classified these organisms by the slide-agglutination technique in which the T antigen was shown to play the main role. On the other hand, Lancefield (1941) introduced her scheme of classification according to the M-protein precipitation with the specific serum. In practice most cultures are T-agglutinable, but it is usually not possible to identify an M antigen in more than about one-half of cultures with current available antisera (Williams & Maxted, 1953; Williams, 1954, 1957; Parker, 1967). Therefore, Williams & Maxted (1953) adopted a combination of the two systems i.e. they typed their strains first by T-agglutination and then examined them with a limited selection of M antisera determined by the T-agglutination pattern. Strains agglutinated by some T antisera include only strains with a single M antigen e.g. T-type 1 strains include members only of M-type 1, but others, notably members of the T-agglutination complexes, include streptococci with several M antigens e.g. the 3/13/B3264 complex includes members of M-types 3, 13, 33, 41, 52 and 53. For a discussion of the advantages of the

two typing systems for different purposes see Parker (1967).

C. Serological tests of the patient's serum

Serological tests are essential aids in the confirmation of recent streptococcal infection or in establishing the diagnosis of sequelae such as rheumatic fever.

1. Antibodies to extracellular antigens

The most commonly used test for both diagnostic and epidemiological purposes is the estimation of ASO in sera. Antibody responses in patients to other extracellular antigens e.g. streptokinase, hyaluronidase, deoxyribonuclease, NADase (DPNase) are less frequently used.

The properties of streptolysin O have been discussed in some detail on p.27. For the determination of ASO, Williams (1958) recommended a modification of the method of Johnson (1955) based on that of Rantz and Randall (1945). In this modification, the titre expressed in Todd units (T.U.) is taken as the reciprocal of the highest dilution of serum that gives complete haemolysis in the presence of 1 T.U. of toxin. A convenient method which is less elaborate in serum dilution and more economic in the use of serum has been introduced by Gooder and Williams (1959). The end-point is the dilution of serum giving 50% haemolysis measured visually or by a colorimeter. This

method has been adopted in this work and will be described in detail in Part Two (page 83).

The antibody response to streptolysin O results generally from infection with group A streptococci and less frequently with groups C and G. ASO may be detected as early as one week after the onset of infection, and usually reaches its maximum level within 3 weeks and then starts to decline, but there are considerable individual variations. A negative ASO titre does not exclude infection, and observations have shown that up to 30% of patients may fail to produce antibody to streptolysin O. In the general population only 10-15% show high titres i.e. 250 T.U. and over; such high titres in normal individuals depend on several factors such as age, occupation, geographical location etc. So, higher titres are more frequent in children than in adults, and in adults in military service more than in civilians. Thus a significant rise in ASO titre is diagnostic of streptococcal infection. Furthermore, the presence of ASO at sufficiently high titres (500 T.U. per ml or over) in single specimens of sera may be taken as evidence of recent streptococcal infection (Wannamaker & Ayoub, 1960). Nevertheless, under

certain circumstances false high titres may occur e.g. in contaminated serum, or serum from patients with hepatitis which contains lipoprotein inhibitors of streptolysin O in large amounts. Cholesterol in normal serum levels does not inhibit the lysin. To remove lipoproteins absorption of the serum with e.g. dextran sulphate can be made before the test (Hälen, 1963). The streptolysin O should be used in the reduced form and tested against a standard serum of known titre in parallel with the sera being tested. Moreover, the titre may vary according to the system of dilution adopted (Wannamaker & Ayoub, 1960).

The magnitude of the ASO level also depends on various factors e.g. nature and severity of infection and the infecting strain. Rheumatic patients generally give more elevated titres than patients convalescent from suppurative streptococcal infections but this finding is by no means diagnostic of rheumatic fever, since the ASO response is only indicative of recent streptococcal infection. Other factors include previous experience of streptococcal infection which explains high initial titres in some individuals as secondary responses. The response in infants is poorer than in older children and adults. The use of drugs such

as cortisone and bactericidal antibiotics e.g. penicillin, may suppress the ASO response in patients treated with them.

Owing to the failure of some patients to give an ASO response tests for the determination of antibodies to some of the other extracellular antigens notably hyaluronidase, streptokinase, DPNase and DNase B are available and may be used in such circumstances. In fact the use of more than one test increases the proportion of patients giving high titres that are of diagnostic value in streptococcal diseases.

2. Antibodies to cellular antigens

The detection of antibodies to cellular antigens has not been widely practised, because, until recently, convenient and reliable methods have not been available. Most attention has been directed to antibodies to the M-protein, because these antibodies are believed to confer immunity, but some information is available about antibodies to the group-specific polysaccharide. The mucopeptide and teichoic acid (polyglycerophosphates) are shared by many Gram-positive bacteria, and antibodies to them are not necessarily strictly specific to group A streptococci. The T antigens show a wide range of cross-reactions among different serotypes and are not

thought to play a part in pathogenesis.

Tests for measuring group-specific antibodies are now available. Haemagglutination tests with tanned human group O erythrocytes have been employed by Schmidt & Moore (1965) and Goldstein & Caravano (1967). Streptococcal cell walls were also used in an indirect Coombs test for the detection of group A specific antibodies, and directly for the detection of group A-var antibodies by Karakawa, Osterland & Krause (1965). These methods will be discussed fully in Part Four in comparison with a latex agglutination test developed in this work.

Several methods have been used for the detection of M antibodies. The most important and most widely used is the bactericidal test (Todd, 1927 a & b). The original test of Todd has been modified by several workers (pages 18-19). The test depends on the enhanced bactericidal activity of the serum after streptococcal infection caused by the appearance of M-protein antibodies. In the direct test whole blood from convalescent patients is incubated with a streptococcus, and in the indirect test convalescent serum is added to blood from a non-immune individual and incubated with a streptococcus. The modification of the indirect

method described by Moxted (1956) has been used in this investigation, and will be described in detail in Part Two. The test is highly specific and sensitive, but it is also laborious and difficult to perform on a large scale. Man and monkeys are practically the only suitable source of blood for the indirect bactericidal test. The strains of streptococci should be of high virulence, and passage through mice or through blood may be necessary before use.

Another reliable test, which is also of biological nature, is the long-chaining test (Stollerman & Ekstedt, 1957). It depends on the formation of long chains by group A streptococci in the presence of the homologous antiserum. In the presence of normal rabbit serum or protein-enriched broth the chains are usually short and consist of 4-12 cocci. The number of cocci per chain may increase by up to ten times the initial number when the homologous serum is added. Each of fifty chains randomly chosen from different microscopic fields is examined, and the average number of cocci per chain is taken as the mean chain length. The result is expressed as the chain index, which is the ratio of the mean chain length of streptococci grown in homologous serum to their mean chain length when grown in the presence of normal rabbit serum. An

increase of three times in the chain length is indicative of the presence of the relevant type-specific antibody in significant amounts. However, long chaining may occur in the absence of M-protein and in the presence of R antigens when the organisms are tested in R antisera (Stollerman, 1967). Spontaneous long chaining may also occur in Todd-Hewitt broth without protein enrichment. In general, the test requires the use of a highly virulent strain and antiserum free from antibiotics and antiseptic preservatives. Furthermore, the pH of the medium must be adjusted between 7.5-8.0 before inoculation.

The third biological test for the M-protein antibodies is the mouse protection test. Mice are passively protected by the administration of antiserum and then challenged with a lethal dose of homologous streptococci. The limitations of this test include the difficulty of selecting strains of sufficient virulence for mice. Numerous mice are required for each test, and this results in an extravagant use of antiserum.

Non-biological methods for the detection of M-protein antibodies are also available. These employ carrier particles with adsorbed purified or partially purified antigen, which are used in

agglutination reactions. The first of these tests, which is widely used, is the haemagglutination test (Boyden, 1951). To facilitate adsorption of the antigen, erythrocytes are first treated with tannic acid. The technique has been adopted to detect M-protein antibodies by several workers referred to in page 18. Spontaneous agglutination of the cells is a major disadvantage, together with intra-species cross-reactions. Haemolysis of the red cells may occur if excess antigen or old cells are used.

To overcome these difficulties, inert non-biological particles have been recently introduced as antigen carriers in agglutination tests. Bentonite flocculation was described by Bozicevich, Bunim, Freund & Ward (1958) for the diagnosis of rheumatoid arthritis and was employed by Lian & Pierce (1961) to detect M-protein antibodies. The use of latex particles is more popular than bentonite. It was originally used in a test for the rheumatoid factor by Singer & Plotz (1956) and has since been extensively used for other serological tests e.g. in histoplasmosis (Carlisle & Saslaw, 1958) tuberculosis (Duboczy & White, 1966), and farmer's lung (Murray, Pepys & Brighton, 1967). Latex agglutination tests to detect M-protein antibodies were first introduced by Molina & Saletti

(1961). The method was also used by Bray et al (1966) and Brighton (1967) for the same purpose. For a specific reaction to be obtained in the latex agglutination test, an antigen should be used that is at least serologically pure. A fuller discussion of the test will be given below.

VII OBJECTIVES OF THE PRESENT INVESTIGATION

The detection of antibodies to the two main cellular antigens viz. the group-specific polysaccharide and the type-specific M-protein is a promising field of study, since it may lead to the development of simple specific techniques that can be used freely in various aetiological, epidemiological and clinical studies of streptococcal diseases. In the field of prevention, handy and accurate methods for the assessment of immunity are important and will be greatly needed if active immunisation is contemplated.

The use of carrier particles in an agglutination reaction was aimed at in this work. To develop such a method it was necessary to obtain reasonably pure antigens for adsorption on to inert, non-biological particles. For this purpose polystyrene latex particles from a commercial source were used. Therefore, with the pure antigens and the inert particles of non-biological origin it was possible to develop a latex agglutination test which provided a simple and sensitive method for the detection of antibodies to cellular antigens in human sera.

The latex agglutination test, therefore, has been applied for the detection of group-specific antibodies as part of this work in the first place. It has not been

used in this respect before. Purified extracts obtained by various standard methods have been employed for adsorption. The full account of the experiments is given in Part Two. The test is shown to be applicable in the case of groups other than group A streptococci.

The use of latex agglutination as mentioned above has already been adopted by several workers for the detection of the type-specific M-protein antibodies. In this work the same principles and procedures concerning the test are followed except that the M-protein fraction differs from fractions used by other workers in that it is derived from a supernate after precipitation of an acid extract with 60% ammonium sulphate and further purified by repeated precipitation at pH 5.5 and washing in absolute alcohol (see Part Three).

A comparison between this fraction and the conventional fraction precipitated by 60% ammonium sulphate is given. It has been tested against rabbit sera and sera from patients suffering from streptococcal diseases. Its specificity has been verified by indirect bactericidal tests.

All the experimental details of tests on both group-specific polysaccharide and M-protein will be given in Part Two. The results will be presented in Part Three, while a full discussion of the findings will follow in Part Four.

PART TWO

MATERIALS AND METHODS

Group-specific polysaccharides and type-specific M-proteins were extracted and purified from intact streptococcal cells or from cell walls. They were used for the detection of antibodies in sera from hyperimmunised rabbits and also in human sera collected from patients and controls by the use of a latex agglutination test which has been established by several experiments described below.

I MATERIALS

A. Cultures of streptococci

1. Cultures used for the extraction of group-specific polysaccharides

Strains of streptococci maintained in the Streptococcus Reference Laboratory, Colindale, London, were used for the preparation of group-specific polysaccharides:-

Group A: (i) Type 1, SF/130 glossy (100079)
(ii) Type 2/44/Rb4, N.C.T.C. 8322
(iii) Type 4, 4990, N.C.T.C. 8326
(iv) Type 6, T6 glossy, N.C.T.C. 8709
(v) Type 3, Richards, N.C.T.C. 8191
(vi) Type 12, SF/42 (100081)

Other groups: (vii) Group A-var., T27/32/3

(viii) Group B, O9OR, N.C.T.C. 9993
(ix) Group C, Chestle, N.C.T.C. 8543
(x) Group G, Valente, N.C.T.C. 9603
(xi) Group L, SHC/16, (10238)

2. Cultures used for the preparation of the type-specific M-proteins

Strains of group A streptococci of the following types maintained in this laboratory were used:-

- Type 1: SF/130/13, N.C.T.C. 8198
- " 2: T2/44Rb4 matt, N.C.T.C. 8322
- " 3: T3M, B930/24, (100064)
- " 4: 4990, N.C.T.C. 8326
- " 5: SB RSI. 776
- " 6: S43/78, N.C.T.C. 8302
- " 9: 101/4 matt, (100067)
- " 12: R53/1077 Nephritogenic (100085)
- " 14: Lowe, N.C.T.C. 8199
- " 18: J17C, N.C.T.C. 8320
- " 22: T22/83/2 matt, (100071)
- " 25: T25/34 PZH small
- " 49: C300/1 and C274/1 (two strains)

In addition freshly isolated strains of group A types 4 (R67/1826), 12(R67/2531-Steve) and 49(R67/2327) were used as substitutes of stock strains of the same types which failed to produce sufficient amounts of M-proteins.

B. Sera used for detection of antibodies to cellular antigens

1. Antisera from hyperimmunised rabbits

These had been prepared in this Laboratory for the grouping and typing of streptococci. They were as follows:-

(a) Grouping sera. Grouping antiserum for group A streptococci was prepared by immunising rabbits with heat-killed trypsinised type 1, SF/130, cells. Also grouping antisera for groups A-var, B, C, D, E, F, G, H, K, L, M, N, O, P and Q were available. They were all prepared by immunising rabbits with heat-killed trypsinised cells except in the case of group B where formalised untreated cells were used, and groups M and O where heat-killed cells without trypsin treatment were used. Antiserum for group D was prepared by immunising rabbits with cells disintegrated in a Mickle's disintegrator.

(b) Typing sera. Type-specific antisera for typing of group A streptococci by M-precipitation were prepared by immunising rabbits with untrypsinised culture of M-types 1, 2, 3M, 3R, 4, 5, 6, 9, 12, 14, 15, 18, 19, 22, 23, 24, 26, 28R, 29, 30, 46, 48, 49 and 51. The group-specific

antibodies were removed by absorption with type 6 glossy (but antisera for type 6 were absorbed with type 44 cells). Special absorptions were carried out on certain antisera viz. types 9, 15 and 49 were re-absorbed with type 11 cells, type 3R antiserum with type 3M cells and vice versa, type 18 antiserum with type 36 cells, type 22 with type 11 and type 13 cells, and types 24, 26, 29, 46 and 48 with type 4 cells.

(c) Unabsorbed typing sera. These were prepared as in (b) with the same strains but no absorption was carried out and therefore they contained the group-specific antibodies as well as the type-specific antibodies.

(d) Normal rabbit sera. Sera from 3 unimmunised rabbits were pooled and used as negative controls in the serological tests.

2. Human sera

Considerable difficulty was experienced in obtaining suitable sera from patients who had suffered from streptococcal infections. When a bacteriological diagnosis of acute streptococcal infection is made, antibiotic therapy is usually begun immediately, and infection may be eradicated before an antibody response occurs; and when a streptococcal infection has remained

untreated sufficiently long for an antibody response to take place evidence of bacteriological confirmation is often lacking.

The following sera were available and were used for detection of group-specific antibodies, and in some cases, also for type-specific antibodies. The ASO was detected in nearly all of them.

(a) Sera sent from Public Health and hospital laboratories in south-east England for the estimation of ASO from patients thought to be suffering from a streptococcal infection or one of its sequelae. In each case the sender had been circularised and asked to confirm that the case was of suspected streptococcal infection and to give the evidence upon which the conclusion was based and the supposed date of onset. The sera included were all from cases where such confirmation was obtained, or which the information submitted with the specimen left little doubt that a clinical diagnosis of streptococcal infection, rheumatic fever or acute glomerulonephritis was justified. It was clear from the replies that nearly all of the sera had been collected often in the second week of the supposed infection or later, and often very much later. In the latter

case, however, the exact date of onset was seldom determined accurately. Group A streptococci had seldom been isolated during the acute stage of the infection.

The 73 sera included single specimens from 61 patients and 6 pairs collected at intervals of 4 to 15 weeks.

(b) Sera from patients from whom group A streptococci had been isolated in the laboratory of Burnley General Hospital; the patients had been seen in consultation by the paediatrician and were seldom suffering from acute infection at the time. There were 3 paired and 11 unpaired specimens of sera in this group.

(c) Sera from young men in a closed institution who were suffering from an acute upper respiratory tract infection and from whom group A streptococci were isolated. These sera composed 25 paired specimens. The first sera of each pair were collected within 2 or 3 days of the supposed date of onset of the infection, and the second serum in each pair was collected 2 to 5 weeks later. All of these patients received antibiotic treatment as soon as streptococci were isolated.

(d) Five pairs of sera from American Indian

children with acute glomerulonephritis following skin infection with type 49 group A streptococci were kindly supplied by Professor L.W. Wannamaker, University of Minnesota, U.S.A.

Also one paired specimen was obtained from a worker in this Laboratory who had suffered from repeated throat infections and from whom type 12 group A streptococci were isolated during the most recent attack.

(e) Eighty-nine sera were obtained from specimens sent to the Wright-Fleming Institute for Wasserman reaction and were used as controls. Four sera from volunteers in this Laboratory were added to this group.

II METHODS

A. Preparation of streptococcal cells

1. Streptococcal cells used for the extraction of group-specific polysaccharides

Lyophilised cultures were resuscitated in Oxoid Todd-Hewitt broth for about 6 hr and used to inoculate 50 ml amounts of the same medium. After overnight incubation in air at 37°C the 50 ml cultures were tested for purity by subculture onto blood agar plates. Two-litre amounts of Oxoid Todd-Hewitt broth were then inoculated from the 50 ml cultures and incubated in air at 37°C for 48-72 hr and then killed by heating in a water-bath at 60°C for 30 min. Thereafter the bottles were left overnight at room temperature, and next morning the deposits were collected after removal of the supernatant broth by a suction pump, and were washed thrice in sterile normal saline. These deposits provided intact cells which were either used as such or further degraded into cell walls where needed.

2. Streptococcal cells used for the extraction of type-specific M-proteins

Oxoid Todd-Hewitt broth was used with added neopeptone to give a high yield of M-protein by inhibiting the production of proteinase in the culture

(Elliot & Dole, 1947). All the procedures followed for preparation of the cells for extraction were as described above. No cell walls were prepared.

B. Preparation of cell walls

For the extraction of group A polysaccharide cell walls were prepared either in a Mickle's disintegrator (Salton & Horne, 1951) or by means of an X-press (Edebo, 1960) as follows.

1. Mickle's disintegrator

Washed cells from 2-litre cultures were resuspended in 100 ml amounts of sterile distilled water. To each of the two Mickle's cups was added 10 ml of the suspension and an amount of No. 12 glass beads to form a third of the height of the mixture in the cup. A drop of octyl alcohol was added to the mixture to prevent frothing. Disintegration was allowed to continue for 40 min at maximum oscillation. Gram-stained films of the mixture were examined at the end of the period for the absence of Gram-positive cocci and for the presence of Gram-negative sheets of cell walls. Forty minutes was found to be a suitable period for effective disintegration. The cell walls were separated from intact cells and large particles by centrifuging the final disintegrated suspension at 2,500 r.p.m. for 20 min. The cell

walls were still suspended in the supernatant at the end of this time. To separate them from dissolved cellular material they were recentrifuged at 10,000 r.p.m. for 30 min. The deposit of cell walls obtained was washed twice in cold sterile saline and redeposited at the same high speed of centrifugation.

2. The X-press method

The principle of this method is that cells frozen at -25°C are driven through a hole a few millimetres in diameter at a pressure of 2000 atmospheres. This pressure transforms ice crystals from one form to another (ice I to ice III) thus reducing the volume by about 20%. Disintegration of the cells results from this change (Edebo, 1960, 1961). The process is carried out in a device that consists of a cylinder divided equatorially by a disc with a small central hole. This method was found to be lengthy and did not yield more bulk of cell walls than the Mickle's disintegrator. Since only a small deposit of cells from one batch of 2 li broth culture was disintegrated by this method, the resulting cell walls were added to the bulk of cell walls prepared in the Mickle's disintegrator.

C. Preparation of cellular antigens

1. Extraction and purification of group-specific polysaccharides

Intact cells or cell walls of streptococci were treated with one of the following methods for the extraction of group-specific polysaccharides:-

(a) Hot formamide. Extraction in hot formamide was carried out according to the method of Fuller (1938). The deposit from a two-litre broth culture either as intact cells or as cell walls was resuspended in 4 ml formamide. The suspension was heated at 160°C in an oil-bath for 15 min. Undissolved cells were deposited by centrifugation at 2,500 r.p.m. for 20 min and re-extracted in hot formamide. The crude extracts were pooled and 25 ml of acid-alcohol (0.5% HCl in absolute alcohol) was added to every 10 ml of crude extract. The precipitate was separated by centrifugation at 2,500 r.p.m. for 20 min and discarded; the supernatant fluid was treated by the addition of 50 ml acetone to every 25 ml. The precipitate formed after acetone treatment was collected and re-dissolved in buffered saline pH 7.2 (normal saline to which is added 0.005 M di-sodium hydrogen phosphate and the pH corrected to 7.2 by N HCl). The presence of the polysaccharide in this solution was detected by the ring precipitin reaction of

Lancefield (1933) with group antiserum. Thereafter it was purified by one of the following two methods: (i) two further treatments with acid-alcohol and acetone as described above, (ii) shaking in chloroform in the presence of amyl alcohol (McCarty, 1952). One volume of chloroform was added to 2 volumes of extract, followed by 1/5 volume of amyl alcohol. The mixture was shaken briskly by hand and allowed to stand for 5 min on the bench before being centrifuged at 2,500 r.p.m. for 20 min. The impurities collected at the interface between the chloroform and the extract. The extract was removed with a pipette, and the process was repeated until no more material formed at the interface. This was usually achieved after the third time. The addition of amyl alcohol was omitted in most occasions and its absence did not affect the purity of the product. Polysaccharide from group A type 1 streptococci was purified by both of the above methods, but polysaccharides from other types of this group (types 2, 3, 4, 6, and 12) and from other groups of streptococci viz. groups A-var, B, C, G and L were purified

only by repeated acid-alcohol and acetone precipitation.

(b) Streptomyces albus enzymes. The group-specific polysaccharide was extracted by digestion with Streptomyces albus enzymes according to the method of Maxted (1948). Cells from 2-litre culture were resuspended in 25 ml of the enzyme preparation and incubated in a water-bath at 56°C for 5 hr. The digest was then cooled and centrifuged at 2,500 r.p.m. for 20 min. The supernatant fluid was collected and dialysed against saline buffer pH 7.2 overnight in the cold. Then it was shaken in chloroform as described above to remove the proteins. The deproteinised extract was further purified by three acid-alcohol and acetone precipitations (see above). Deproteinisation was also carried out on another batch of the crude extract by precipitation in 50% saturated ammonium sulphate as follows:-

Saturated ammonium sulphate was prepared by adding 1,000 g of ammonium sulphate to one litre of distilled water. This was shaken thoroughly and left overnight at room temperature for saturation or alternatively it was heated in a water-bath at 50°C for 2-4 hr. 50% saturated

ammonium sulphate was made up by adding equal volumes of the saturated solution and distilled water. The pH was adjusted to 7.2 by adding 0.88 ammonia dropwise while the reading was made on a pH meter. The extract was dispensed in dialysis bags and immersed into the 50% ammonium sulphate solution. For every 10 ml of extract 100 ml of the solution was used. The dialysis was allowed to continue overnight in the cold. Thereafter, the precipitated proteins were deposited by centrifugation at 2,500 r.p.m. for 20 min. and the supernatant fluid containing the extract was collected and dialysed against four changes of distilled water in the cold to get rid of the ammonium sulphate. Further purification was carried out by acid-alcohol acetone precipitation as above.

(c) Hot acid. Intact cells from 2-litre culture were resuspended in 15 ml N/5 HCl and boiled in a water-bath. After cooling, neutralisation was carried out by means of N/2 NaOH with phenol red as indicator. The suspension was then centrifuged at 2,500 r.p.m. for 20 min. The supernatant fluid was collected while the deposited cells were re-extracted in the same way. The two supernatant fluids were pooled. Deproteinisation was initially

carried out in 50% ammonium sulphate as above. The supernatant fluid containing the extract was dialysed in distilled water in the cold and further deproteinised by shaking in chloroform. Purification was again carried out by repeated precipitations in acid-alcohol and acetone.

2. Extraction and purification of the type-specific M-proteins

Extraction with hot acid was carried out according to the method of Lancefield (1928 a) described in the preceding paragraph. After neutralisation, the supernatant fluid from two extractions was pooled and proteins were precipitated by two treatments with ammonium sulphate as follows: (a) the first precipitation was carried out in 30% ammonium sulphate by dialysis (30 ml saturated solution added to 70 ml distilled water and pH corrected to 7.2 by the addition of 0.88 ammonia). The precipitate formed after overnight dialysis was separated by centrifugation and designated P30 fraction while the supernatant was designated S30 fraction. P30 fractions were freed from ammonium sulphate by dialysis in distilled water and tested serologically for type-specific M-protein and group-specific polysaccharide (see below);

(b) the second precipitation was carried out on the S30 fraction; 60% ammonium sulphate was used (60 ml saturated solution and 40 ml distilled water and pH corrected to 7.2). After overnight dialysis the thick precipitate was collected and designated P60, while the supernatant solution was designated S60. Both were dialysed against distilled water to remove the ammonium sulphate. The P60 was the fraction which had been adopted as the type-specific M-protein in the first stages of this work (see results in Part Three). After dialysis in distilled water, the pH of the solution P60 was adjusted to 7.5 to dissolve any precipitated part of this fraction during distilled water dialysis. Then it was tested chemically and serologically (see below). These tests revealed the presence of group-specific polysaccharide, which was removed by washing the P60 fraction three times in 4 volumes absolute alcohol (Barkulis & Jones, 1957). The final P60 fraction was again tested serologically and chemically (see results in Part Three).

The S60 fraction was also cleaned from ammonium sulphate in distilled water. Its pH was re-adjusted to 7.5, and it was tested chemically and serologically. For details of further studies on this fraction see results in Part Three.

D. Chemical analysis

The chemical nature and purity of each of the two cellular antigens was investigated by the detection and estimation of its nitrogen content and its rhamnose content according to the following methods:

1. Estimation of nitrogen content

Nitrogen content of the extracts was estimated by a micro-Kjeldahl technique in which the ammonia was recovered in a Markham's apparatus (Markham, 1942; Kabat & Meyer, 1961). Duplicate samples of 0.25 ml of each extract were transferred to thick test tubes, and 1 ml of a digestion solution (concentrated sulphuric acid saturated with copper sulphate) was added to each; digestion was allowed to continue for 2 hr on an electric heater. The solution was cooled and transferred to the Markham apparatus. It was neutralised by 10 ml of freshly prepared 50% NaOH, and steam was bubbled through it. The steam was condensed in a cold-water jacket, and the ammonia solution was collected in a measuring cylinder. From each specimen 50 ml distillate was collected. A blank buffer control was also tested in duplicate.

The distillates were treated with 2 ml Nessler's reagent per sample. After development of the colour the optical densities of the distillates were measured

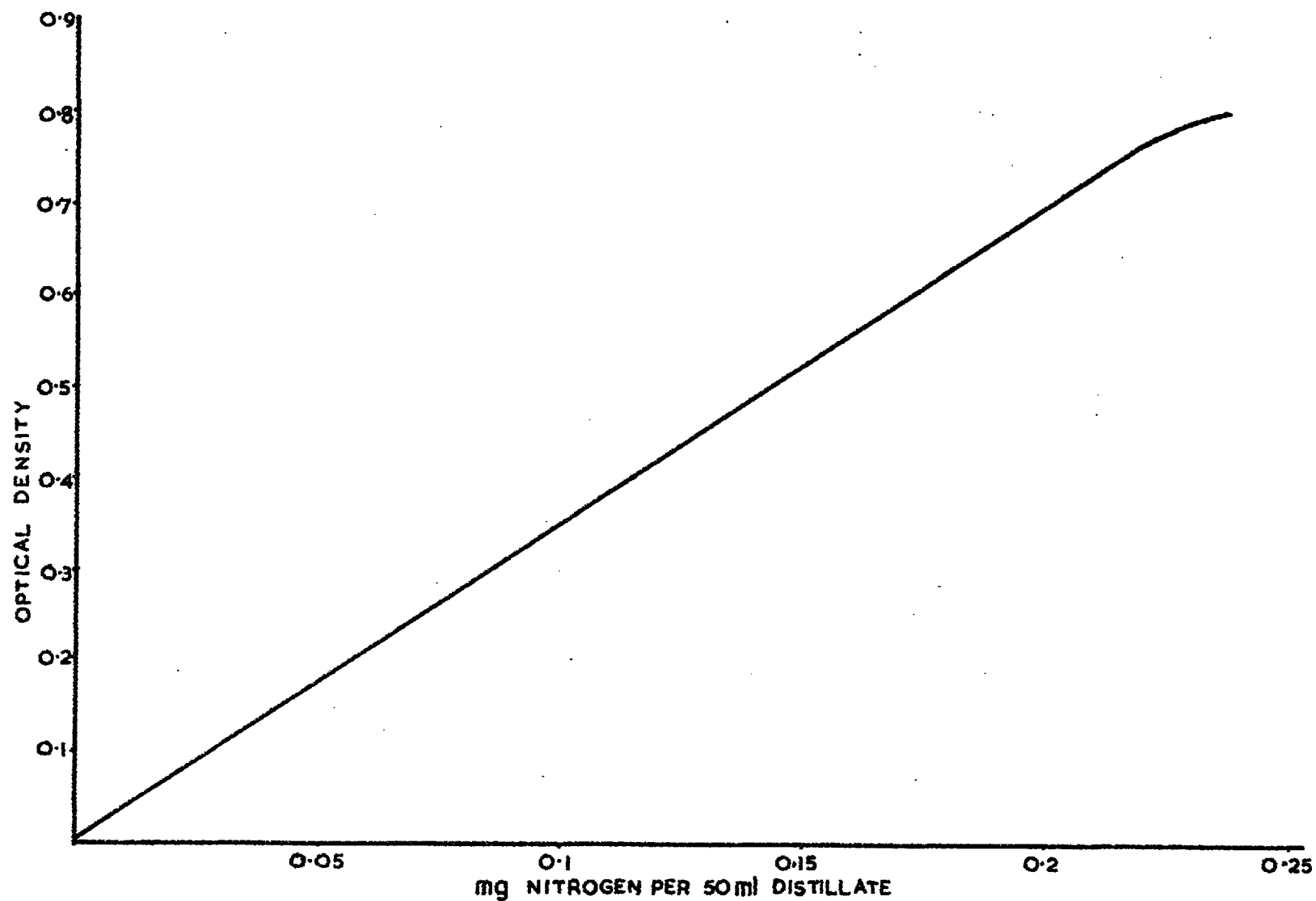


Figure 1: Calibration curve for nitrogen determination. (See text.)
This calibration is in use for routine work in this Laboratory.

in 1 cm cells in a S.P. 600 spectrophotometer, at a wavelength of 420 μ . The nitrogen content of each sample was read off a calibration of optical densities against the nitrogen content of 50 ml distillates prepared from known standard solutions (Figure 1). The average of the duplicate specimens was taken and corrected by subtraction of the value obtained for the control. This corrected value gave the estimated nitrogen content of the extract per 0.25 ml, and it was therefore multiplied by 4 to obtain the nitrogen content per ml. In polysaccharide preparations this was very small and could not result from gross protein contamination (see results in Part Three). In the case of M-proteins the values were higher and conversion to protein content per ml was made by multiplying each value X6.25. This conversion factor was obtained from Documenta Geigy Scientific Tables, page 126, 1956.

2. Estimation of rhamnose content

Rhamnose content was estimated by the cysteine-sulphuric acid reaction of Dische and Shettles (1948) as described by Dische (1955).

A preliminary test was carried out on known rhamnose solutions for the construction of a standard calibration curve. Solutions containing 40, 35, 30,

Table 1. The optical densities of known solutions of rhamnose measured by the cysteine-sulphuric acid reaction

Specimen No.	Rhamnose content	O.D. 396	O.D. 428	O.D. 396 - O.D. 428
1 (distilled water)	0	0.00	0.00	0.00
2	5	0.15	0.05	0.1
3	10	0.24	0.08	0.16
4	15	0.39	0.13	0.26
5	20	0.42	0.12	0.3
6	25	0.51	0.11	0.4
7	30	0.53	0.10	0.43
8	35	0.52	0.11	0.51
9	40	0.74	0.15	0.59

O.D. = optical density. 396 and 428 wavelength in μ

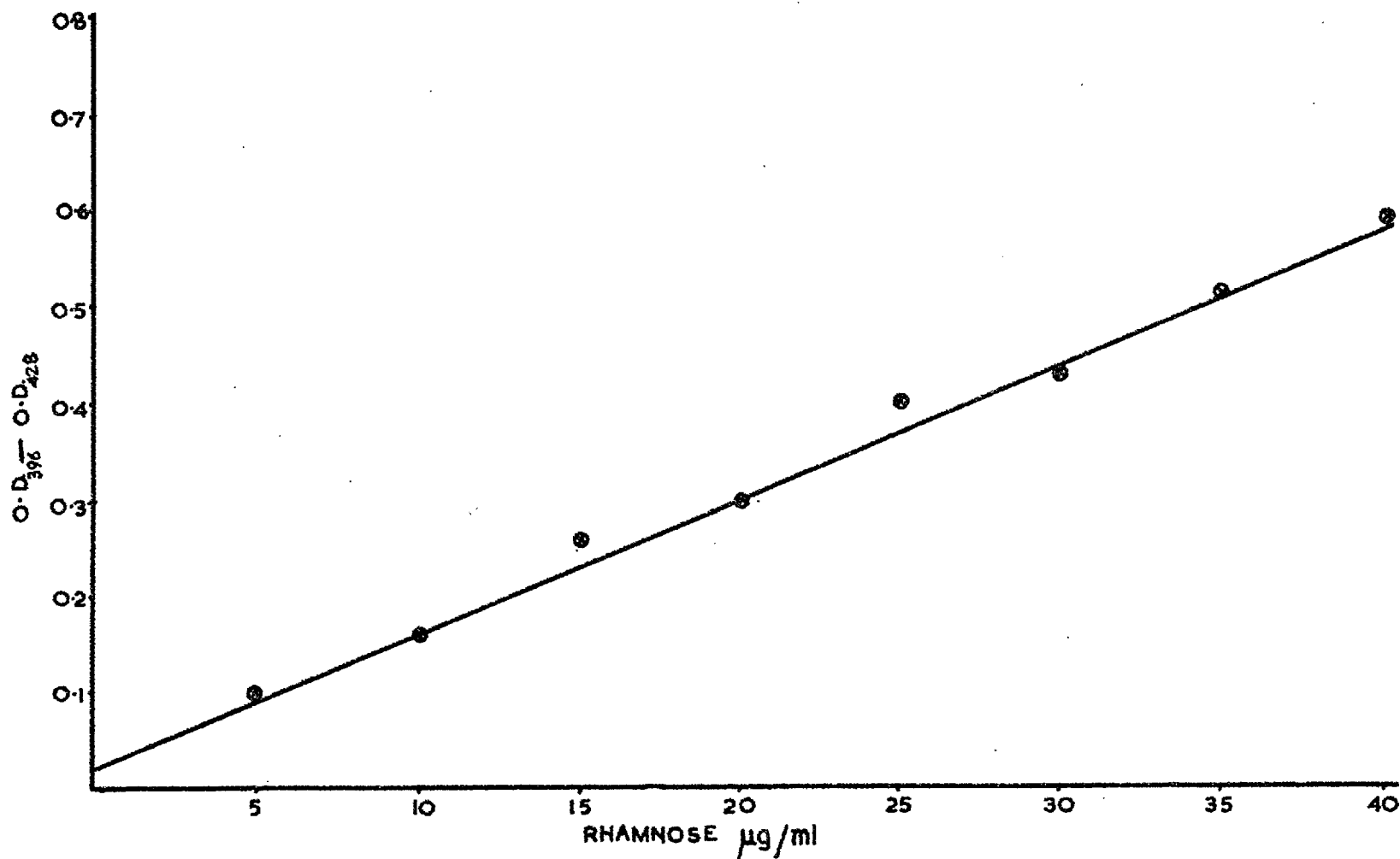


Figure 2: Calibration curve for rhamnose concentrations at values of O.D.396 minus O.D.428, after allowing 3 hr for the development of the colour (see Table 1).

25, 20, 15, 10, 5 μg per ml rhamnose were prepared. Volumes of 1 ml of each solution were transferred to thick-walled test tubes and mixed with 4.5 ml volumes of a mixture of 6 parts of concentrated H_2SO_4 plus 1 part of distilled water. After one minute the tubes were shaken briefly in ice water and then placed in tap water for 2 min and finally in boiling water for 3 min. Then, after again cooling in tap water, 0.1 ml volumes of 3% cysteine hydrochloride solution were added and the tubes were shaken. Colour was allowed to develop for 3 hr. Optical densities were read in an S.P.600 spectrophotometer at wavelengths 396 μ and 428 μ . A blank of distilled water was also included. The results were as shown in Table 1.

Both hexoses and rhamnose give coloured solutions in this reaction. The optical density given by hexoses is identical at wavelength 396 μ and 428 μ , but the optical density at 396 μ is greater than that at 428 μ with rhamnose. Therefore, rhamnose can be estimated by finding the difference between the optical densities at these two wavelengths. From the data in Table 1 the calibration curve in Figure 2 was constructed and used to work out the rhamnose content of the extract.

The extracts were tested by the same procedure.

Polysaccharide extracts were diluted to contain approximately 30 $\mu\text{g/ml}$ as rhamnose. This was estimated by calculating the total rhamnose content in the extract; the polysaccharide content of each extract was adjusted to 10 mg/ml , and therefore the rhamnose content should be 6 mg/ml (the rhamnose content of group-specific polysaccharide was reported by e.g. Krause (1963), to be 60%). The dilution of the polysaccharide extracts to contain 30 $\mu\text{g/ml}$ rhamnose was necessary because values of rhamnose higher than 40 $\mu\text{g/ml}$ gave non-linearity in the calibration curve constructed from testing the known solutions. For this reason, accurate estimations were only made with such dilute solutions. In all tests a solution of 20 $\mu\text{g/ml}$ rhamnose was included as a control. A blank control of distilled water was also included (see results in Part Three).

E. Serological tests

1. The ring precipitin test (Lancefield, 1933)

This test was performed in grouping pipettes by superimposing a drop of extract on a drop of antiserum. It was performed to detect group-specific polysaccharides in extracts with grouping antisera. A positive test is indicated by a ring of precipitate at the interface between the antigen and the antiserum. An immediate

reaction was graded as 4+, a reaction developing in the first min as 3+, in the second min as 2+, in 3-5 min as 1+, and after 5 min it was considered negative.

2. Capillary pipette precipitation test

Preliminary tests for M-protein in extracts were performed in capillary pipettes (Swift, Wilson & Lancefield, 1943). The antiserum was run in by capillary action making a column about 2 cm high in the pipette, and the extract was drawn up as a column of the same height after the serum. The pipettes were fixed upright on a plasticine holder and incubated at 37°C for 2 hr. A first reading of the reaction was made after incubation, but the final reading was made after overnight settlement in the cold. A thick precipitate rising to midway or more of the height of the serum-extract mixture was graded as 4+, $\frac{3}{4}$ of that was graded 3+, $\frac{1}{2}$ of that height was 2+, while $\frac{1}{4}$ was 1+. Less than 1+ was considered a trace reaction. Absence of precipitation indicated a negative result.

3. Double gel-diffusion tests (Ouchterloney, 1953)

Agarose gel on microscope slides was used for double gel-diffusion tests. The slides were cleaned

by boiling in a suitable detergent and rinsed three times in distilled water. They were dried with filter paper or clean towels. Then they were placed on a horizontal surface and a few drops of 0.8% agarose in buffer, pH 7.2, were spread on each slide and allowed to dry completely to form a base layer for the gel. Molten agarose was then transferred to the slides by a pipette as 2.5 ml volumes on each slide and allowed to set. To bore the holes in the gel a template was used together with a metal canula. Cut agarose was sucked by a suction-pump through a pipette.

The antigen was placed in the centre well and the antisera in the peripheral wells. The slides were kept in a moist chamber (a sandwich box with moist filter paper or cotton wool) overnight. Lines were inspected and either photographed or drawn.

4. Immunoelectrophoresis

A micro-technique in gel-slides was adopted according to the method of Grabar & Williams (1953) modified by Scheidegger (1955). The gel-slides were prepared as for gel-diffusion tests with 0.8% agarose dissolved in barbiturate buffer pH 8.5. The buffer was made up by dissolving 20.62 g sodium barbitone and 3.684 g diethylbarbituric acid in one litre of

distilled water, and the pH was corrected to 8.5 by the addition of N/1 HCl. The same buffer was also used in the electrophoresis tank. On each slide a centre trough and a hole on either side were made with a gel-punch. The antigens were placed in the holes and the slides were run at a constant voltage of 200 for varying periods (15 min to 45 min). After electrophoresis, the antisera were run into the troughs and the slides were left at room temperature overnight in a moist chamber. The lines were again recorded either by photographing or by drawing.

5. Latex agglutination test

This test was developed to measure antibodies to both group-specific polysaccharides and type-specific M-proteins. The biologically inert latex particles were used as antigen carriers. The procedures were as follows:-

(a) Latex particles. A commercial preparation of polystyrene latex particles, standard suspension of particle size 0.81 μ (Difco Bacto Latex 0.81) was used as antigen carrier. The suspension was stored at refrigerator temperature.

(b) Adsorption of antigen to latex particles.

To one volume of the standard suspension an equal volume of the appropriate antigen solution (for

suitable concentrations see results in Part Three) was added and mixed gently for 30 sec. Then the suspension was further diluted X50 and incubated at 37°C for 2 hr with polysaccharide extracts and 1 hr with M-protein extracts. After incubation, it was chilled to refrigerator temperature and merthiolate was added as preservative to a final concentration of 1:10,000.

(c) The agglutination test. Serial dilutions of the sera were made with buffer (phosphate buffer saline pH 7.2 described above) as 0.2 ml volumes in a clean W.H.O. haemagglutinating plate, each serum in one row. Two drops of the antigen prepared as in (b) above were added to each dilution with a 50-dropper pipette. The plate was thoroughly agitated to mix and left overnight at room temperature. In the morning the plate was again agitated and left for a further 2 hr. Then it was gently but firmly agitated forwards and backwards, and sideways. Agglutination was shown by the aggregated particles settling to the sides of the cup and rolling towards the bottom in ring formation. The intensity of the agglutination was graded as: 4+ curdy agglutination in clear fluid, 3+ coarse agglutination in clear

fluid, 2+ moderate agglutination in clear fluid and 1+ fine agglutination in partially clear fluid. In all tests, the last cup in each row of dilutions contained buffer and antigen only as control to exclude spontaneous aggregation. In subsequent tests a known positive serum was titrated with the test sera. Three normal rabbit sera were also titrated individually, but later they were pooled and used as a negative control. The reciprocal of the highest dilution of serum showing agglutination was recorded as the titre of that serum. When testing for group-specific antibodies in human sera it was noticed that the quality of agglutination reaction was improved when the plates were allowed to stand at refrigerator temperature for some time. Therefore, after reading the titres as previously described the plates were kept in the refrigerator until the end of the working day or until next morning. However, the end-point showed no change from the first reading.

(d) Cleaning of the plates. Spontaneous agglutination of the latex particles may occur if inadequately cleaned plates are used. Dried proteins from various sources e.g. serum, red

blood cells, micro-organisms, are the main sources of error. Another difficulty in obtaining good results is also the drying of the latex particles themselves. It is therefore of great importance not to allow used plates to dry up and they should be washed in running tap water cup by cup as soon as the results have been recorded. The washed plates are further cleaned by immersing in a suitable detergent solution overnight. In this work the detergent Labrite, obtained from British Hydrological Corporation, Deep Park Road, S.W.19, was used as a 1% solution in tap water. The plates are rinsed in running tap water cup by cup and then in distilled water. Immediate drying of the cups is achieved by inverting the plate and beating it against a clean towel on the bench several times followed by wiping the surface with the same towel.

6. Absorption tests

In a series of tests for establishing the specificity of the latex agglutination in detecting group A specific antibodies, group A antiserum was absorbed with homologous cells and also with heterologous cells of groups A-var, B, C, G and L. The cells were

prepared as previously described. They were further treated with crystalline trypsin to remove any surface proteins that might mask the group reactions. Trypsinisation was carried out by the addition of 0.5% solution of the enzyme as one volume to 20 volumes of the cell suspension. Incubation at 37°C was allowed for 2-3 hr. The cells were then washed and packed in Kahn test tubes by centrifugation to a height of 1 cm. The serum was added to an equal height and the cells re-suspended in it. Absorption was allowed to continue at 37°C for 2 hr and thereafter repeated once.

7. The indirect bactericidal test

A modification of the method described by Maxted (1956) was used.

(a) Sera. Both human sera and M antisera from hyperimmune rabbits were used. Antisera from rabbits were absorbed with homologous and heterologous extracts by precipitation in small tubes. Equal volumes of extract and serum were mixed, incubated for 3-4 hr at 37°C and the resulting precipitate was separated by centrifugation. Absorbed and unabsorbed sera were used as 1:2 and 1:10 dilutions. Human sera were used neat. The merthiolate preservative in rabbit sera was

neutralised by the addition of 1:50 thioglycollic acid (0.02 ml to 1 ml serum).

(b) Bacterial cultures. The M-strains (see above) were grown in 2-3 ml Todd-Hewitt broth with neo-peptone overnight. In the morning fresh amounts of broth were inoculated from the overnight culture and incubated at 37°C for 3 hr. Serial 10-fold dilutions of the young culture were made in sterile broth saline (5 ml Todd-Hewitt broth in 100 ml normal saline). The number of colony forming units in each broth saline was determined by Miles & Misra count.

(c) Blood. Human blood from a subject previously shown not to have bactericidal activity for the types under investigation was drawn aseptically in the required amount and mixed with heparin (10 units per 0.05 ml distilled water to every 1 ml of the blood). The blood was used immediately.

(d) The bactericidal test. Sterile freeze-drying tubes were prepared in racks and to each was added 0.3 ml of the heparinised human blood followed by a drop of the serum to be tested from a 50-dropper pipette. The appropriate culture dilutions are also added with a 50-dropper pipette as one

drop per tube. The tubes are then sealed in a bunsen flame and incubated at 37°C in a rotator which makes 5 revolutions per min. Subculture was carried out at the end of 3 hr by mixing 0.02 ml blood delivered by a 50-dropper pipette with 1 ml sterile saline in a Petri dish. Blood agar at 50°C was poured into the Petri dish and mixed well with the blood. After the agar had set, the poured plates were incubated at 37°C overnight, and the colonies were counted.

(e) Controls. The test included normal rabbit serum as a negative control and also some tubes were inoculated with the dilutions of the organisms but no serum of any kind was added. A heterologous organism was included in each test as a control.

8. Antistreptolysin O (ASO) estimations

ASO levels in human sera were estimated by the method recommended by Goeder & Williams (1959).

(a) General. The streptolysin O was an unreduced commercial preparation (Burroughs Wellcome). It was reconstituted in ASO buffer and reduced by 1:50 thioglycollic acid (1 part of the acid was mixed with 20 parts of buffer and 4% NaOH was added dropwise in the presence of a universal indicator (BDH) until the colour became blue-

green; the volume was completed to 50 parts by addition of buffer). The buffer (ASO buffer) was prepared by dissolving 1 g of bovine plasma albumen (Armour) in 500 ml of 0.85% NaCl; to this was added 270 ml of 3.12% $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ and 126 ml 7.6% $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, and the volume was completed to 1 l by the addition of distilled water. Its final pH was 6.3-6.5.

A standard antiserum was used for the standardisation of the streptolysin O. A dilution of 1:100 of the antiserum was made and from it further dilutions of 1:150, 1:200 and 1:250 were also made in eight sets as 1 ml volumes per tube. Each 4 sets were placed in a rack in order of dilution from left to right and in 4 rows from top to bottom. Streptolysin O dilutions 1:50, 1:45, 1:40 and 1:35 were added each in one row in both racks. The racks were kept in the refrigerator for 2 hr. Horse erythrocytes washed and standardised in buffer to 8% were added to each tube as 0.5 ml volumes. The tubes were incubated in a water-bath at 37°C for 30 min. Then they were centrifuged, and 1 ml volumes of the supernatant were added to 2 ml volumes of distilled water, and the haemoglobin content was read in the SP 600

spectrophotometer at 520 μ . A calibration for percentage haemolysis on the spectrophotometer scale was available. The titre of the antiserum for each dilution of the streptolysin was read off a standard curve for the 50% end-point. The streptolysin O dilution that gave the closest titre to 200 Todd units/ml was used for all ASO tests on patients' sera.

(b) Testing of the patients' sera. Each serum was diluted 1:50, 1:100, 1:200, 1:400 and 1:800 in ASO buffer in final volumes of 1 ml. Sera were inactivated for 30 min at 56°C. The reduced standardised streptolysin O was added to each tube as 0.5 ml (containing 1 unit of toxin) and mixed. The tubes were put in the refrigerator for 2 hr. Then 0.5 ml of the standard 8% red cells were added to each tube. The tubes were incubated in a water-bath at 37°C for 30 min shaking after 15 min. The approximate 50% end-point was located and each tube on either side of it was centrifuged. One ml of the supernatant was added to 2 ml of distilled water; the exact haemoglobin percentage was read in the spectrophotometer as above and hence the accurate ASO titre was determined.

PART THREE

RESULTS

I DETECTION OF STREPTOCOCCAL GROUP-SPECIFIC ANTIBODIES

A. Preparation of group A specific polysaccharide

Group A specific polysaccharide was extracted by the three main methods viz. acid extraction (Lancefield, 1928), formamide extraction (Fuller, 1938) and Streptomyces albus enzymes extraction (Maxted, 1948). The crude extract in each case was purified as described in Part Two (pages 65-69) and the following preparations were obtained:-

1. The formamide-extracted polysaccharide

Extraction in hot formamide was carried out on intact killed cells, as well as on cell walls, of group A type 1 streptococci (SF/130). Each crude extract was divided into two parts. One part was purified by two further treatments with acid-alcohol and acetone while the other part was purified by shaking in chloroform. In the end four purified preparations were obtained viz. two differently purified preparations from intact cells and two from cell walls. The concentration of polysaccharide in each preparation was adjusted as follows: poly-saccharide extracted from intact cells and purified

Table 2. Nitrogen content of formamide-extracted polysaccharides from intact group A streptococcal cells and from group A cell walls, purified by either acid-alcohol and acetone precipitations or by shaking in chloroform. The nitrogen was estimated by the micro-Kjeldahl technique.

Starting Material	Method of purification	Optical density *		Nitrogen content, mg per ml ++				Percentage Nitrogen
		Specimen 1	Specimen 2	Specimen 1	Specimen 2	Average of 1 & 2	Corrected average ②	
Intact cells	(a)acid-alcohol and acetone	0.05	0.049	0.052	0.048	0.05	0.042	3.3
	(b)chloroform	0.062	0.027	0.060	0.020	0.04	0.032	2.8
Cell walls	(a)acid-alcohol and acetone	0.03	0.04	0.028	0.042	0.035	0.027	2.7
	(b)chloroform	0.040	0.030	0.04	0.030	0.035	0.027	2.7
Control (Buffer)	-	0.000	0.002	0.000	0.016	0.008	-	-

* The optical density is that of 50 ml distillate treated with Nessler's reagent and read in the SP 600 spectrophotometer in silica cells and at wavelength 420 μ .

++ This is obtained by multiplying the value in 0.25 ml by 4 (see text).

② This is the result of subtracting the average nitrogen content in control buffer from the average nitrogen content of specimens 1 & 2.

by acid-alcohol and acetone precipitation was in a concentration of 12.5 mg/ml, while that purified by chloroform treatment was 12.0 mg/ml. Both extracts from cell walls were in concentration of 10.0 mg/ml. Before the estimation of nitrogen content was carried out dilutions of 1/10 were made from each preparation.

Each of the four polysaccharide preparations was examined chemically, and also immunologically to reveal the degree of its purity as a single antigen. The chemical tests included estimation of the nitrogen content and the rhamnose content. Immunological tests included the ring precipitin test of Lancefield (1933), gel-diffusion tests, and immunoelectrophoresis.

(a) Nitrogen content. Eight specimens were tested. They consisted of a pair from each of the four polysaccharide preparations. Two controls of buffer without polysaccharide were also added. The results of the readings of the optical densities and their corresponding nitrogen values obtained after testing the duplicate specimens and controls are shown in Table 2. The nitrogen contained in 0.25 ml of each dilute preparation was read off from the calibrations of optical densities against nitrogen content

of 50 ml distillate derived from the digestion of the preparation (Figure 1), allowance being made for the nitrogen content of the control buffer. The preparation from intact cells purified by acid-alcohol and acetone precipitation showed a nitrogen content of 3.3% while that purified by chloroform had a nitrogen content of 2.8%. Preparations from cell walls showed nitrogen content of 2.7%. McCarty (1952) obtained values of nitrogen content ranging from 3% to 5%, for polysaccharide extracted by Streptomyces albus enzyme digestion of cell walls. Schmidt (1952) obtained a nitrogen content of 2.7% in a group A polysaccharide extracted by a pepsin digestion method. Thus it would appear that simple shaking in chloroform or repeated acid-alcohol and acetone precipitation when applied on a formamide-extracted polysaccharide from intact cells or cell walls resulted in a reasonably pure preparation.

(b) Rhamnose content. It has been mentioned in Part Two (page 74) that to obtain accurate values of rhamnose its concentration in the preparation should not exceed 40 µg/ml because

Table 3. Rhamnose content of formamide-extracted polysaccharides from intact group A streptococcal cells and from cell walls, purified either by acid-alcohol and acetone precipitation or by shaking in chloroform. Rhamnose was estimated by the cysteine sulphuric acid reaction of Dische & Shettles (1948).

Starting Material	Method of purification	Optical density			Observed Rhamnose content $\mu\text{g/ml}$	Per cent Rhamnose content
		396 μ	428 μ	O.D.-O.D. 396 428		
Intact cells	(a)acid-alcohol and acetone	0.50	0.075	0.42	28.2	53.8
	(b)chloroform	0.60	0.125	0.48	32.7	54.0
Cell walls	(a)acid-alcohol and acetone	0.515	0.100	0.415	28.1	56.2
	(b)chloroform	0.50	0.09	0.41	27.5	54.1
Controls	1. 20 $\mu\text{g/ml}$ rhamnose	0.275	0.00	0.275	18	90
	2. Distilled water	0.000	0.00	0.00	00	00

greater concentrations give non-linearity of the curve. To achieve such a concentration each of the four preparations was diluted in distilled water to 1:200 thus giving polysaccharide concentrations of 62.5 $\mu\text{g/ml}$, 60.0 $\mu\text{g/ml}$, 50 $\mu\text{g/ml}$ and 50 $\mu\text{g/ml}$. The first two are the preparations from intact cells and the last two from cell walls (see above). The theoretical rhamnose content of these preparations calculated according to the approximate percentage of this sugar in group A polysaccharide (60% rhamnose) should, therefore, range from 30 $\mu\text{g/ml}$ to 37 $\mu\text{g/ml}$. A control rhamnose solution of 20 $\mu\text{g/ml}$ concentration was included in the test together with a distilled water control. The results are shown in Table 3. The observed value for the rhamnose content of each preparation in $\mu\text{g/ml}$ was read from the graph (Figure 2) against the relevant difference between optical densities at wavelength 396 μ and 428 μ . These values were used in the calculation of the percentages of rhamnose in the various preparations (Table 3). The percentage of rhamnose ranged from 53.8 to 56.2 and there was no significant difference between preparations from intact cells and those

Table 4. Ring precipitin tests with group A antiserum and formamide extracts prepared from intact cells and from cell walls of group A streptococci; the extracts were purified by either acid-alcohol and acetone precipitation or by shaking in chloroform.

Polysaccharide preparation	Antigen dilutions											
	Neat	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
Extract from cells												
(a) acid-alcohol & acetone purified	4+	4+	4+	4+	3+	2+	2+	2+	1+	-	-	-
(b) chloroform purified	4+	4+	4+	4+	3+	2+	2+	1+	-	-	-	-
Extract from cell walls												
(a) acid-alcohol & acetone purified	4+	4+	3+	3+	3+	3+	2+	-	-	-	-	-
(b) chloroform purified	4+	4+	4+	2+	2+	2+	2+	2+	1+	-	-	-

4+ : immediate strong reaction, 3+ : strong reaction within 30 sec., 2+ : moderate reaction, 1+ : weak reaction, - : no reaction.

from cell walls irrespective of the method of purification. Since the rhamnose estimations performed in this work appeared to underestimate the true rhamnose content by about 10%, the rhamnose content of the preparations of group A polysaccharide agree well with those reported by Krause (1963).

(c) Ring precipitin tests. Serial dilutions were made from each of the four preparations starting at 1:2 up to 1:2048. Each of these dilutions was tested by the ring precipitin test (Lancefield, 1933) against group A antiserum (grouping serum). The results are shown in Table 4. They all showed good reactions even at high dilutions. No reactions were given in normal rabbit sera.

(d) Double gel-diffusion tests. The test was carried out on agarose-gel slides. All polysaccharide preparations were tested against grouping sera of groups A to Q. In addition, group A-var antiserum, unabsorbed group A type 1 antiserum and absorbed group A type 1 antiserum (typing sera) were tested. A pool of three normal rabbit sera was included as control. A

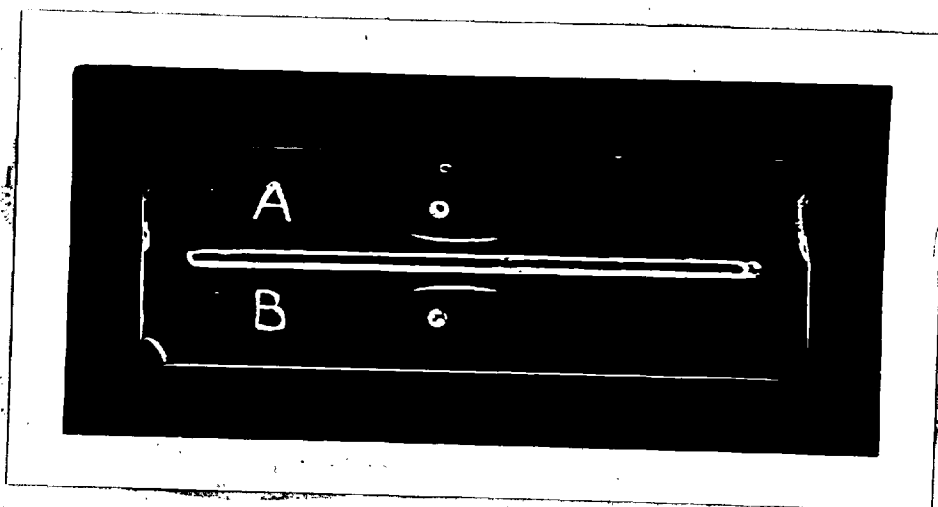


Figure 3: Group A polysaccharide extracted by hot formamide from type 1 cell walls and tested by immunoelectrophoresis with group A antiserum. Well A contained polysaccharide purified by repeated acid-alcohol and acetone precipitation, and well B contained polysaccharide purified by shaking with chloroform. The centre trough contained unabsorbed type 1 antiserum. A single line of precipitation, with negligible mobility, was given by each preparation after running at 200 volts for 45 min.

single line was given by the homologous group A antiserum (grouping serum) and also by group A type 1 unabsorbed serum but no reaction was seen with the group A type 1 absorbed or group A-var antisera. Furthermore, none of the heterologous group antisera gave any reaction with any of the four polysaccharide preparations.

(e) Immunoelectrophoresis. Group A specific polysaccharide is an inert substance which is electrophoretically immobile. To demonstrate this, each preparation was run at constant voltage (200 volts) for either 20 min or 45 min. This was followed by the addition of group A type 1 unabsorbed serum in the serum trough. Single arcs of precipitation were seen between the trough of the antiserum and the antigen wells (Figure 3). This test further confirmed the specificity and purity of these preparations by the absence of any second lines that could indicate the presence of any antigen of different electrophoretic properties.

In summary the group A polysaccharide purified from the formamide extract contained a minimal amount of nitrogen in agreement with results by other workers (see page 88) but a high rhamnose content. It was

Table 5. Nitrogen content of polysaccharide extracted from group A streptococcal cells by treatment with Streptomyces albus enzymes and purified by either shaking in chloroform followed by acid-alcohol and acetone precipitations or by precipitation with ammonium sulphate followed also by acid-alcohol and acetone precipitation.

Polysaccharide preparation	Optical density		Nitrogen content (mg per ml)				Per cent nitrogen
	Specimen 1	Specimen 2	Specimen 1	Specimen 2	Average of 1 & 2	Corrected average	
Preparation purified by chloroform and acid-alcohol-acetone	0.055	0.07	0.56	0.064	0.06	0.052	5.2
Preparation purified by 50% ammonium sulphate and acid-alcohol-acetone	0.055	0.05	0.056	0.042	0.049	0.041	4.1
Buffer control	0.035	0.035	0.008	0.008	0.008	-	-

also serologically pure and specific as demonstrated by ring precipitin tests, gel-diffusion tests and immunoelectrophoresis.

2. Polysaccharide extracted by Streptomyces albus enzymes

Cells of group A type 1 streptococci were treated with Streptomyces albus enzymes as described in Part Two (page 67). The extract was purified by two methods viz. one part was first shaken in chloroform several times and then further purified by acid-alcohol and acetone precipitation three times; the other part was first precipitated with 50% ammonium sulphate and then purified with acid-alcohol and acetone three times. Both fractions were tested chemically for nitrogen content and serologically by ring precipitin tests, gel-diffusion tests and immunoelectrophoresis.

(a) Nitrogen content of enzyme-extracted polysaccharide. The concentration of the polysaccharide in the two differently purified preparations was adjusted to 10.0 mg/ml. Again the same micro-Kjeldahl technique was employed. As before, dilutions of 1:10 (1 mg/ml) were used for the test as 0.25 ml in duplicate for each preparation as well as the buffer control. The results are shown in Table 5. The nitrogen content of the

Table 6. Ring precipitin tests with group A antiserum and polysaccharide extracted by Streptomyces albus enzymes from group A streptococcal cells and purified by either shaking in chloroform followed by acid-alcohol and acetone precipitation, or by precipitation in ammonium sulphate also followed by acid-alcohol and acetone precipitation.

Polysaccharide preparation	Degrees of reactions given by antigen dilutions:											
	Neat	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
Chloroform, acid-alcohol & acetone	4+	4+	4+	4+	4+	4+	3+	3+	2+	2+	-	-
Ammonium sulphate acid-alcohol & acetone	4+	4+	4+	4+	4+	4+	4+	2+	2+	2+	-	-

purified enzyme-extracted group A polysaccharide ranged from 4.1% to 5.2%. This is a higher value than that for the purified formamide-extracted polysaccharide. Chemical tests to determine rhamnose content were not carried out.

(b) Ring precipitin tests. Testing of a range of serial dilutions of the two preparations against group A antiserum revealed high reactivity of polysaccharide extracted by Streptomyces albus enzymes. Table 6 shows the details of the results. Good reactions were given at dilutions higher than those of the formamide-extracted polysaccharide (cf Table 4). The tests were repeated against normal rabbit serum but no reactions were given.

(c) Immunoelectrophoresis. This test was carried out in the same way as for the formamide-extracted polysaccharide. The preparations were run at 200 volts (constant voltage) for periods of 20 min only. Group A antiserum and group A type 1 unabsorbed antiserum were tested against both preparations. In all occasions two distinct arcs were given by each extract. The first arc showed no mobility and was the same as that obtained for the formamide-extracted polysaccharide.

Table 7. Summary of the chemical and serological properties of the various polysaccharide preparations extracted by the three main methods from group A type 1 streptococci.

Polysaccharide preparation	Test				
	Nitrogen content (%)	Rhamnose content (%)	Ring precipitin tests	Gel-diffusion tests	Immuno-electrophoresis
Formamide-extracted polysaccharide	2.7-3.3	53-56	reactions good up to dilutions 1:128 to 1:256	single lines with homologous sera	single immobile line
<u>Streptomyces albus</u> enzymes-extracted polysaccharide	4.1-5.2	N.D.	good reactions up to dilutions of 1:512	N.D.	2 lines: one immobile second is mobile
Acid-extracted polysaccharide	3.6	N.D.	good reactions up to dilution of 1:32 only	single lines with homologous sera	N.D.

N.D. = Not done.

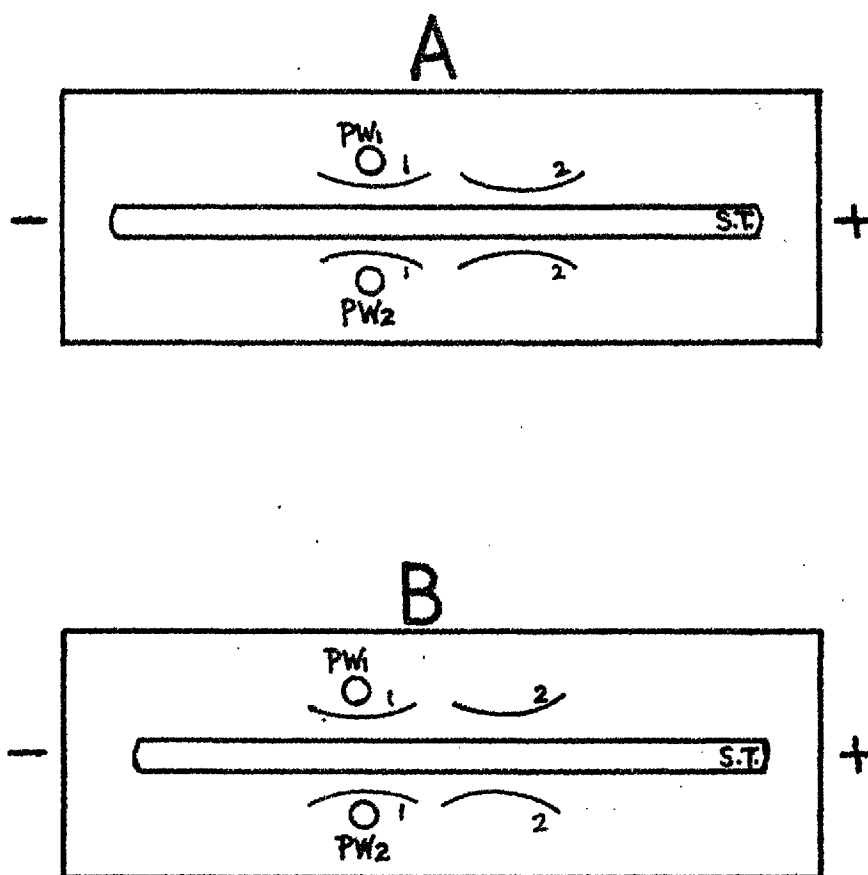


Figure 4: Group A polysaccharide extracted by Streptomyces albus enzymes and tested by immunoelectrophoresis.
P.W. = well containing the polysaccharide.
S.T. = trough containing group A antiserum.
In both preparations A and B P.W.1 contained polysaccharide purified by ammonium sulphate, while P.W.2 contained polysaccharide purified by chloroform. In preparation A, S.T. contained group A antiserum (grouping serum). In preparation B, S.T. contained group A type 1 unabsorbed serum. Line 1 represents the polysaccharide reaction and line 2 represents the mucopeptide reaction.

The second arc was quite mobile and its movement was directed towards the positive pole (Figure 4). Since Streptomyces albus enzymes also attack the mucopeptide and expose the peptide moiety which is the antigenic determinant of that substrate (Karakawa et al, 1966b), this second arc is presumably due to the mucopeptide.

3. Polysaccharide extracted by hot acid

Lancefield's hot acid extraction was carried out on cells of group A type 1 strain SF/130. The extract was purified by first precipitation of the proteins in 50% saturated ammonium sulphate. The supernatant fluid was freed from this salt by dialysis in distilled water and then shaken in chloroform for further deproteinisation. It was then further purified by treatment with acid-alcohol and acetone. The nitrogen content of this extract after three acid-alcohol and acetone treatments was found to be 3.6%. No estimation of rhamnose content was made. Ring precipitin tests showed good reactions up to a dilution of 1:32 of the extract. Gel-diffusion tests showed no apparent cross-reactions.

A summary of the properties of the three polysaccharide extracts is given in Table 7. The formamide-

extracted polysaccharide was, therefore, the most chemically pure and serologically specific of the three extracts.

B. The latex agglutination test on rabbit antisera

Each of the preparations of polysaccharide was then adsorbed to latex particles in suspension and used as antigen. The following account deals with results obtained after testing different polysaccharide preparations. Hyper-immune rabbit sera prepared against group A and other groups were used all through these tests.

1. The use of latex particles coated with group A specific polysaccharide

The procedure of adsorption of the polysaccharide to the latex particles has already been described in Part Two (Page 77). In general, the method described by Molina and Saletti (1961) was adopted. A screening experiment was first made by adsorbing each of the four preparations of the formamide-extracted polysaccharide at concentrations of 10 mg/ml to latex particles and testing them against a group A antiscrum. A suspension of latex particles in buffer in the same final dilution was included as control to exclude agglutination in the absence of the polysaccharide. The control latex suspension gave no agglutination reaction in the test sera. Strong agglutination

Table 8. Latex agglutination test on group A antisera (grouping serum) by the use of latex particles sensitised with each of the four preparations of formamide-extracted polysaccharides.

Polysaccharide preparation adsorbed to latex particles	Agglutination reactions in serum dilutions									
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	NRS 1:10	Saline buffer
Extracted from cells										
(a) acid-alcohol & acetone purification	4+	3+	3+	2+	2+	2+	2+	1+	-	-
(b) chloroform purification	4+	3+	3+	2+	2+	2+	1+	-	-	-
Extracted from cell walls										
(a) acid-alcohol & acetone purification	4+	3+	3+	2+	2+	2+	-	-	-	-
(b) chloroform purification	3+	3+	3+	3+	3+	3+	1+	-	-	-
Buffer control without polysaccharide	-	-	-	-	-	-	-	-	-	-

4+ : curdy coarse agglutination in clear fluid, 3+ : coarse agglutination in clear fluid, 2+ : moderate agglutination in clear fluid, 1+ : fine agglutination in more or less clear fluid, - : no agglutination.

reactions were given by each of the antigens against the serially diluted serum to titres varying from 320 to 640 (reciprocals of dilutions) as shown in Table 8. These results strongly suggested the validity of the adsorption of the polysaccharide on the latex particles and their possible use in a specific agglutination test to detect antibodies to this antigen in hyperimmune rabbit sera. The next step was to determine the most suitable concentration of the antigen that could be adsorbed to latex particles so that they agglutinated effectively in homologous sera.

(a) Determination of a suitable concentration of polysaccharide for adsorption to latex particles

One of the four preparations (polysaccharide from formamide-treated intact cells, purified by acid-alcohol and acetone treatment) was diluted serially from 1:2 to 1:64. Since the initial concentration was already adjusted to 10 mg/ml, subsequent doubling dilutions contained, 5, 2.5, 1.25, 0.6, 0.3 and 0.15 mg/ml approximately. Latex particles were coated with each preparation. Reasonably good agglutination was given by suspensions prepared from polysaccharide at concentrations of 0.6 mg/ml and higher but with greater dilution of the material the reaction was very weak. However, the strongest agglutination was given

Table 9. Determination of a suitable concentration of group A specific polysaccharide to be adsorbed on to latex particles for use in the latex agglutination tests.

Concentration of group A specific polysaccharide	Degree of reaction in serum dilutions									
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	NRS 1:10	Saline buffer
10 mg/ml	4+	3+	3+	2+	2+	1+	-	-	-	-
5 mg/ml	3+	3+	2+	2+	2+	1+	-	-	-	-
2.5 mg/ml	3+	3+	2+	2+	2+	±	-	-	-	-
1.25 mg/ml	3+	2+	2+	2+	1+	±	-	-	-	-
0.625 mg/ml	3+	2+	2+	1+	1+	±	-	-	-	-
0.3125 mg/ml	2+	2+	1+	1+	1+	±	-	-	-	-
0.15625 mg/ml	2+	2+	2+	1+	1+	±	-	-	-	-

N.B. For explanation of signs see footnote for Table 8.

by particles coated with the undiluted polysaccharide (10 mg/ml: see Table 9). All subsequent latex agglutination tests employing group-specific polysaccharide were therefore carried out with particles coated with preparations at concentrations of 10 mg/ml.

(b) Latex agglutination tests on homologous and heterologous rabbit antisera

To investigate the specificity of the latex agglutination test, further tests on group A antiserum (grouping serum), group A type 1 absorbed and unabsorbed antiserum (typing sera) and antisera to all other groups, including group A-var, were tested with latex suspensions prepared from the four formamide-extracted preparations, two enzyme-extracted preparations and a hot acid extract (see above). A pool of three normal rabbit sera was also tested as a negative control. Reactions at significant titres (640-1280) were given by all formamide-extracted preparations with group A antiserum and group A type 1 unabsorbed antiserum. Minor reactions were given by this preparation in group A type 1 absorbed antiserum, group A-var antiserum and group O antiserum. The enzyme-

Table 10. Agglutination by antisera of latex particles sensitised with group A polysaccharide prepared in various ways. The figures given are the reciprocals of agglutination titres.

Antisera	Formamide-extracted polysaccharide from intact cells & purified by acid-alcohol & acetone precipitation				Streptomyces albus enzymes-extracted polysaccharide purified by chloroform & acid-alcohol & acetone treatment		Acid-extracted polysaccharide purified by 50% ammonium sulphate, chloroform & acid-alcohol & acetone precipitation
	chloroform treatment	acid-alcohol & acetone precipitation	chloroform treatment	acid-alcohol & acetone precipitation	50% ammonium sulphate & acid-alcohol & acetone treatment		
Group A antiserum (grouping serum)	640	320	320	320	1280	1280	640
Unabsorbed group A type 1 antiserum (typing serum)	640	1280	640	640	10240	5120	1280
Absorbed group A type 1 antiserum (typing serum)	10	20	10	10	40	40	10
Group A-var antiserum	10	10	10	10	320	80	20
Groups B,C, D,E,F,G,H, K,L,M,N,P, O antisera	-	-	-	-	Group B: 10 " C:160 " E: 40 " G: 80	Group C: 40 " G: 10	Group C: 10 " G: 10
Group O antiserum	10	20	10	10	40	40	40
Normal rabbit serum	-	-	-	-	-	-	-

extracted preparations gave reactions with the homologous sera to higher titres (1280-10240) than did the formamide extracts, but they exhibited marked cross-reactions especially with group A-var. In addition, they showed cross-reactions with groups B, C, E and G (see Table 10). The ammonium sulphate treated enzyme-extracted polysaccharide had less cross-reactions than the extract deproteinised with chloroform. The acid-extracted polysaccharide also showed reactions with the homologous sera to the same level of titres as those with formamide extracts but exhibited some cross-reactions particularly with groups C and G. However, none of the preparations tested reacted to any degree in normal rabbit serum.

It is obvious from these results that the formamide-extracted preparations gave serologically more specific reactions and were more easy to purify than extracts made either with hot acid or with Streptomyces albus enzyme digestion. No further attempts were made to improve the purity of the latter two preparations and the formamide extracts were used in all subsequent experiments, including the testing of human sera.

Table 11. Reactions of formamide-extracted polysaccharides from group A streptococci of six different serological types, adsorbed to latex, with antisera to streptococci of homologous and heterologous groups.

Source of antigen	Group A type 1		Group A type 2		Group A type 3		Group A type 4		Group A type 6		Group A type 12	
Antiserum	Titre	P.R.	Titre	P.R.	Titre	P.R.	Titre	P.R.	Titre	P.R.	Titre	P.R.
Group A (1) (grouping serum)	640	4+	640	4+	640	4+	320	4+	640	4+	320	4+
Group A (2) type 1, unabsorbed	1280	3+	1280	3+	1280	3+	2560	3+	640	3+	1280	3+
Group A (2) type 1, absorbed with type 6 glossy (typing serum)	20	-	10	-	40	-	10	-	20	-	20	-
Group A-var (grouping serum)	10	-	10	-	10	-	10	-	10	-	10	-
Groups B, C, D, E, F, G, H, K, L, M, N, P & Q (grouping sera)	-	-	-	-	-	-	-	-	-	-	-	-
Group O antiserum (grouping serum)	10	-	10	-	-	-	20	-	-	-	-	-
Normal rabbit sera	-	-	-	-	-	-	-	-	-	-	-	-

P.R. = Precipitin reaction with undiluted serum.

- = less than 10

(1) = prepared with trypsinised vaccine.

(2) = prepared with untrypsinised vaccine.

(c) The use of formamide-extracted polysaccharide from group A streptococci of types other than type 1.

In this experiment group A specific polysaccharide preparations were obtained from strains of group A of types 2 (2/44/Rb4), 3 (Richards), 4 (4990), 6 (T6 glossy) and 12 (SF/42). Intact cells were extracted in hot formamide and the extracts purified by repeated acid-alcohol and acetone precipitations. Each preparation was adjusted in concentration to 10 mg/ml and adsorbed on to latex particles. These five preparations together with the preparation from group A type 1 cells (see above) were tested in parallel by the use of latex agglutination and the ring precipitin tests against group A antiserum (grouping serum), group A type 1 absorbed and unabsorbed antisera (typing serum) and antisera to all other groups including group A-var, together with a pool of normal rabbit sera. Similar reactions were given by all preparations (Table 11). Specific reactions were given with homologous sera. Cross-reactions with group A-var, and group O were also noted. In general the results given here were typical of the formamide-extracted preparations obtained above (compare Table 10). Thus, the

Table 12. Agglutination test with latex particles coated with group A polysaccharide and unabsorbed antisera of various types of group A streptococci.

Antiserum	Antiserum of type:												
	1	2	3M	3R	5	6	9	11	12	14	15	17	18
Latex agglutination Titre*	2560	<10	2560	160	-	320	160	2560	-	-	40	2560	2560
Precipitin reaction	3+	-	3+	1+	-	2+	1+	3+	-	-	-	3+	2+

Antiserum	Antiserum of type:										NRS
	19	22	23	24	25	26	28	Group A antiserum†	Absorbed type 10		
Latex agglutination Titre	2560	2560	2560	-	1280	-	20	640	10	-	
Precipitin reaction	2+	3+	3+	-	2+	-	-	2+	-	-	

* The titre is the reciprocal of the highest dilution of serum showing agglutination.

† Grouping serum

∅ Absorbed to remove group specific antibodies

** Normal rabbit sera (pooled)

- Less than 10

specificity of the latex agglutination test was not affected by extracting the group-specific polysaccharide from different types of group A streptococci.

(d) The detection of group A specific antibodies in unabsorbed sera. Unabsorbed sera were collected from rabbits immunised with untrypsinised cells of group A types 1, 2, 3M, 3R, 5, 6, 9, 11, 12, 14, 15, 17, 18, 19, 22, 23, 24, 25, 26 and 28. They were tested by latex agglutination and ring precipitin tests with a formamide extract of a type 1 group A streptococci that had been purified with acid-alcohol and acetone. Group A antiserum (grouping serum), group A type 1 antiserum (typing serum) and normal rabbit serum were included as controls. The results are shown in Table 12. Unabsorbed sera of types 2, 5, 12, 14, 24 and 26 were all negative for group-specific antibodies by both tests. The remaining sera were all positive by both tests and latex agglutination titres ranged from 160 to 2560. The control group A antiserum was positive, while the absorbed group A type 1 serum and the normal rabbit serum were negative as tested by both tests. These

Table 13. Agglutination of latex particles coated with group A polysaccharide in group A antiserum absorbed with streptococcus of homologous and heterologous groups.

	Group A specific antiserum absorbed with cells of					Group specific antiserum (unabsorbed) *
	A	A'	B	C	L	
Titre	10	320	640	640	640	640

* Grouping serum

A' = A-var.

sera had been prepared from M-types and contained variable amounts of group-specific antibodies as shown by the ring precipitin tests with the purified group-specific polysaccharide.

(e) Absorption tests. To investigate the specificity of the latex agglutination test further, samples of group A antiserum (grouping serum) were absorbed with homologous cells (group A type 1) and with the heterologous but immuno-chemically related groups A-var and C. Absorption with the unrelated groups B and L was also carried out. All the cells used for absorption were heat-killed and trypsinised. The latex agglutination test was carried out on each absorbed sample by the use of group A polysaccharide-coated latex particles. Table 13 shows the results of these absorption tests. The coated latex particles failed to agglutinate in significant titre in group A antiserum absorbed with the homologous group A cells. On the other hand they agglutinated in the samples of serum absorbed with heterologous cells and also in the control unabsorbed sample of group A antiserum.

Table 15. The group-specific antibody response in rabbits during the course of immunisation with group A vaccines. The latex agglutination test was used to detect both group A and group A-var antibodies in these rabbits.

Rabbit No.	Initial serum		1st serum specimen after start of immunisation		2nd serum specimen		3rd serum specimen		4th serum specimen		5th serum specimen	
	group A anti-body titre	group A-var anti-body titre	group A anti-body titre	group A-var anti-body titre	group A anti-body titre	group A-var anti-body titre	group A anti-body titre	group A-var anti-body titre	group A anti-body titre	group A-var anti-body titre	group A anti-body titre	group A-var anti-body titre
1	<20	<20	320	40	320	80	2560	160	5120	160	5120	160
2	<20	<20	40	<20	320	160	2560	160	5120	160		
3	<20	<20	160	40	320	80	640	80	1280	80		
4	<20	<20	80	40	160	40	320	40	320	40		
5	320	80	2560	320	2560	320	2560	320	2560	320	2560	320
6	320	40	2560	320	2560	640	2560	640	5120	640	5120	640
7	<20	<20	<20	<20	<20	<20	<20	<20				

Table 14. Detection of antibodies in homologous and heterologous group antisera with latex particles coated with polysaccharides of groups A, A-variant, B, C, G and L.

Group	Titre with homologous antiserum	Titres with heterologous antisera
A	640	A-var: 10, O: 10-20
A-var	320	A: 160, C: 80, G: 20
B	640	Nil
C	640	A-var: 20, G: 20
G	320	A-var: 20, C: 80
L	160	Nil

2. The use of the latex agglutination test to detect group A-var and other group-specific antibodies

Group-specific polysaccharides of groups A-var, B, C, G and L were extracted in hot formamide and purified by repeated acid-alcohol and acetone precipitations. Double gel-diffusion tests showed specific reactions with homologous antisera and no cross-reactions with heterologous antisera. Specific reactions were also given by these polysaccharides tested with the ring precipitin tests. The results in Table 14 were those given by the latex agglutination test of each polysaccharide. The homologous antisera gave high titres with their respective antigens. Cross-reactions were noted amongst groups A-var, C and G. On the other hand group A antisera gave high titres with group A-var antigen.

3. The use of the latex agglutination test in a study of the development of group A and group A-var antibodies in immunised rabbits

Sera from 7 rabbits were collected at weekly intervals. Initial sera before the commencement of immunisation were also tested. Table 15 shows the results of the study of the appearance and development of group A and group A-var antibodies. One of the rabbits (rabbit 7) was immunised with group B cells

and was, therefore, included as a control. Two other rabbits (rabbits 5 and 6) were receiving booster doses of group A vaccines of M-types 41 and 49 after a rest of three months. The remaining four rabbits, viz. rabbits 1, 2, 3 and 4 started their immunisation for the first time. The vaccines used for their immunisation were cells of group A streptococci of M-type 29 and T-types 14, 17 and 22 respectively. The boosted rabbits showed a typical secondary response for group A antibodies giving a rise from a titre of 320 to 2560 in the first week and continued to rise in the case of rabbit 6 to 5120. Rabbit 1 showed a dramatic rise in group A antibodies after the initial injection to a titre of 320 which reached its maximum after the third week. Another high titre, 160, after the first week was given by rabbit 3, the fourth specimen of which gave the maximum titre. Rabbits 2 and 4 showed low primary responses (titres 40 and 80) and again the maximum titre was reached after the third week. The appearance and development of group A specific antibodies was paralleled by a similar appearance and development of group A-var specific antibodies but at correspondingly lower titres as shown in Table 15, e.g. rabbits 2, 3 and 4 showed low titres after the

first week (20-40) which also reached their peaks after the third week but at a lower level than those of group A antibodies (titres 160-1280). Neither group A nor group A-var antibodies were detected in sera from the control rabbit 7 immunised with group B cells. Thus it was evident that both group A and group A-var antibodies were formed following immunisation of rabbits with group A cells alone and that their first appearance at detectable levels occurred in the second week after the initial dose of vaccine. The maximum titres were generally reached after the third week. These findings are in agreement with those of Karakawa, Lackland and Krause (1966_a).

C. The detection of group A and A-var antibodies in human sera by latex agglutination tests

The latex agglutination test for group A or group A-var antibodies was performed on all the human sera listed under Materials and Methods. In addition ASO tests were performed on all except one of the sera from patients suspected of suffering from streptococcal disease or its sequelae, and on all of the Wassermann test sera from which sufficient amounts were available for the test.

As stated on page 58, it had been found difficult to obtain a large collection of sera from untreated patients

Table 16. Results of latex agglutination tests on 147 sera from patients (including 30 "acute-phase" sera), and on 89 control sera (collected for the Wassermann test) for the detection of group A and group A-var antibodies.

Description of sera	No. of sera tested	Group A antibodies				Group A-var antibodies				ASO	
		% sera reacting at titres of				% sera reacting at titres of				% sera reacting at titres of	
		<1/20	1/20	1/40 & 1/80	1/160 & over	<1/20	1/20	1/40 & 1/80	1/160 & over	200 & less	201 & over
"Normal" controls (WR patients)	89	60.7	7.8	28.0	3.3	71.9	10.1	14.6	3.3	98 †	2 †
All sera from patients	147	7.5	3.4	16.4	72.5	11	7.5	20.6	60.3	48.3	51.7
"Suspected streptococcal sera" *	117	9.5	4.3	17.2	69.0	13.8	7.8	20.7	58	43.6	56.4
(a) Upper respiratory tract infection	68	10.3	3.0	16.2	70.6	11.8	8.8	20.6	58.8	50	50
(b) rheumatic fever	32	9.4	6.2	22	62.5	9.4	9.4	25	56.2	37.5	62.5
(c) acute glomerulonephritis	17	6	6	11.8	76.5	29.4	0	17.6	53	41.2	58.8

* excluding "acute-phase" sera.

† only 45 sera were tested.

who had undoubtedly suffered from group A streptococcal infection. There were 147 sera from patients for whom there was a strong clinical suspicion of streptococcal infection, but 30 of them were acute phase sera collected soon after the onset of illness and before an antibody response was to be expected (25 from young men in a closed group, and 5 from American Indians in Minnesota). The remaining 117 sera were taken to constitute a group in which there was a large proportion of established streptococcal infection; this group, which shall be referred to as "suspected streptococcal sera", included ASO sera and second sera from the closed group and from the American Indians; 68 were from patients who had had respiratory symptoms, 32 from patients with a provisional clinical diagnosis of rheumatic fever, and 17 from patients thought to have acute glomerulonephritis (Table 16). In all, the 117 sera came from 103 patients; it was considered justifiable to include pairs of sera from 9 patients, because none of the sera had been collected at the onset of disease.

1. Studies on the group antibody response in 147 patients' sera and 89 control sera

The latex agglutination tests carried out on all the specimens of sera from patients revealed the

presence of group A and group A-var antibodies in about 90% (Table 16). On the other hand, 60.7% and 71.9% of the control sera were negative (titres of less than 20) for group A antibodies and A-var antibodies respectively. Amongst the patients' sera only 7.5% were negative. When the acute-phase sera had been excluded the "suspected streptococcal sera" were found to give rather similar results to those found in all sera from patients with suspected streptococcal disease. This unexpected finding will be considered later, when the results obtained on the pairs of sera are given. The "suspected streptococcal sera" were studied in more detail and were subdivided into 3 groups; the first consists of 68 sera from patients with streptococcal upper respiratory tract infection, the second of 32 sera from rheumatic patients and the third of 17 sera from patients with acute glomerulonephritis (Table 16). They all showed the presence of group A and group A-var antibodies in 70% - 90% of cases.

High antibody titres (160 and over) were obtained in the majority of the patients' sera at proportions ranging from 53% to 76% (Table 16). Moderate antibody titres (40 - 80) were shown by 11% to 25%, while low

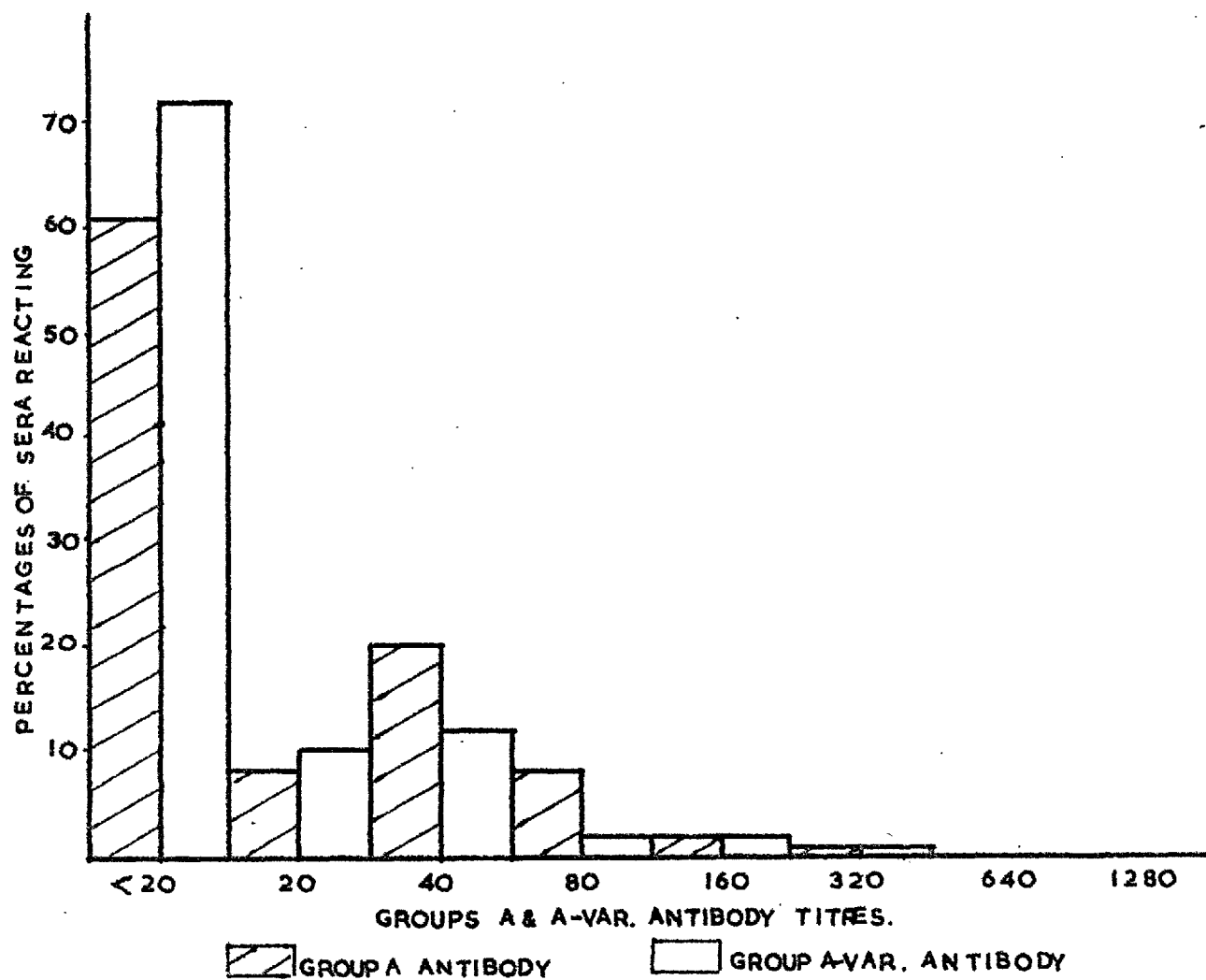


Figure 5: Titres of control sera tested by latex agglutination for group A and group A-var antibodies.

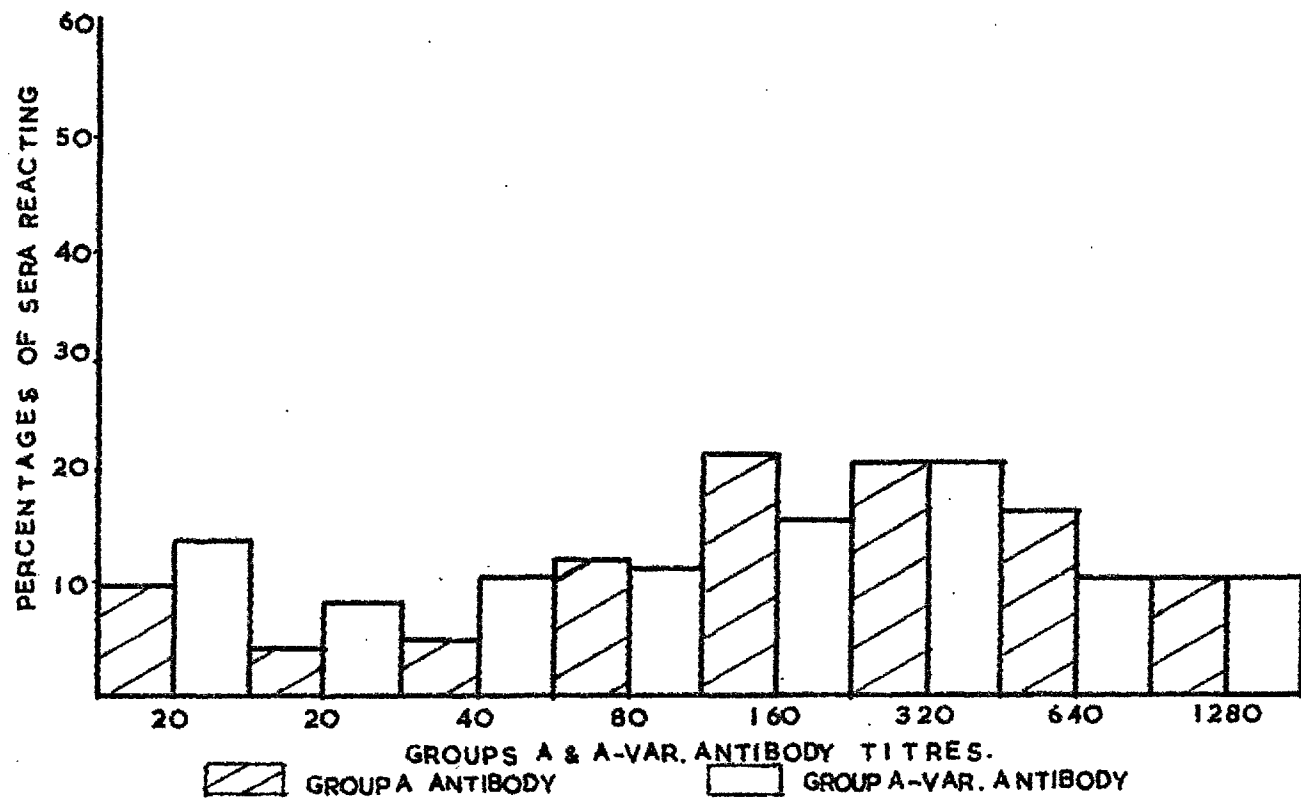


Figure 6: Latex agglutination titres of groups A and A-var antibodies in 117 sera of patients suspected of suffering from various streptococcal diseases.

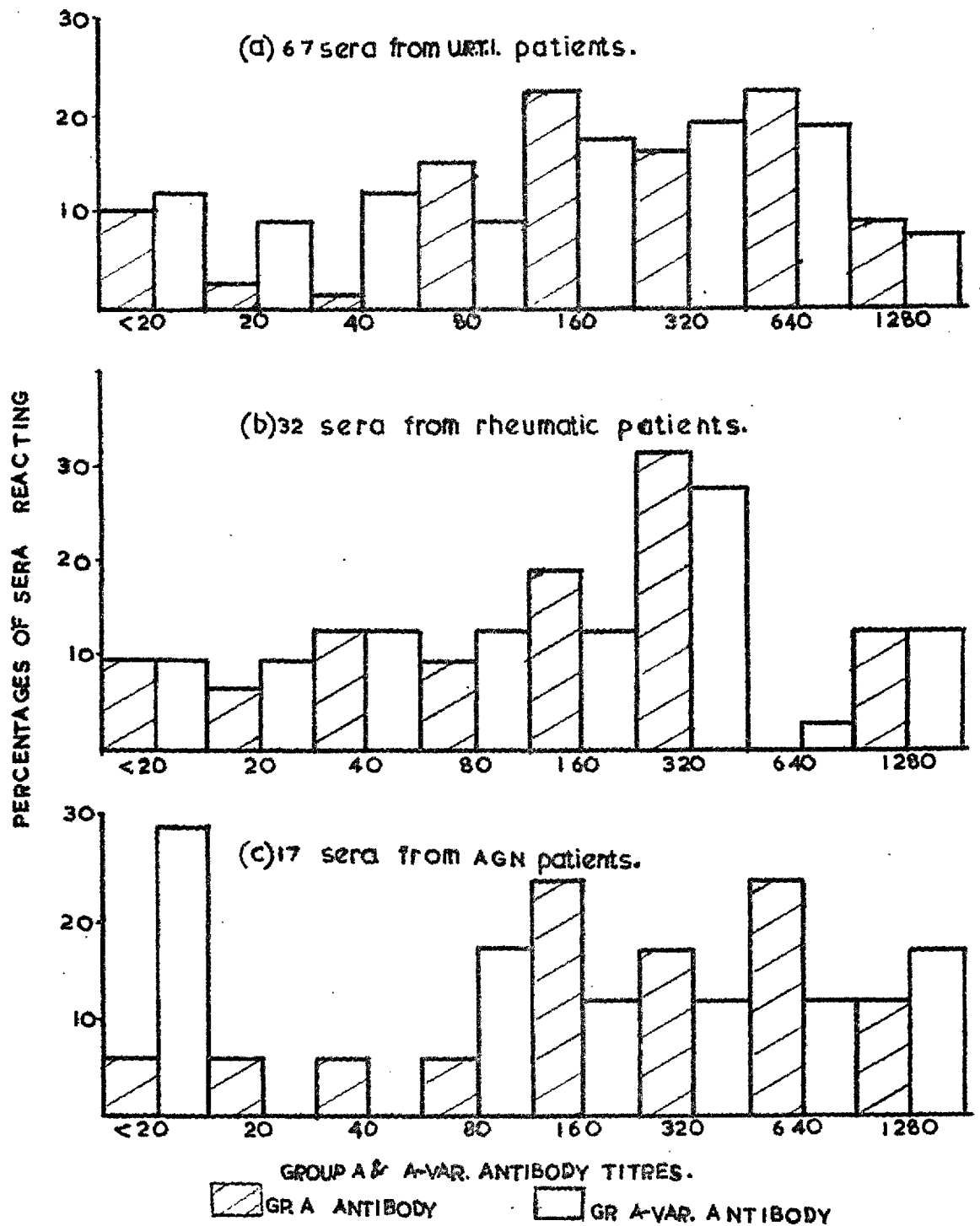


Figure 7: group A and A-var. antibody titres of sera from patients suspected of suffering from streptococcal U.R.T.I., rheumatic fever and acute glomerulonephritis tested by latex agglutination.

titres (20) were shown by 3% to 9% of the sera.

In contrast ASO titres of 200 T.U./ml or less were observed in 98% of the control sera while 40% to 50% of the patients' sera showed ASO titres of 201 T.U./ml and over.

It is convenient to consider and compare the results of the test and control sera in more detail. Results from control sera showed that most of these sera reacted to titres of 40 or less as illustrated in Figure 5 viz. the individuals with no apparent or suspected streptococcal infections either gave negative group antibody reactions or reacted to low titres but rarely to high titres. Figure 6 shows the magnitude of the reactions of groups A and A-var antibodies as titres plotted against the percentages of 117 sera from patients suspected to be suffering from streptococcal diseases. The majority of the sera reacted to titres from 80 to 1280 while the others gave titres of 40 or less. However, few sera (10%) gave titres of 1280, but none gave titres higher than that. Similar findings are shown in Figures 7a, b and c for sera from streptococcal upper respiratory tract infection, rheumatic and nephritic patients respectively.

Furthermore, ASO titres of control and patients' sera plotted in the same manner showed a similar

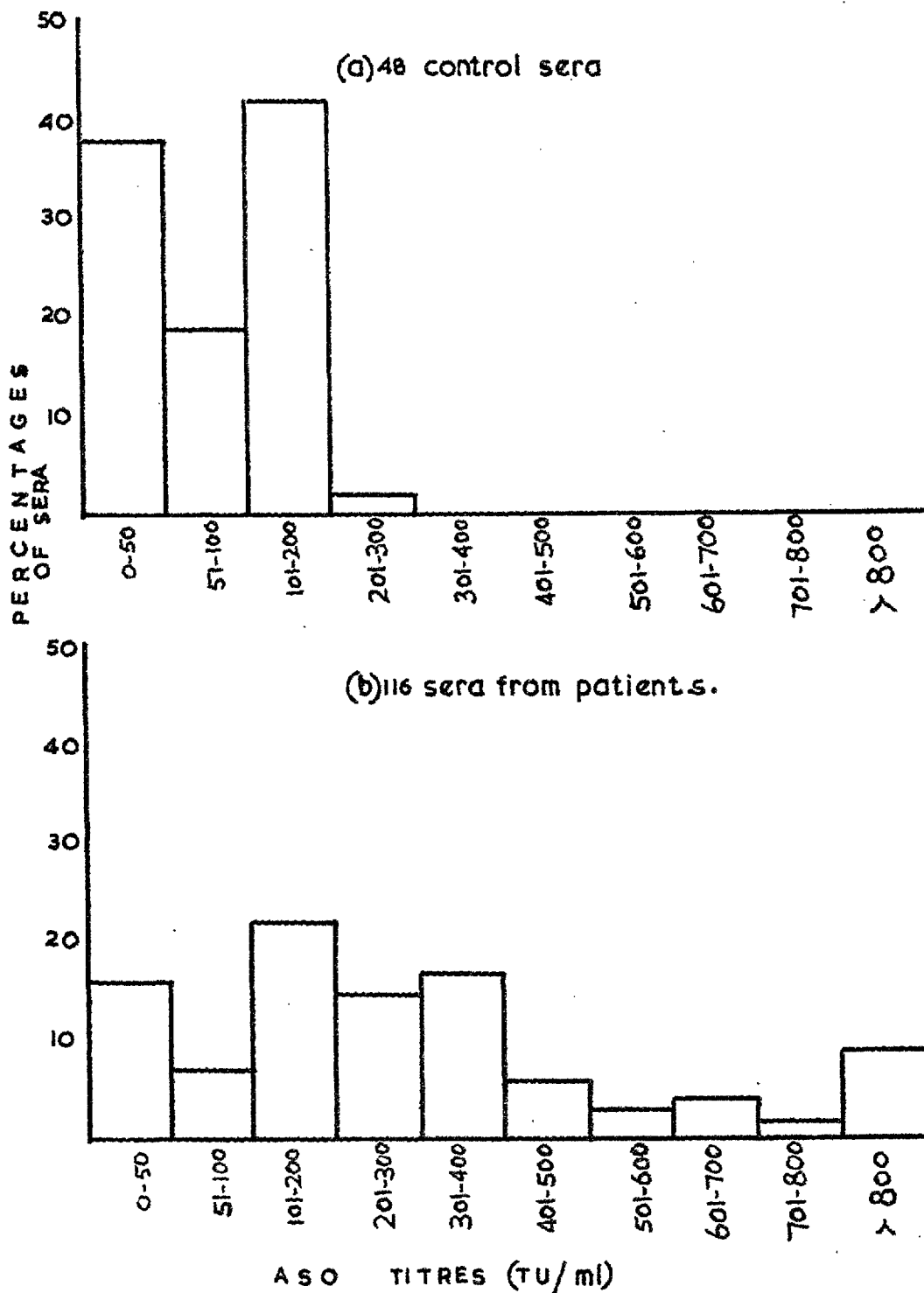


Figure 8: A.S.O. titres of control sera and sera from patients suspected of suffering from various streptococcal diseases.

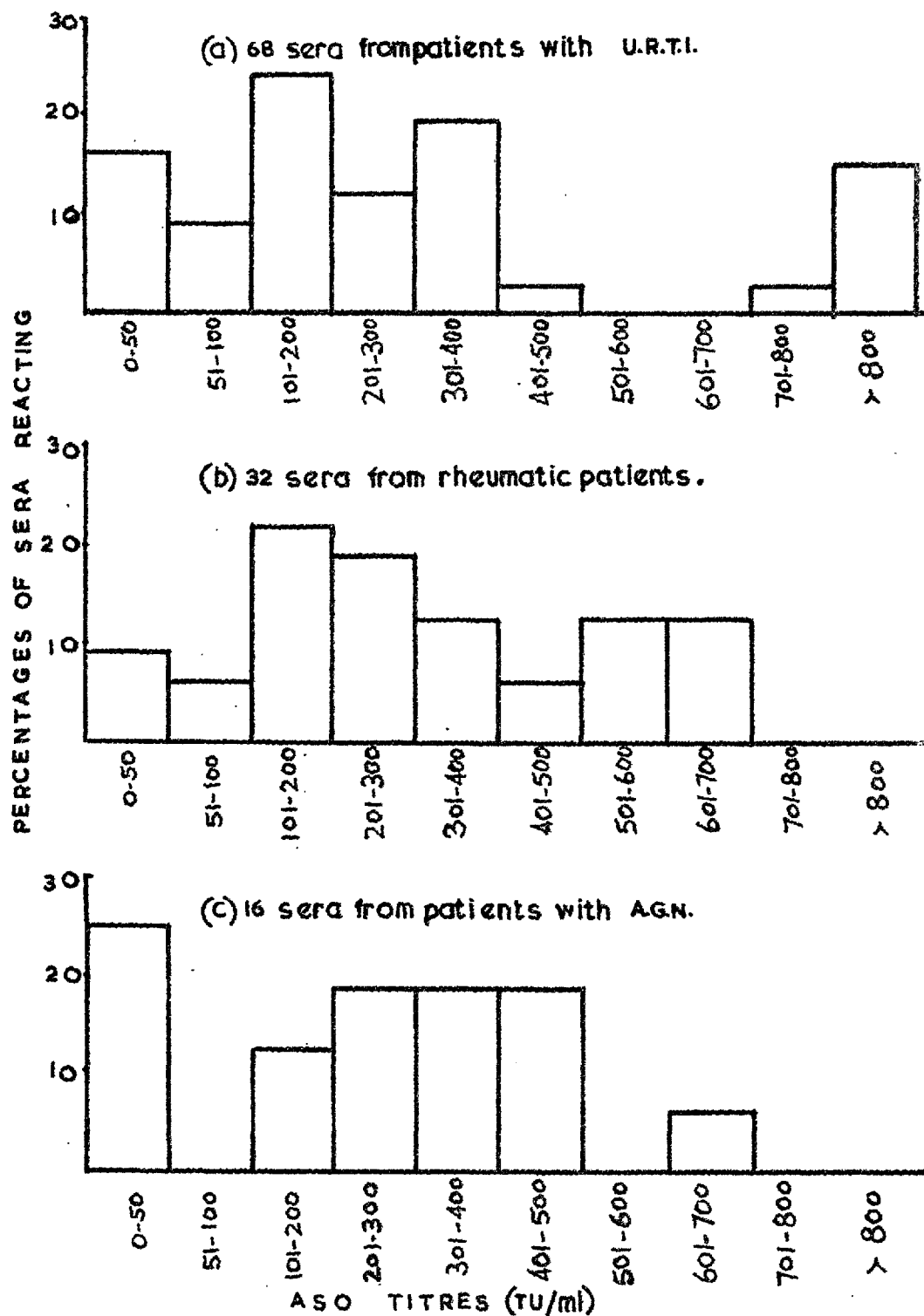


Figure 9: A.S.O. titres of sera from patients suspected of suffering from various streptococcal diseases.

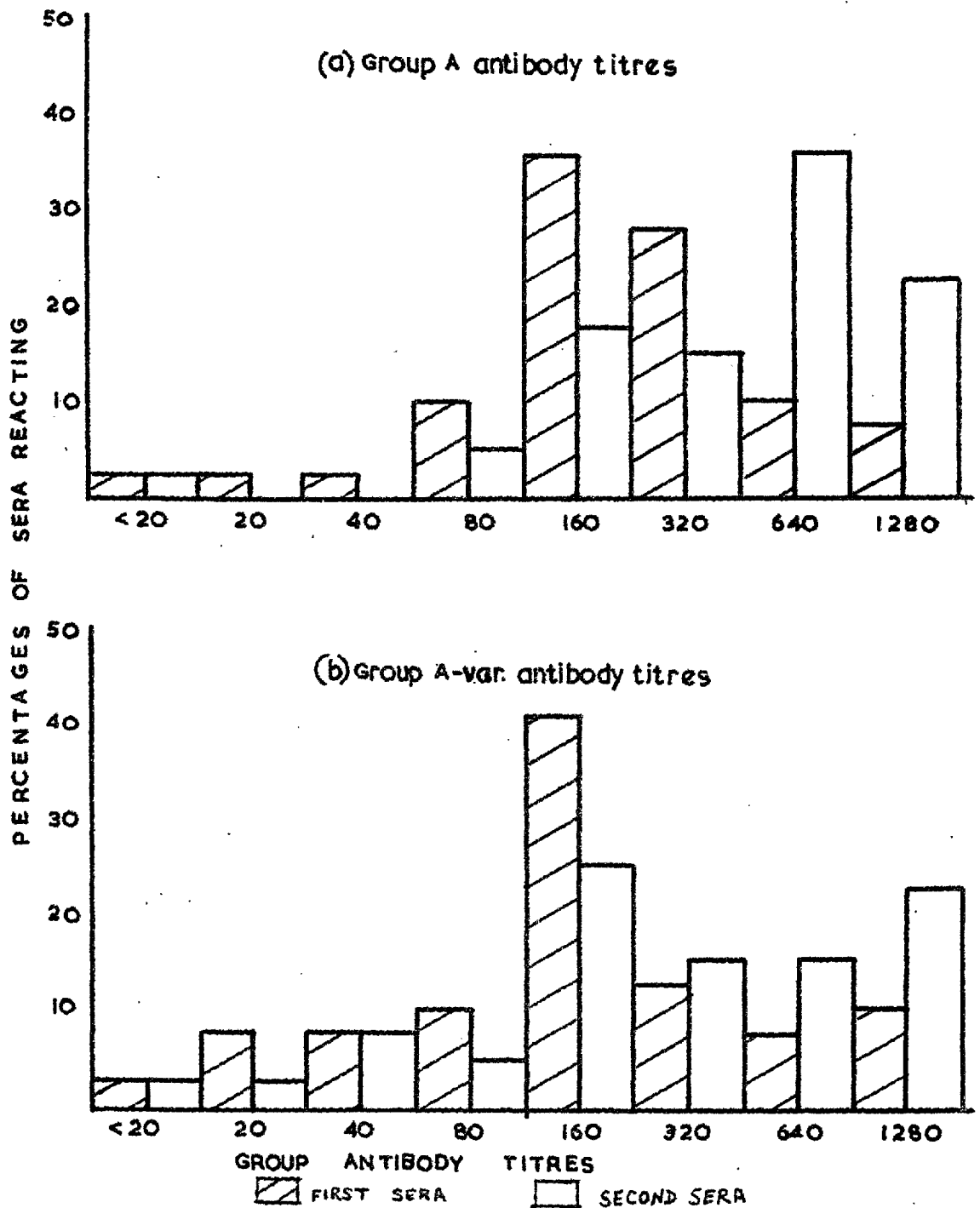


Figure 10: Latex agglutination titres of group-specific antibodies in 39 paired sera from patients suffering from various streptococcal diseases.

distribution indicating a response to these antibodies in patients. Figure 8a shows ASO titres obtained from 48 control sera and it is evident that over 95% of them showed titres of 200 T.U./ml and less but only 4% showed titres of 201 T.U./ml or more. In contrast, over 55% of 116 sera from patients gave titres of 201 T.U./ml and over. A more detailed study of the ASO response of these 116 sera revealed that similar contrast existed between sera from patients with upper respiratory tract infection, rheumatic patients and nephritic patients on the one hand and the control sera on the other hand as illustrated by Figures 9a, b, c and 8a.

2. Studies on paired sera from patients suffering from various streptococcal diseases

All the paired sera available (39 pairs) from patients suspected of suffering from various streptococcal diseases were studied and their group antibody (A and A-var) titres were plotted to show the titres of the first sera and those of the second sera. The majority of the latter showed little significant difference in the magnitude of titres from the former. In fact most of the first sera had high titres as illustrated in Figures 10a and b.

In 25 of the 39 pairs, the first serum was thought

Table 17. Group-specific antibody and ASO titres of 14 pairs of sera of patients suffering from various streptococcal diseases.

Patient No.	age	sex	Onset in relation to 1st serum	Interval between 1st & 2nd sera	Group A anti-body titre		Group A-var antibody titre		ASO titre	
					1st serum	2nd serum	1st serum	2nd serum	1st serum	2nd serum
3	20	F	1 week before 1st serum	5 weeks	< 20	< 20	< 20	< 20	100-200	100-200
34	5	F	1½ " " " "	3 " "	640	640	1280	1280	180	160
35	28	M	1 " " " "	11½ "	1280	1280	1280	1280	240	400
120	30	M	3 " " " "	5 "	160	1280	160	320	480	> 800
1381	5	M	3½ " " " "	4½ "	20	640	20	640	310	> 800
1370	26	M	17 " " " "	13 "	320	1280	320	1280	670	490
1380	15	F	8 " " " "	14½ "	320	1280	320	1280	260	650
1399	11	M	4 " " " "	11½ "	80	1280	640	1280	510	400
1493	5	F	3 " " " "	6 "	160	1280	160	1280	300	650
39 *	6	M	5 weeks after 1st serum	6 "	160	320	160	320	240	200
40 *	6	F	4 " " " "	6 "	1280	1280	1280	1280	200	N.D.
41 *	3	F	10 " " " "	10½ "	640	640	640	640	40	480
42 *	8	F	11 " " " "	13 "	320	640	160	640	240	400
43 *	10	F	2 years " " "	2 years	320	640	320	1280	500	400

* Sera from these patients were kindly supplied by Prof. L.W. Wannamaker, University of Minnesota, U.S.A., with ASO titres already determined. F = female, M = male, N.D. = not done.

to have been collected within 2-3 days of the onset of a streptococcal infection, and a group A streptococcus was isolated at this time (25 from the upper respiratory tract and 5 from the skin). First, in most sera the rise in antibody titre was shown to be either absent (equal titres) or negligible as illustrated in Figure 11 (group A antibodies) and Figure 12 (group A-var antibodies). Second, most of the initial sera had high titres viz. higher than expected, ranging from 160-1280 (Figures 11 and 12). Third, it was clearly obvious that titres of group A-var antibodies were generally lower than those of group A antibodies (compare Figures 11 and 12), although some were equal.

Results from the remaining 14 pairs of sera are shown in Table 17. The first sera of these pairs were taken from 2 years before the illness to one week after it, and again most of them showed high titres. However, patient 3 failed to give any antibody response in both first and second sera. Patient 1381 gave a titre of 20 in the first serum taken $3\frac{1}{2}$ weeks after onset and gave a sharp rise to a titre of 640 $4\frac{1}{2}$ weeks later.

Table 19. Low, moderate and high titres of group A-var antibodies in 146 sera of patients correlated to low, moderate and high ASO titres of the same patients.

ASO titres	Group A-var antibody titres		
	20 & less	40 & 80	160 & over
200 & less	20	17	33
201 - 400	4	11	28
401 & over	1	2	30

Table 18. Low, moderate and high titres of group A antibodies in 146 sera of patients correlated to low, moderate and high ASO titres of the same patients.

ASO titres	Group A antibody titres		
	20 or less	40 & 80	160 & over
200 & less	13	13	44
201-400	1	5	37
401 & over	0	3	30

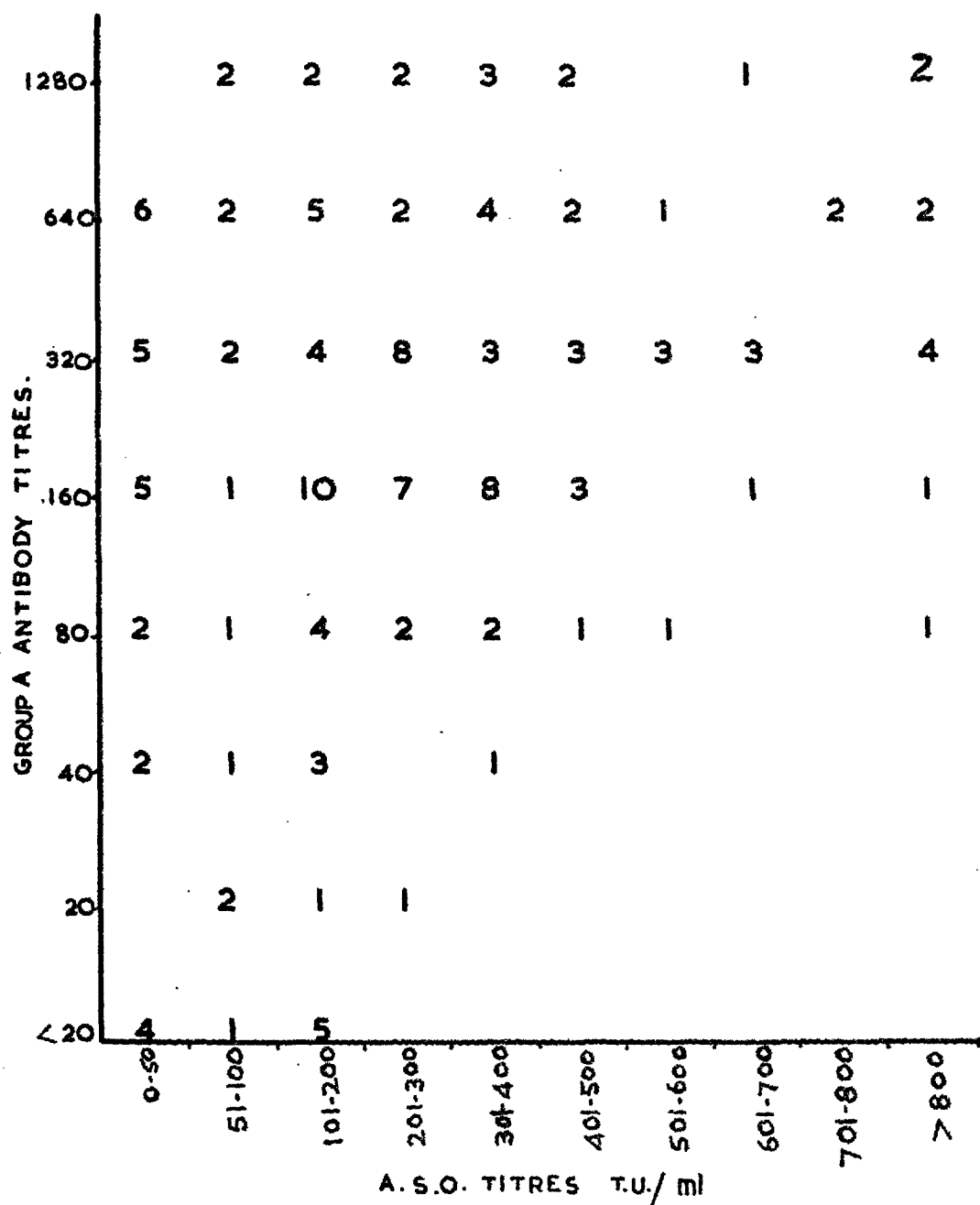


Figure 13: Correlation of group A antibodies to ASO in 146 sera from patients suspected of suffering from streptococcal diseases.

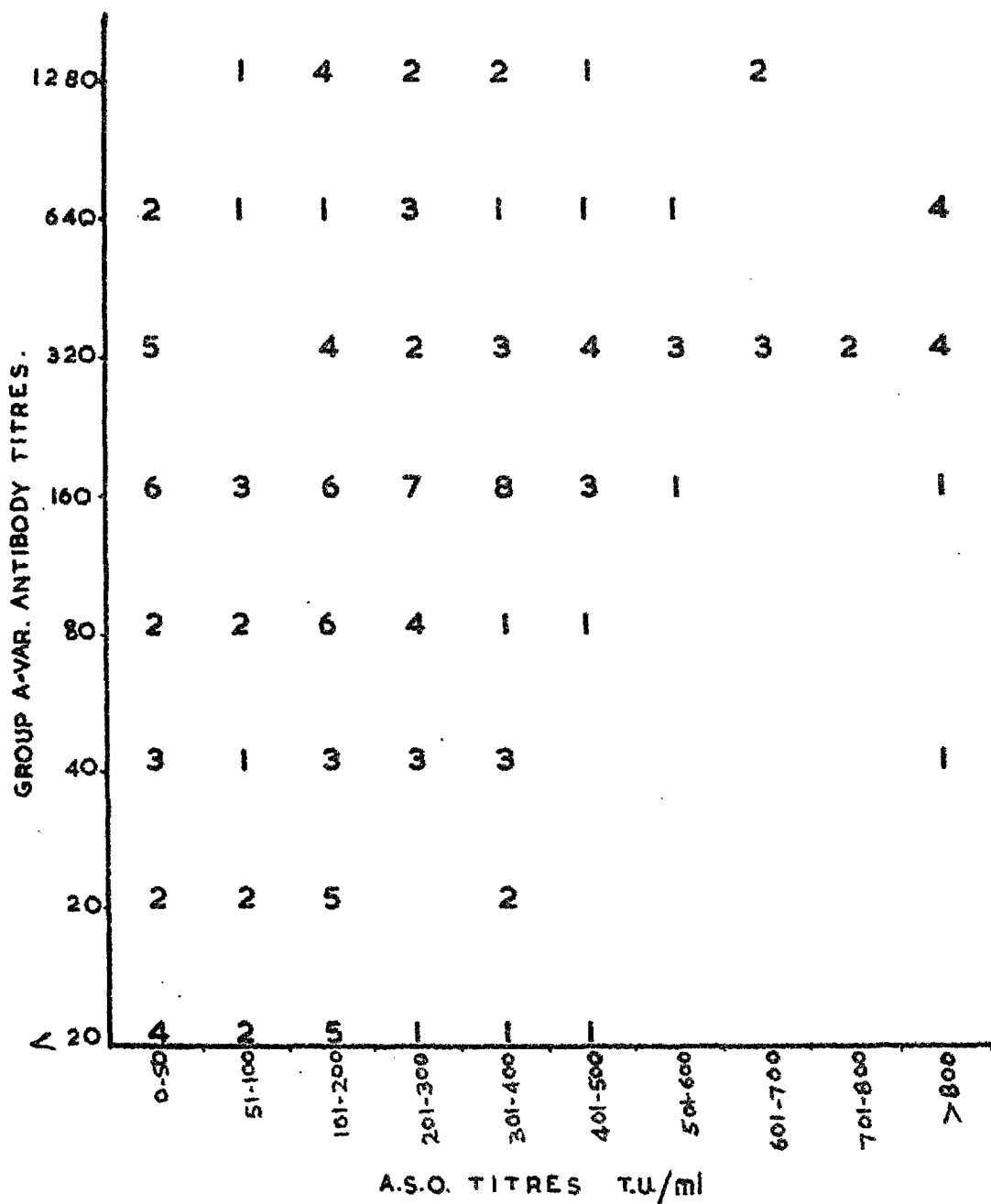


Figure 14: Correlation of group A-var antibodies to ASO in 146 sera from patients suspected of suffering from streptococcal diseases.

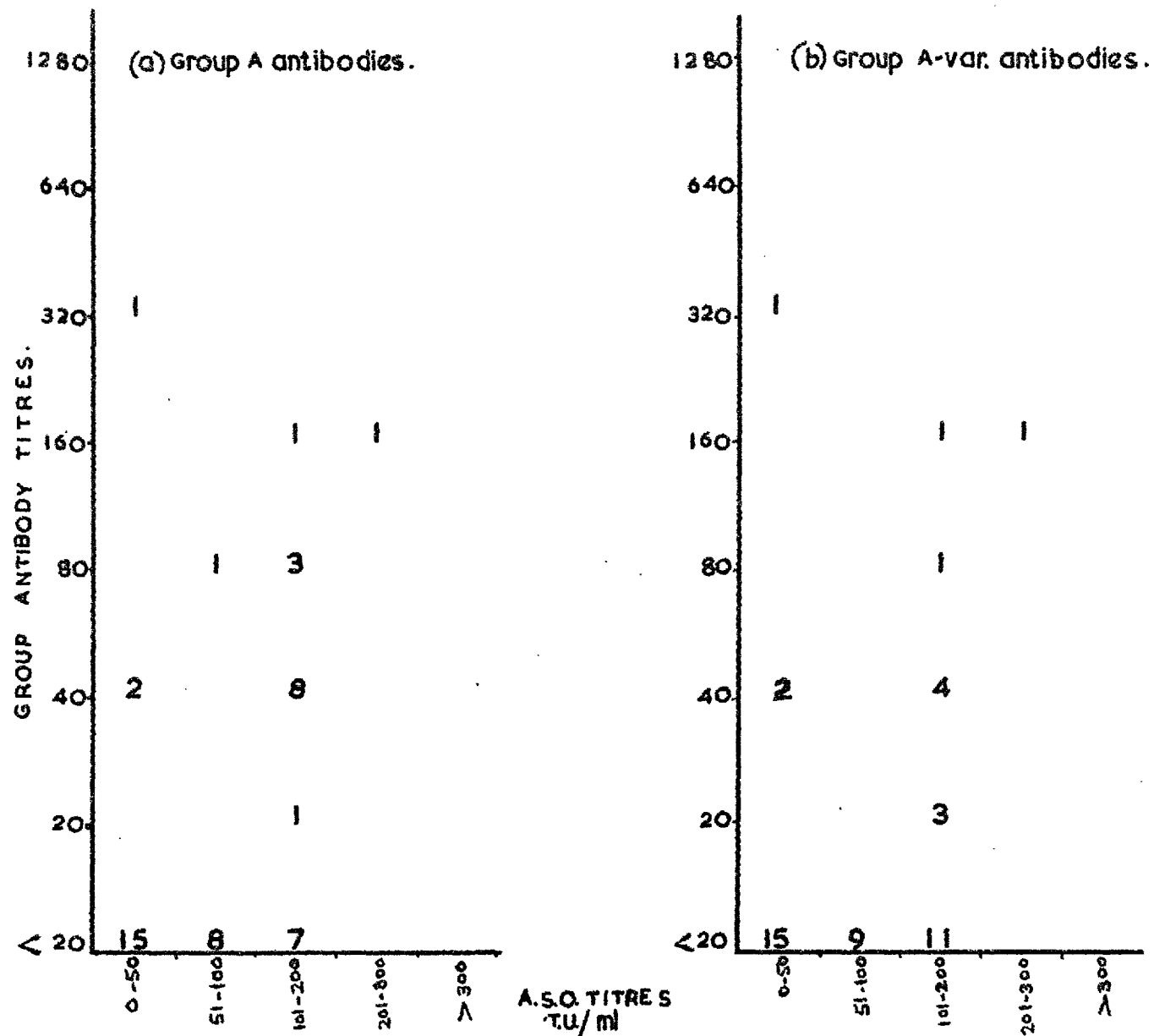


Figure 15: Correlation of groups A and A-var antibodies to ASO in 48 control sera.

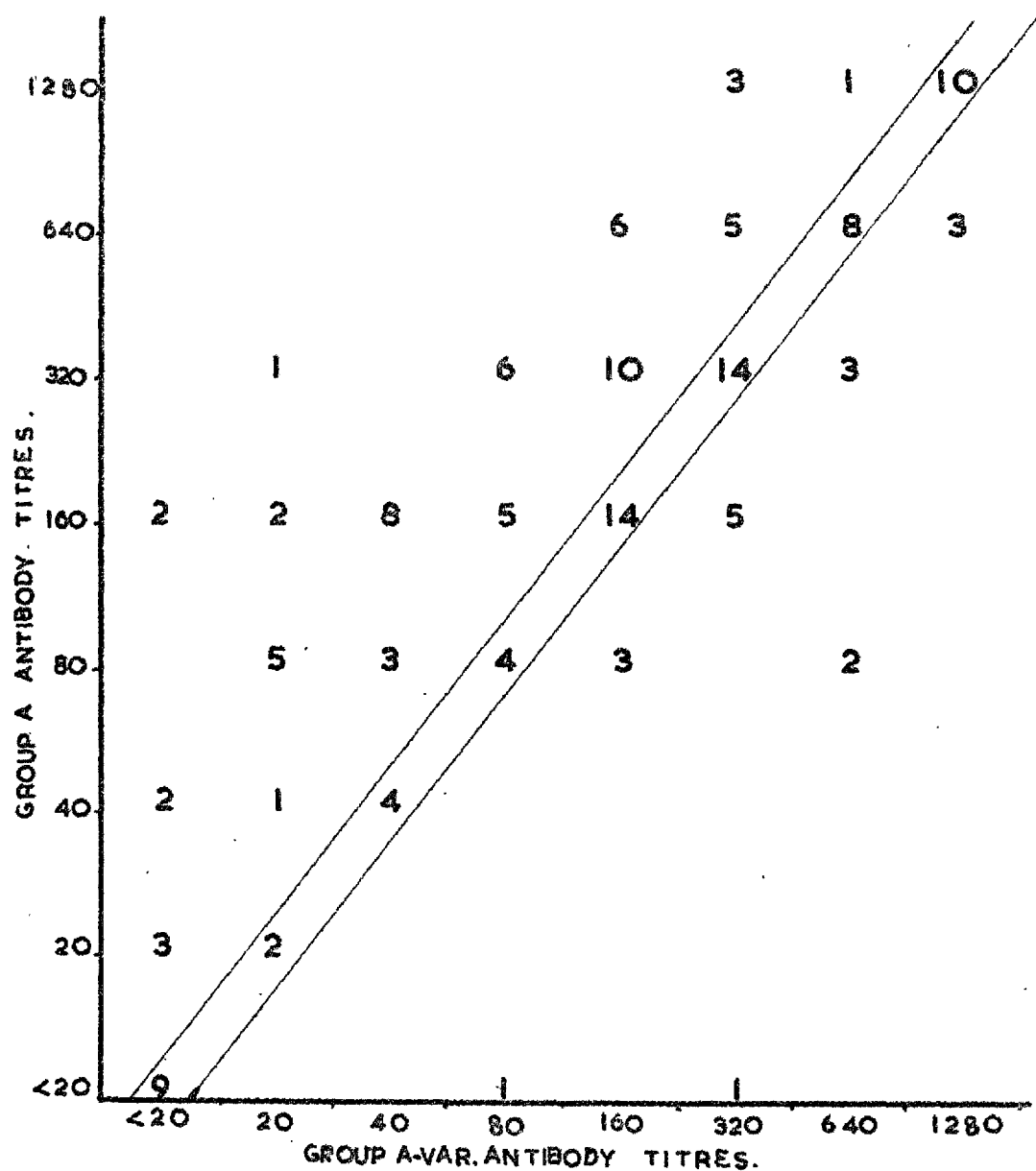


Figure 16: Correlation of group A antibody titres to group A-var antibody titres in 146 sera from patients with suspected streptococcal diseases. Sera with equal titres of groups A and A-var antibodies are included between the two parallel lines.

3. Correlation between group A, group A-var and
ASO titres in individual sera

Finally, a comparison was made of the results obtained in the various tests on individual sera. Figures 13 and 14 compare the titre of the latex agglutination test for group A and A-var antibodies respectively with the ASO result on sera from patients thought to be suffering from streptococcal disease, and Figure 15a and b gives similar findings on the control sera.

The results for the patients' sera are summarised in Tables 18 and 19, which group the results for each test into "low", "moderate" and "high". In general, the higher the ASO titre, the more likely it is that antibody for group A and group A-var will be detected in large amounts. On the other hand, high titres for both group A and group A-var antibody are more frequently found in the absence of a significant ASO titre. This finding, taken together with the relative rarity of high titres for any of the three antibodies in the control sera, provides suggestive evidence that the test for group A and group A-var antibodies is a sensitive indicator of streptococcal infection.

In Figure 16, the results of tests for group A and group A-var antibodies are compared. In general,

Table 20. Details of studies on 4 patients whose sera reacted either to group A antibodies or group A-var antibodies alone when tested by the latex agglutination test.

Patient	Age (yrs)	Sex	Diagnosis	Onset in relation to 1st serum	Group A antibody titres	Group A-var antibody titres	ASO titres
67/R/89	21	F	Recurrent S.T.	unknown	<20	80	100-200
67/R/48	20	F	Recurrent R.F.	"	<20	320	50
201	36	F	Acute G-N	2 weeks	160	<20	460
345	12	M	" "	2 "	160	<20	380

S.T. = sore throat, R.F. = rheumatic fever, G-N = glomerulonephritis

F = female, M = male,

the correlation was good. Moreover, group A antibody titres in most patients tended to be equal to or one dilution more or, occasionally, less than the titres of group A-var antibodies. However, Figure 16 also shows that 2 of the sera gave high group A antibody titres (160) and negative (<20) group A-var antibody titres; on the other hand 2 other sera gave titres of 80 and 160 for group A-var antibodies and negative titres for group A antibodies. Table 20 contains the details of results on these 4 sera. The 2 sera which reacted for only group A-var antibodies showed ASO titres of 100-200 T.U./ml for one and 50 T.U./ml for the other. The 2 sera which reacted only to group A antibodies gave ASO titres of 460 T.U./ml and 380 T.U./ml. These findings will be discussed further in Part Four.

Table 21. Homologous and heterologous (control)
M antisera from hyperimmunised rabbits
used in the capillary precipitation
tests for M-proteins.

M-types	Homologous M antiserum	Heterologous M antisera (controls)
1	1	3M, 3R
2	2	6
3M	3M	3R, 1
4	4	24, 26, 28R, 29, 46, 48
5	5	12, 15
6	6	2
9	9	18
12	12	5, 15
14	14	49, 51
18	18	17, 19, 23, 30
22	22	28
49	49	14, 51

II DETECTION OF STREPTOCOCCAL TYPE-SPECIFIC M ANTIBODIES

A. Preparation of group A streptococcal type-specific M-proteins

Lancefield's acid extracts from killed intact group A streptococcal cells of stock strains of types 1, 3, 4, 5, 6, 12, 25 and 49 were prepared as previously described in Part Two (page 69). After treatment with ammonium sulphate solutions (30% and 60%) three fractions were obtained:-

(i) P30 fraction viz. the precipitate that formed after treatment with 30% ammonium sulphate.

(ii) P60 fraction which resulted from treatment of the supernatant separated from P30 with 60% ammonium sulphate.

(iii) S60 fraction viz. the supernatant fluid collected after the treatment of the supernatant separated from P30 with 60% ammonium sulphate.

The three fractions were tested for the presence of the type-specific M-protein by the capillary pipette precipitation method, and for the group-specific polysaccharide by the ring precipitin reaction. The control heterologous sera tested for M antibodies with each of the above types and other types of group A streptococci used in this work are shown in Table 21 for reference. Chemical analysis for both protein and polysaccharide were also

Table 22. To show the results of the capillary pipette precipitation tests on fractions P60, S60 and P30 separated by ammonium sulphate fractionation from an acid extract of intact cells of certain serotypes of group A streptococci.

Serotype	Crude extracts reactions with		P60 fractions reactions with		S60 fractions reactions with		P30 fractions reactions with	
	Homolo- gous sera	Heterolo- gous sera (controls)	Homolo- gous sera	Heterolo- gous sera (controls)	Homolo- gous sera	Heterolo- gous sera (controls)	Homolo- gous sera	Heterolo- gous sera (controls)
1	2+	-	2+	-	1+	-	Tr.	-
3	3+	Type 3R: 1+ Type 1: -	2+	Type 3R: 1+ Type 1: -	2+	Type 3R: + Type 1: -	Tr.	-
4	+	-	Tr.	-	-	-	-	-
5	2+	-	1+	-	1+	-	Tr.	-
6	4+	-	3+	-	2+	-	1+	-
12	3+	-	2+	-	1+	-	Tr.	-
25	2+	-	1+	-	Tr.	-	-	-
49	1+	-	Tr.	Type 14:Tr. Type 51:Tr.	Tr.	Type 14:Tr. Type 51:Tr.	-	-

4+ = precipitation column $1\frac{1}{2}$ cm high, 3+ = precipitation column 1- $1\frac{1}{2}$ cm high,
 2+ = " " between $\frac{1}{2}$ cm & 1 cm high, 1+ = precipitation column $\frac{1}{4}$ - $\frac{1}{2}$ cm high,
 Tr. (trace) = $\frac{1}{4}$ cm high, - = negative.

carried out on fractions P60 and S60. The results of all these tests were as follows:

1. Serological tests

Preliminary serological tests were carried out to reveal the extent of the type-specific reactivity of each of the three fractions and the degree of contamination with the group-specific polysaccharide. The results of capillary precipitation tests for the type-specific M-proteins are shown in Table 22. The two fractions P60 and S60 contained significant amounts of the type-specific antigen. On the other hand, fraction P30 was the least reactive of the three fractions and gave only trace or negative reactions with the homologous type-specific antisera. A cross-reaction was obtained with type 3 crude extract and its fractions P60 and S60 tested with type 3R antiserum. Cross-reactions were also obtained with type 49 fractions P60 and S60 reacting in types 14 and 51 antisera. Furthermore, type 4 (strain 4990) crude extract showed only a weak reaction in the homologous antiserum, while its P60 fraction showed a trace reaction and its S60 fraction gave a negative reaction. Also type 49 (strains C300/1 and C274/1) gave similar weak reactions (Table 22). In order to obtain a better yield of M-proteins for these two types freshly isolated strains

Table 24. Results of ring precipitin reactions for group A specific polysaccharide obtained by testing fractions P60, S60 and P30 separated by ammonium sulphate fractionation from acid extracts of certain types of group A streptococci.

Serotype	Ring precipitin reactions of group A antiserum in the presence of			
	Crude extract	P60 fraction	S60 fraction	P30 fraction
1	4+	Tr.	4+	Tr.
3	4+	+	4+	Tr.
4	4+	Tr.	4+	Tr.
5	4+	+	4+	Tr.
6	4+	+	4+	Tr.
12	4+	Tr.	4+	Tr.
25	4+	+	4+	Tr.
49	4+	Tr.	4+	Tr.

4+ = immediate reaction, 3+ = reaction in the 1st minute, 2+ = reaction in the second minute, 1+ = reaction in the third minute, Tr. = reaction in 3-5 minutes (trace)
- = negative.

Table 23. Results of the capillary precipitation test for M-proteins on fractions P60, S60 and P30 separated from acid extracts of freshly isolated types 4 and 49 group A streptococci obtained by ammonium sulphate fractionation.

Serotype	Crude extracts reacting with		P60 fractions reacting with		S60 fractions reacting with		P30 fractions reacting with	
	Homolo- gous sera	Heterolo- gous sera (controls)	Homolo- gous sera	Heterolo- gous sera (controls)	Homolo- gous sera	Heterolo- gous sera (controls)	Homolo- gous sera	Heterolo- gous sera (controls)
4	2+	-	1+	-	1+	-	-	-
49	2+	-	1+	Type 14: 1+ Type 51: 1+	1+	Type 14: 1+ Type 51: +	Tr.	-

N.B. For abbreviations and signs see footnote for Table 22.

which gave strong precipitation reactions in routine typing were extracted (type 4 strain R67/1826, and type 49 strain R67/2327). The results of testing the new extracts are shown in Table 23.

The extracts from the new strains of types 4 and 49 gave a better yield of M-proteins as shown by the improvement in reactivity demonstrated in Table 23. The cross-reactions of type 49 M-types 14 and 51 antisera were also enhanced.

So far, both P60 and S60 fractions from all types of group A streptococci tested showed high serological reactivity for the presence of M-proteins. However, the P60 was found to be less contaminated with group A specific polysaccharide. This was well demonstrated by the results of the ring precipitin reactions shown in Table 24. The S60 fraction obviously contained the bulk of the polysaccharide as shown by the strong reactions in group A antiserum. As recommended by Barkulis and Jones (1957) further removal of the polysaccharide from fraction P60 was effected by 2-3 washes in 4 volumes absolute alcohol (see page 70). This procedure quickly removed the small amounts of polysaccharide without affecting the M-protein reactivity. This was shown by testing the purified P60 by both

Table 25. Results of capillary tube precipitation tests for M-proteins and ring precipitin reactions for group-specific polysaccharide on alcohol-purified P60 fractions of certain types of group A streptococci.

P60 fractions	M-precipitation tests		Group precipitin reactions
	Homologous sera	Heterologous sera (controls)	
Type 1	2+	-	-
" 3	2+	Type 3R: 1+, Type 1: -	-
" 4	1+	-	-
" 5	1+	-	-
" 6	3+	-	-
" 12	2+	-	-
" 25	1+	-	-
" 49	1+	Type 14: 1+, Type 51: 1+	-

N.B. For explanation of signs see footnotes of Table 22.

Table 26. The protein and polysaccharide contents of fractions P60, S60 and P30 obtained by ammonium sulphate precipitation from acid extracts of some types of group A streptococci.

Serotype	Protein content in mg/ml				Polysaccharide content in µg/ml		
	Unpurified P60	Purified* P60	S60	P30	Unpurified P60	Purified* P60	S60
1	3.1	3.6	3.1	1.4	15	-	> 200
3	5.3	5.7	4.0	0.4	29	-	99.3
4	5.3	3.9	1.3	1.1	17	-	> 200
5	3.0	3.4	3.4	1.2	30	-	> 200
6	3.2	3.7	2.9	1.1	35	-	109
12	3.4	3.0	2.2	0.9	12	-	174
25	3.3	3.2	3.1	0.8	20	-	> 200
49	3.3	3.6	3.7	0.9	10	-	162

- = not detectable

* treated with absolute alcohol to remove the group-specific polysaccharide.

capillary tube precipitation tests and ring precipitin reactions (Table 25). In addition to their reactions with homologous antisera, P60 fractions of types 3 and 49 retained the cross-reactions discussed above (compare Tables 22, 23 and 25).

2. Chemical analysis

Further comparison of fractions P60, S60 and P30 was carried out by determining their respective protein content by the micro-Kjeldahl technique. The polysaccharide content of fractions P60 and S60 was estimated but not that of P30 which only gave trace precipitin reactions (Table 24). The results obtained showed slightly higher protein content of P60 fraction than S60 fraction but low protein content of P30 fraction (Table 26). However, type 4 gave a protein content of P60 fraction which was four times that of the S60 fraction. On the whole, the unpurified P60 fractions had very low polysaccharide contents as compared with the S60 fractions, whose polysaccharide concentrations ranged from 60 $\mu\text{g/ml}$ to over 200 $\mu\text{g/ml}$. These results confirmed the absence of serological interference by the group-specific polysaccharide in all tests carried out on the purified (alcohol treated) P60 fractions.

In summary, the P60 fractions and S60 fractions

of types 1, 3, 4, 5, 6, 12, 25 and 49 contained the bulk of M-protein and gave good reactions with the respective type-specific antisera; but the S60 fraction was grossly contaminated with the group-specific polysaccharide. On the other hand, the small amounts of polysaccharide in the P60 fractions were easily removed, and their reactivity in the grouping serum was abolished, by washing in absolute alcohol without affecting the type-specific reactivity. For these reasons the P60 fraction was used in preference to the S60 fraction in preliminary latex agglutination tests carried out on hyperimmune type-specific rabbit sera.

B. The determination of type-specific M-antibodies in hyperimmune rabbit sera by the agglutination of latex particles carrying P60 fractions of M-proteins

The P60 fractions of M-proteins from streptococci of types 1, 3, 4, 5, 6, 12, 25 and 49, purified by washing in absolute alcohol and tested as above, were dissolved in distilled water and adsorbed to latex particles. Each fraction was tested against its homologous antiserum and the appropriate control heterologous antisera employed in the routine typing of group A streptococci by capillary tube precipitation tests. All the homologous and heterologous antisera used here were those absorbed and prepared for typing purposes (see Part Two). Homologous and control

Table 27. Results of latex agglutination tests with purified P60 fraction of some group A streptococcal types tested against absorbed typing sera.

Type-specific P60 fraction	Agglutination titres of M sera		Quality of agglutination of M sera	
	Homologous	Control heterologous	Homologous	Heterologous
1	80	Type 3M: 20, Type 3R: 80	1+	1+
3	1280	" 3R: 80	4+	2+
4	20	" 24: 40, " 26: 40, " 28R: 80	1+	1+
5	640	" 15: 640	3+	3+
6	5120	" 2: 20	4+	2+
12	40	" 5: 80, " 15: 160	1+	3+
25	-	-	-	-
49	2560	" 14: 640 " 51: 320	4+	4+

N.B. For explanation of signs see Part Two, page 78.

heterologous sera used in testing each type-specific P60 fraction were as shown on Table 21.

It should be noted that the P60 fractions used for types 4 and 49 were those prepared from the new strains R67/1826 and R67/2327 respectively.

The technique for the latex agglutination test was as described for the detection of the group-specific antibodies, but it was found that the quality of agglutination was improved considerably by shaking the plates in a mechanical horizontal rotatory shaker for 10-30 min at a speed of 125 r.p.m. before leaving them overnight at room temperature. Refrigeration of the plates was not necessary. Moreover, adsorption of the antigen to latex particles was effectively achieved after incubation at 37°C. for only one hour.

The results of the latex agglutination tests on the P60 fraction showed considerable disagreement with those obtained by capillary tube precipitation on the same fraction (Table 27). Whereas in precipitation tests the homologous sera showed a reasonable degree of reaction, in latex agglutination tests some of these sera showed low titres and a poor quality of agglutination (1+); type 1 specific serum gave a titre of 80 with the homologous P60 fraction and the quality of agglutination was equivalent to 1+ (fine agglutination). Type 12 specific serum gave a titre

of 40 and 1+ agglutination reaction. Type 4 specific serum reacted very weakly to a titre of 20. However, type 25 antiserum failed to react with the homologous antigen. All other type-specific sera reacted satisfactorily with their homologous antigens to high titres (640-5120) and gave reaction of adequate strength ($>2+$). Moreover, there were more cross-reactions in control heterologous sera revealed by the latex agglutination test than by the precipitation test. All the control sera tested gave cross-reactions by the latex agglutination test. In some instances the titres given by the heterologous sera were equal or even higher than those given by the homologous sera e.g. heterologous control sera of types 1, 4, 5, 12.

Since the type 25 extract did not react with the homologous antiserum, a freshly isolated strain of type 25 R67/2784 was obtained from a routine specimen and extracted in hot acid as before. Fractionation in 30% and 60% ammonium sulphate was carried out and the P60 fraction was purified by washing in absolute alcohol. The crude extract and the three fractions obtained were tested as before for the type-specific M-proteins by capillary tube precipitation and for the group-specific polysaccharide by the ring precipitin reaction. Finally the P60 fraction was used in a latex agglutination test against the homologous type 25 antiserum and the control heterologous type 17 antiserum.

Table 28. Results of M-precipitation tests and group-specific reactions on crude extracts and fractions P60, S60 and P30 of M-protein of type 25 R67/2784, together with results of latex agglutination test on purified P60 fraction.

Antigen fraction	M-precipitation reactions		Group-specific reactions	Latex agglutination titres	
	Homologous serum type 25	Control serum type 17		Homologous serum type 25	Control serum type 17
Crude extract	2+	-	4+		
Unpurified P60	1+	-	1+		
Purified P60	1+	-	-	-	-
S60	1+	-	4+		
P30	-	-	Tr.		

N.B. For explanation of signs see footnote for Tables 22 and 24.

The results of all these tests are shown in Table 28. No practical differences were noted between the new P60 fraction and the previous P60 fraction of type 25 M-protein. Again there was no agglutination of the latex particles carrying the purified P60 fraction in the homologous or the control antiserum.

In view of these results and the considerable number of cross-reactions exhibited by almost all of the types tested against absorbed typing sera it appeared that the P60 fraction was unlikely to be useful in the latex agglutination test with human sera.

C. The preparation and purification of highly specific fractions of M-proteins from some serotypes

The S60 fractions had been shown to contain large amounts of protein that reacted specifically with type-specific antisera in the capillary tube M-precipitation test. As will be described below, removal of the group-specific polysaccharide from these fractions results in the isolation of M-proteins that are highly specific in their reactions with homologous antisera. When tested with heterologous antisera only a few of them will show cross-reactions at considerably low titres compared with the P60 fractions when both were tested with the latex agglutination.

1. The removal of polysaccharide from S60 fractions

The iso-electric point of M-protein was defined

by Lancefield & Perlman (1952 a) as pH 5.3. This fact was utilised to precipitate the protein from solution leaving the greater part of the polysaccharide in solution. The pH of the solution was adjusted to 5.5 by the addition of $\frac{N}{5}$ HCl dropwise and checking with indicator paper after each drop. At this pH a flocculant heavy precipitate formed. If the pH was overshoot and the solution became too acid (pH < 5.0), it could be readjusted to pH 5.5 by the addition of $\frac{N}{2}$ NaOH or $\frac{N}{5}$ NaOH. The precipitate was separated by centrifugation at 2500 r.p.m. for 20 min. The deposited precipitate was redissolved in a minimal volume of distilled water to which a drop of $\frac{N}{2}$ NaOH was added to assist quick solution of the material. It was then neutralised with $\frac{N}{5}$ HCl to pH 7.5 and tested for the presence of the group-specific polysaccharide. Usually at this stage a 2+ reaction was given (see footnote of Table 24). The pH was readjusted to 5.5 as above to re-precipitate the M-protein; 4 volumes of absolute alcohol were added, and the mixture was shaken. The alcohol enhanced the precipitation. The precipitate was allowed to settle for an hour or two, centrifuged, redissolved and neutralised as above. Again it was tested for group-specific polysaccharide and in most cases it gave a 1+ reaction. After the second or the

Table 29. Protein and polysaccharide content of purified S60 fractions of U-proteins from acid extracts of some types of group A streptococci fractionated by ammonium sulphate.

	S60 fractions of serotypes							
	1	3	4	5	6	12	25	49
Protein content mg/ml	1.2	1.2	1.4	1.08	0.9	0.8	1.3	1.25
Polysaccharide content µg/ml	-	-	-	-	-	-	-	-

third wash in alcohol the group reaction completely disappeared and was not observed even after allowing the ring precipitin test to stand for 2-3 hr. At this stage the purified S60 fractions were collected ready for chemical analysis and serological tests.

2. Chemical analysis

The protein content of the purified S60 fractions was estimated by the micro-Kjeldahl technique and the polysaccharide content was determined by the method described previously. The results are shown on Table 29. After purification, the amounts of protein in S60 fractions were lower than before purification (compare Tables 26 and 29). In fact, estimation of the protein content of the supernatant of type 1 S60 fraction resulting from the initial precipitation at pH 5.5 before washing with alcohol showed that its protein content was 1.0 mg/ml. So, it may be that a part of the protein remains attached to the polysaccharide. On the other hand, chemical analysis revealed the absence of the polysaccharide from all the purified S60 fractions, thus verifying the elimination of group-specific reactions after the second or third washing in absolute alcohol. This emphasized the validity of the ring precipitin reaction,

Table 30. Capillary tube M-precipitation and group-specific ring precipitin reactions carried out on purified S60 fractions of M-proteins from acid extracts of some types of group A streptococci.

Test	Antiserum	S60 fractions of serotypes:							
		1	3	4	5	6	12	25	49
M-precipitations	Homologous	Tr.	1+	Tr.	Tr.	1+	Tr.	Tr.	Tr.
	Heterologous	-	-	-	-	-	-	-	type 14: Tr. " 51: Tr.
Ring precipitin test	Grouping serum	-	-	-	-	-	-	-	-

N.B. For explanation of signs see footnotes of Tables 22 and 24.

when observed for long periods (2-3 hr), as a guide to the presence or absence of the group-specific polysaccharide.

3. Serological tests for specificity

The specificity of the purified S60 fractions was established by the following serological tests.

(a) Capillary tube M-precipitation tests. The purified S60 fractions were found to precipitate poorly in the presence of the homologous antisera (Table 30) in comparison with the purified P60 fractions (Table 25). It was also observed that the cross-reaction of type 3M with type 3R was not given by type 3 fraction S60. However, the cross-reactions between types 49, 14 and 51 persisted. The results of testing the purified S60 fractions for the presence of the group-specific polysaccharide were also included in Table 30, and as mentioned previously they were all negative. Table 30 should be compared with Table 24.

(b) Gel-diffusion tests. These tests were carried out according to the method described on page 75. The purified S60 fractions were used concentrated five times and unconcentrated. Precipitin lines were not detectable in either

Table 31. Results of latex agglutination tests with purified S60 fractions of M-proteins, from some types of group A streptococci, tested against absorbed typing sera.

Type-specific S60 fraction	Agglutination titres of sera		Quality of agglutination	
	Homologous	Control heterologous	Homologous sera	Heterologous sera
1	640	-	3+	-
3	1280	-	4+	-
4	320	-	3+	-
5	2560	-	3+	-
6	2560	-	3+	-
12	320	Type 15: 20	3+	3+
25	-	-	-	-
49	1280	Type 14: 80, Type 51: 80	3+	2+

N.B. For explanation of signs see Part Two, page 78.

case. This further confirmed the poor precipitating ability of this antigen.

(c) The latex agglutination test. This was carried out as before. Purified S60 fractions were adsorbed to latex particles as usual and tested against homologous and control heterologous antisera in the same way as for the P60 fractions. The results are shown in Table 31. All the purified S60 fractions showed highly specific agglutination reactions with homologous antisera to considerably high titres. Most of the cross-reactions with heterologous antisera disappeared; the only cross-reactions observed were given by type 12 S60 in type 15 serum to a titre of 20, and also by type 49 S60 in types 14 and 51 antisera to titres of 80 in both cases. However, type 25 S60 fraction like its P60 fraction failed to agglutinate to any degree. To compare reactions of purified P60 fractions see Table 27.

4. Preparation of S60 fractions from further group A serotypes

From the above results the purified S60 fractions appeared to be highly specific and quite suitable for use in latex agglutination tests. Therefore, it was the aim to detect this specificity in S60 fractions

Table 32. Results of chemical analysis and serological tests on purified P60 and S60 fractions from group A serotypes 2, 9, 14, 18 and 22 streptococci extracted in hot acid and fractionated by ammonium sulphate.

M-protein fraction	Protein content mg/ml	Polysaccharide content ug/ml	Group-specific reaction	M-precipitation reactions		Latex agglutination titres	
				Homologous sera	Control heterologous sera	Homologous sera	Control heterologous sera
Type 2 P60	2.75	Nil	-	1+	-	160	Type 6: 80
S60	1.4	"	-	1+	-	5120	-
" 9 P60	3.4	"	-	2+	-	40	" 18: 20
S60	1.0	"	-	Tr.	-	1280	-
" 14 P60	5.7	"	-	1+	Type 51: 1+	80	" 49: 160, " 51: 320
S60	1.4	"	-	Tr.	" 51: Tr.	1280	" 51: 40
" 18 P60	5.7	"	-	3+	-	80	" 23: 160, " 30: 640
S60	1.25	"	-	1+	-	2560	" 17: 20, " 23: 20, " 30: 640
" 22 P60		"	-	1+	-	80	-
S60		"	-	Tr.	-	320	-

N.B. For explanation of signs see Part Two page 78 and Table 22 footnote.

of more types. So, cells of group A types 2, 9, 14, 18 and 22 were extracted in hot acid and fractionated by ammonium sulphate to obtain both P60 and S60 fractions. These fractions were purified as previously described and tested chemically and serologically. The results are shown in Table 32. The same situation experienced with purified P60 and S60 fractions of types 1, 3, 4 etc presented above was also reproduced here. The P60 fractions of types 2, 9, 14, 18 and 22 gave more protein content than the S60 fractions but both fractions showed absence of polysaccharide chemically and serologically. M-precipitation was given by both fractions, and again the S60 fractions generally gave the weaker reactions. The P60 fraction exhibited cross-reactions in the case of type 14 with type 51 antiserum and this persisted with the S60 fraction of the same type. In latex agglutination the P60 fractions showed weaker reactions in the homologous antisera and gave very low titres (40-160) compared with the S60 fractions which gave strong agglutination to high titres (320-5120) in the homologous sera. Moreover, in the heterologous sera the P60 fractions gave cross-reactions in the case of all types except type 22. The titres of the heterologous

sera tested against types 14 and 18 were much higher than those of the respective homologous sera: the homologous sera in both occasions gave titres of 80 while the heterologous titres ranged from 160 to 640. However, the S60 fraction of type 14 reacted in the heterologous type 51 to a low titre of 40 compared with the homologous titre (1280). Also the S60 fraction of type 18 gave a negligible reaction with types 17 and 23 sera (titres of 20) but its reaction with type 30 antiserum gave a titre of 640 viz. the same titre given by P60 fraction, but it should be noted that the titre of the homologous serum with type 18 S60 is remarkably high (2560) compared with that of type 18 P60 fraction which was only 80.

5. Latex agglutination tests with P60 and S60 fractions on unabsorbed antisera

Before applying the latex agglutination test with the purified S60 fractions on human sera these fractions were tested with unabsorbed hyperimmune rabbit sera. Sera for typing with M-precipitation are absorbed for the removal of the group-specific antibodies in the first place, but also special absorptions with heterologous M-strains are necessary for many type-specific sera (see page 58). Therefore, the application of the

Table 33. Results of latex agglutination tests with purified P60 and S60 fractions of M-proteins of twelve serotypes of group A streptococci extracted in hot acid and fractionated in ammonium sulphate. All sera used were unabsorbed.

Serotype	Titres of type sera tested with P60		Titres of type sera tested with S60	
	Homologous	Heterologous	Homologous	Heterologous
1	320	Type 3M; 640, type 3R: 640	640	-
2	320	" 6: 40	2560	-
3M	1280	" 3R: 320, " 1: 40	5120	-
4	160	" 24: 640, " 26: 1280, " 28: 640	640	-
5	1280	" 12: 160, " 15: 320	2560	-
6	640	" 2: 40	10240	-
9	160	" 18: 20	640	-
12	320	" 5: 320, " 15: 160	1280	Type 15: 80
14	320	" 49: 160, " 51: 80	5120	" 49: 80, type 51: 160
18	320	" 23: 320, " 30: 1280	5120	" 17: 40, " 19: 160, " 23: 160, " 30: 640
22	80	" 28: 20	5120	-
49	5120	" 14: 1280, " 51: 1280	640	" 14: 80, " 51: 80

latex agglutination test on these sera before absorption should give a better assessment of the purified S60 fractions in comparison with the P60 fractions.

Homologous and heterologous unabsorbed sera were tested with latex particles carrying each of the purified P60 and S60 fractions of types 1, 2, 3M, 4, 5, 6, 9, 12, 14, 18, 22 and 49 group A streptococci prepared previously. Since type 25 failed to react in homologous and heterologous absorbed sera tested with both fractions it was omitted from this and subsequent tests. The results for the former types are shown in Table 33. As with absorbed sera the purified S60 fractions also showed high specificity in the unabsorbed sera, and cross-reactions were given by the same types encountered before viz. types 12, 14, 18 and 49 (compare Tables 31, 32 and 33). The heterologous titres due to the cross-reactions were appreciably lower than the homologous titres, which ranged from 640-10240. On the other hand, the P60 fractions exhibited more marked cross-reactions with the unabsorbed than with the absorbed sera (compare Tables 27, 32 and 33) and as usual, all the P60 fractions reacted in heterologous sera. Heterologous titres in some cases were greater than the homologous

titres e.g. type 1 homologous titre was 320 but the heterologous types 3M and 3R sera gave titres of 640; type 4 homologous titre was 160 and heterologous titres ranged from 640 to 1280 and so on. The homologous titres given by the P60 fractions ranged from 80 to 5120 and were generally lower than the homologous titres given by the S60 fractions. In some types the difference between the two sets of homologous titres was very great e.g. type 22 P60 gave a titre of 80 while its S60 fraction gave a titre of 5120, types 14 and 18 P60 fractions gave titres of 320 while their S60 fractions reacted to titres of 5120 etc.

6. Removal of the bactericidal activity of type-specific sera by their homologous S60 fractions

Since the purified S60 fractions showed high specificity in their serological reactions, an investigation was carried out to elicit their relation to antibactericidal activity. This was done indirectly by absorbing type-specific sera with homologous S60 fractions and then testing the absorbed sera for bactericidal activity. The absorption was carried out in small precipitation tubes by adding a volume of the S60 fraction to an equal volume of the homologous serum. The tubes were incubated at 37°C for 5-6 hr and then allowed to stand in the cold overnight.

Table 34 continued

Serotype	Organisms dilutions in saline broth	Controls				Miles & Misra count of test organisms
		No. of organisms surviving in normal rabbit serum (undiluted)	No. of organisms surviving in the absence of serum	No. of heterologous organisms surviving in		
				absorbed sera	unabsorbed sera	
1 ^o	10 ⁻²	C.	C.	C.	C.	C.
	10 ⁻³	C.	C.	C.	C.	U.C.
3M ^o	10 ⁻²	C.	C.	C.	C.	U.C.
	10 ⁻³	C.	U.C.	C.	C.	400
4 ^o	10 ⁻²	C.	C.	C.	C.	U.C.
	10 ⁻³	U.C.	U.C.	C.	C.	365
5	10 ⁻²	C.	C.	C.	C.	U.C.
	10 ⁻³	U.C.	U.C.	C.	C.	318
6	10 ⁻²	C.	C.	C.	C.	U.C.
	10 ⁻³	U.C.	U.C.	C.	C.	380
12	10 ⁻²	C.	C.	C.	C.	U.C.
	10 ⁻³	C.	U.C.	C.	C.	400
49	10 ⁻²	C.	C.	C.	C.	C.
	10 ⁻³	C.	C.	C.	C.	U.C.

* Initial dilution of sera used was 1:3, H.D. = not done, C = confluent growth,
U.C. = colonies discrete but crowded and uncountable.

Table 34. Results of indirect bactericidal tests on type-specific sera absorbed with homologous and heterologous S60 fractions of types 1, 3, 4, 5, 6, 12 & 49 streptococci in comparison with unabsorbed sera.

Serotype	Organisms dilutions in saline broth	Tests					
		No. of organisms surviving in unabsorbed sera		No. of organisms surviving in sera absorbed with homologous S60		No. of organisms surviving in sera absorbed with heterologous S60	
		1:2	1:10	1:2	1:10	1:2	1:10
1 *	10 ⁻²	33	60	U.C.	C.	N.D.	N.D.
	10 ⁻³	4	19	1400	C.	N.D.	N.D.
3H *	10 ⁻²	0	0	U.C.	C.	3	10
	10 ⁻³	0	0	U.C.	U.C.	0	0
4 *	10 ⁻²	800	1500	C.	C.	N.D.	N.D.
	10 ⁻³	160	260	800	2000	N.D.	N.D.
5	10 ⁻²	0	0	C.	C.	N.D.	N.D.
	10 ⁻³	0	0	U.C.	U.C.	N.D.	N.D.
6	10 ⁻²	0	0	C.	C.	0	10
	10 ⁻³	0	0	U.C.	U.C.	0	3
12	10 ⁻²	12	15	790	1500	0	17
	10 ⁻³	0	0	1230	U.C.	2	0
49	10 ⁻²	0	28	C.	C.	0	217
	10 ⁻³	0	0	C.	C.	0	130

* Initial dilution of sera used was 1:3, N.D. = not done, C = confluent growth, U.C. = colonies discrete but crowded and uncountable.

Finally the precipitate was separated by centrifugation and the supernatant was collected and used as the absorbed serum. The absorbed sera were thus obtained diluted 1:2 unless absorption was repeated. Seven type-specific sera were absorbed in this way, namely specific sera of types 1, 3M, 4, 5, 6, 12 and 49. However, types 1, 3M and 4 antisera were absorbed twice and hence their initial dilutions used in the bactericidal tests were 1:3. For each of the absorbed sera a second dilution of 1:10 was made. Therefore, the bactericidal tests were carried out on two dilutions of each sera. In addition, antisera of types 3, 6, 12 and 49 were absorbed with heterologous S60 fractions of types 5, 1, 2 and 14 respectively and then tested for bactericidal activity. The indirect bactericidal test described on page 81, was employed for testing both absorbed and unabsorbed antisera. The controls included were normal rabbit serum, absorbed and unabsorbed serum against a heterologous type of group A streptococci, and also homologous streptococci in blood without any serum.

The results shown in Table 34 revealed that the purified S60 fractions effectively removed the bactericidal activity of their homologous type-specific sera. However, type 4 showed rather appreciable growth in

the presence of the unabsorbed serum and its S60 fraction seemed not to have effected complete removal of the bactericidal activity in spite of the fact that the serum was absorbed twice. This was indicated by the low count in the number of colonies of the 10^{-3} dilution in the presence of 1:2 and 1:10 absorbed serum (800 and 2000 colonies) compared with those growing in the presence of normal rabbit serum (uncountable numbers of colonies). It should be mentioned here that the bactericidal activity of the M-typing serum for type 4, which was used in this work, was known to be poor compared with its strong precipitation reactions with homologous acid extracts. A better result was obtained in the case of type 12; the counts given here were after a single absorption of the serum and obviously they could be much better by a second absorption as in the cases of types 1 and 3M but this was not done due to shortage in type 12 S60 fraction. However, with these counts in the presence of absorbed serum compared with the complete bactericidal activity of the unabsorbed serum the results for type 12 were quite satisfactory. Types 5, 6 and 49 showed complete removal of bactericidal activity after only a single absorption of each homologous serum.

Furthermore, absorption of types 3, 6, 12 and 49 antisera with heterologous S60 fractions 5, 1, 2 and 14 failed to remove their bactericidal activity (Table 34). The results were conclusive for types 3, 6 and 12 absorbed with S60 fractions of types 5, 1 and 2 respectively. In the case of type 49 the absorption was carried out by type 14 S60 fraction. As mentioned before type 49 S60 fraction showed a persistent cross-reaction by both M-precipitation and latex agglutination tests with type 14 antiserum; type 14 S60 fraction also reacted with type 49 serum by latex agglutination (see above). So, close antigenic relationship in the way of the M-protein would seem to exist between the two types. Therefore, it should be reasonable to assume that type 14 might have removed a small part of the bactericidal antibodies of type 49 as exhibited by the effect of dilution viz. the 1:10 diluted type 49 serum absorbed with type 14 S60 fraction gave rather high colony counts (217 and 130) compared with those of the corresponding 1:10 diluted type 49 serum absorbed with the homologous fraction (diffuse growth) and the 1:10 diluted unabsorbed batch of serum (28-0 colonies).

In order to confirm further the specific removal

Table 35: Results of bactericidal tests on series of dilutions of types 5 and 6 specific antisera both unabsorbed and absorbed with their homologous S60 fractions.

Serotype	Organisms dilutions	Colony counts in presence of unabsorbed antiserum dilutions									
		1:2	1:10	1:25	1:50	1:100	1:200	1:400	1:800	1:1600	1:3200
5	10^{-2}	0	0	0	0	12	29	54	260	U.C.	U.C.
	10^{-3}	0	0	0	0	0	3	9	52	U.C.	U.C.
6	10^{-2}	0	0	0	0	0	0	1	3	130	U.C.
	10^{-3}	0	0	0	0	0	0	3	5	68	U.C.

Serotype	Organisms dilutions	Colony counts in presence of absorbed antiserum dilutions									
		1:2	1:10	1:25	1:50	1:100	1:200	1:400	1:800	1:1600	1:3200
5	10^{-2}	C.	C.	C.	C.	C.	C.	C.	C.	C.	C.
	10^{-3}	U.C.	C.	C.	C.	C.	C.	C.	C.	C.	C.
6	10^{-2}	C.	C.	C.	C.	C.	C.	C.	C.	C.	C.
	10^{-3}	U.C.	U.C.	U.C.	C.	C.	C.	C.	C.	C.	C.

C. = confluent, U.C. = uncountable discrete colonies.

of the bactericidal activity by the homologous S60 fraction and to exclude any effect from diluting the serum, the bactericidal activity of absorbed and unabsorbed types 5 and 6 antisera was tested after diluting them 1:2, 1:10, 1:25, 1:50, 1:100 up to 1:3200. The results are shown in Table 35. In the unabsorbed sera of both types 5 and 6 the bactericidal activity was retained at remarkably high dilutions (1:800 and 1:1600). This killing effect on the other hand was completely removed by absorption with homologous fractions even from dilutions as low as 1:2.

D. The determination of type-specific M-antibodies in human sera by the agglutination of latex particles carrying purified S60 fractions of M-proteins

Sera from patients suspected of suffering from streptococcal sore throat, rheumatic fever or acute glomerulonephritis, and control sera, were tested for M antibody levels with the latex agglutination test by the use of purified S60 fractions of types 1, 2, 3M, 4, 5, 6, 7, 9, 12, 14, 18, 22 and 49 of group A haemolytic streptococci. A preliminary screening test was carried out by testing two dilutions (1:40 and 1:80) of each serum against each type. Sera that gave reactions of 2+ or more were further doubly diluted up to 1:10,240 to determine their M antibody

titres. A reaction of 1+ (fine agglutination) in either 1:40 or 1:80 dilution during the screening was taken as the end-point of the serum under test, and no further dilutions were made. In general the latex agglutination test was carried out by exactly the same method as that adopted for the detection of M antibodies in hyperimmune type-specific sera from rabbits.

1. M antibody titres in sera of patients suffering from sore throat and in control sera

This group of patients included 38 patients suffering from sore throat; group A streptococci were isolated from 33 of the patients at about the time of collection of the first (or only) serum specimens. Specimens of sera from 26 of them were paired and 12 were unpaired. The ages of the patients ranged from 2-30 years; 34 of them were males and 4 were females. The infecting group A streptococcus from 25 patients yielding paired sera was isolated and typed. Type 5 M-positive strains were identified in 19 of the cultures, type 12 in 3, type 22 in 2 and type 4 in 1 of the remaining 6 cultures. The serotype from the 26th patient was unknown. Cultures from patients from whom unpaired sera were collected yielded 2 type 12, 1 type 3M, 2 type 5, 1 type 25/Imp.19, 1 type 3/13/B3264 and 1 type 18. Cultures from the

Table 36. Results of latex agglutination tests on 12 unpaired sera from patients with streptococcal sore throat (uncomplicated). The latex particles were coated with purified 360 fractions of M-proteins from some serotypes of group A streptococci.

Patient	Age years	Infecting type			Interval between onset & collection of serum (weeks)	M antibody titres of serotypes											
		M- pro- tein	R anti- gen	T anti- gen		1	2	3M	4	5	6	9	12	14	18	22	49
Daw.(M)	8½	3M	-	3	3	40	40	80	640	40	40	640	40	-	40	160	-
Dav.(M)	4	12	-	12	1½	-	-	-	40	-	40	40	-	-	40	160	40
Ost.(F)	8	12	-	12	2½	-	-	160	320	40	80	40	160	-	40	80	40
Hor.(M)	7	N.A.	N.A.	N.A.	3	320	40	-	320	320	640	160	2560	40	320	40	-
Wit.(M)	13	N.A.	N.A.	N.A.	4	-	-	320	-	160	320	40	320	40	80	80	40
Kel.(M)	16	5	-	5	3½	-	-	160	320	80	320	-	320	-	80	-	160
Ref.(M)	16	5	-	5	3½	-	-	-	-	80	-	-	40	-	-	-	-
* Wol.(F)	11	18	-	18	1	320	-	640	640	640	1280	320	1280	160	160	-	320
She.(F)	8½	-	-	3/13/83264	5	-	-	320	-	160	320	80	640	-	80	-	-
Ham.(F)	2	-	-	25/Imp.19	9	-	-	-	160	-	-	640	1280	-	-	-	-
1381(M)		N.A.	N.A.	N.A.	4	-	-	160	-	40	40	80	40	40	40	40	-
1436(M)	11	N.A.	N.A.	N.A.	2½	-	-	40	-	-	-	-	80	-	-	80	-

* Repeated attacks of sore throat since January 1966 (last attack July 1967).

- = less than 40, (M) = male, (F) = female, N.A. = culture not available.

remaining 4 patients were not available for typing.

Control sera from 73 individuals not suffering from streptococcal infections were collected from routine specimens sent for Wassermann reaction at St Mary's Hospital and from 4 volunteers in this Laboratory. The ages of the subjects here ranged from 18-60 years; 13 of them were females and 60 were males.

The tests were first performed on the collection of 12 unpaired sera as a preliminary to further detailed studies on the paired sera (Table 36). Most sera gave antibody titres for more than one serotype. Serum from patient Ref. gave reactions with only two types (types 5 and 12) and their titres were low (40 and 80), while sera from patients Daw, Hor and Wol reacted each with ten types but Daw serum differed from the other two in showing low titres (40) for most of the types with which it reacted; on the other hand titres for Wol serum were all high (160 and over) but might probably be explained by the repeated attacks of sore throat experienced by this patient over more than one year. Other sera gave reactions with several types between these two extremes. It will be noticed, however,

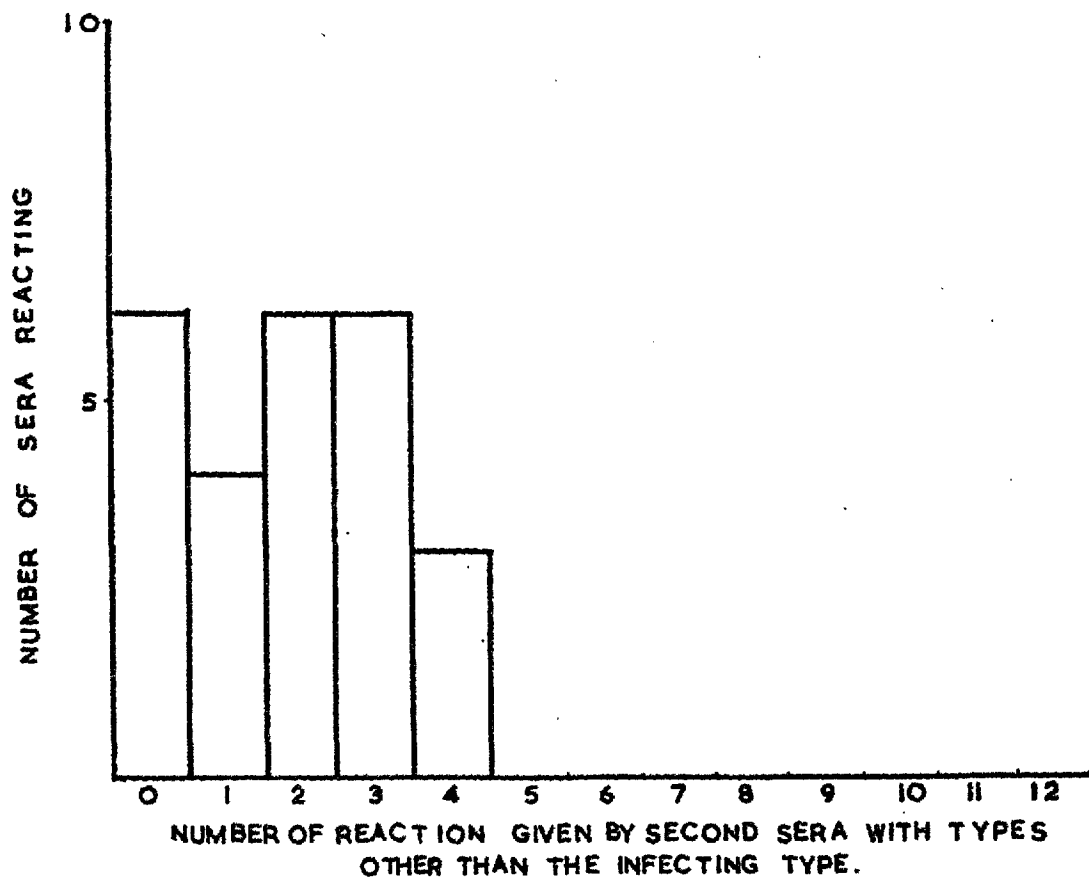


Figure 17: The number of reactions at high titres (160 & over) in second sera from 25 patients obtained by testing with S60 fractions from types other than the type responsible for the infection.

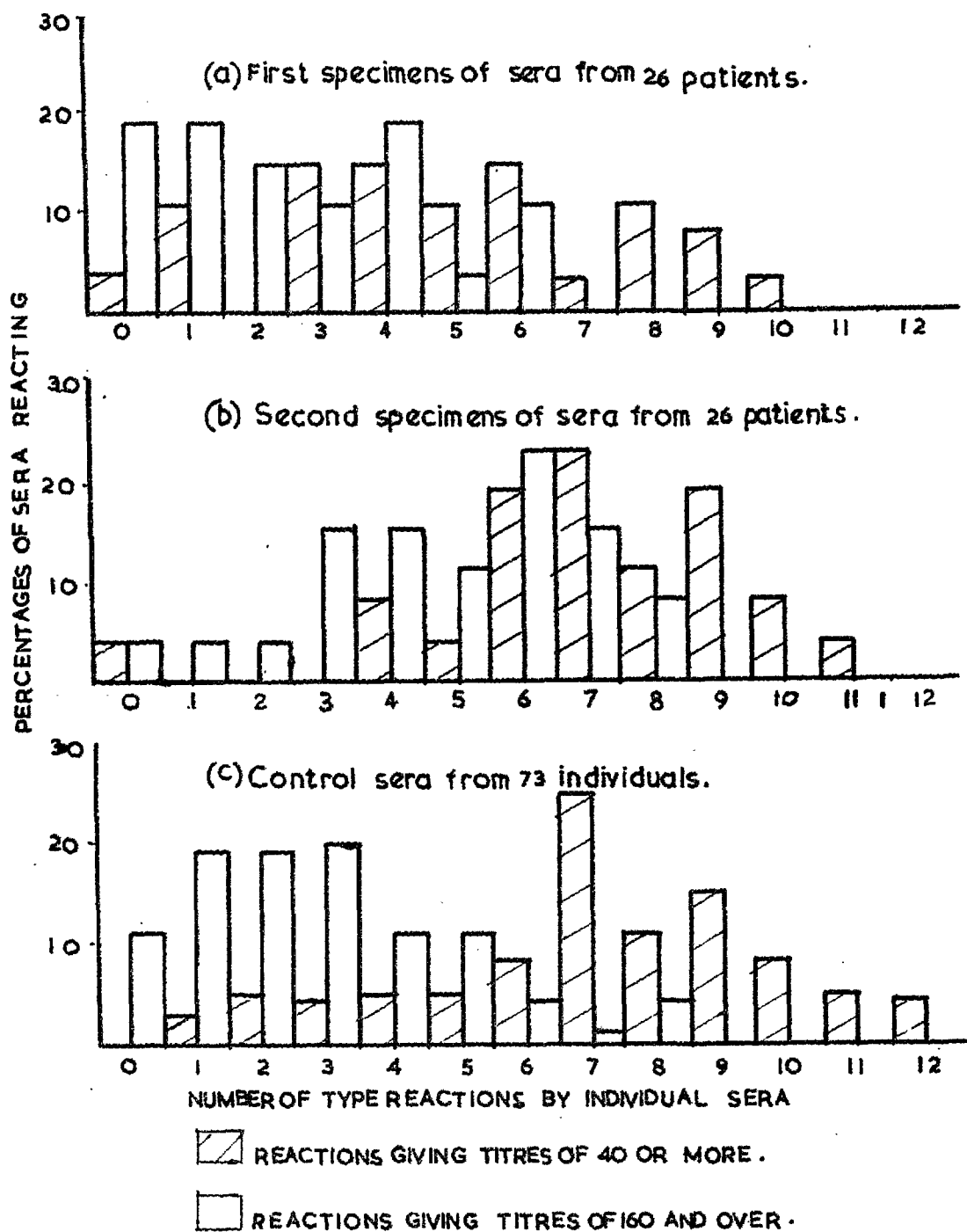


Figure 18: The numbers of type reactions of 26 paired sera from patients with sore throat and of 73 control sera.

Table 37 continued .(4).

Patient	Infecting type	Serum	Titres of M antibodies of H-types											
			1	2	3M	4	5	6	9	12	14	18	22	49
Par.(M) 16 years	22 M+	1st	-	40	160	80	40	80	40	80	-	40	40	-
		2nd	-	-	160	640	40	80	160	40	-	40	320	-
Daw.(M) 16 years	22 M+	1st	-	-	320	640	80	160	80	160	-	40	80	-
		2nd	-	40	160	640	80	80	80	320	-	40	320	80
Ill.(F) 5 years	4 M+	1st	-	40	320	320	-	320	-	640	-	40	160	160
		2nd	-	80	320	640	160	160	-	320	-	80	640	160
McD.(M) 35 years	N.A.	1st	-	40	320	-	40	40	-	40	40	-	40	40
		2nd	-	-	320	-	80	160	-	320	-	40	40	40

(M) = male, (F) = female, N.A. = culture not available.

Table 37 continued .(3)..

Patient	Infecting type	Serum	Titres of M antibodies of M-types											
			1	2	3M	4	5	6	9	12	14	18	22	49
Wol.(M) 16 years	5 M+	1st	-	80	640	320	160	320	-	640	40	160	40	-
		2nd	-	160	320	320	160	320	-	640	40	80	80	-
Spe.(M) 16 years	5 M+	1st	-	-	640	160	160	160	-	160	-	80	80	80
		2nd	80	320	320	160	640	320	-	320	-	80	160	320
Sco.(M) 16 years	5 M+	1st	-	40	80	-	-	80	-	80	-	40	-	-
		2nd	-	160	160	1280	-	320	-	160	-	160	-	-
Abe.(M) 16 years	5 M+	1st	-	80	80	-	-	40	-	40	-	40	40	40
		2nd	-	160	320	-	320	160	320	320	-	80	80	-
Pam.(F) 20 years	12 M+	1st	-	-	-	-	-	40	-	-	-	-	-	-
		2nd	-	-	160	160	-	40	-	160	-	40	80	-
Hal.(M) 16 years	12 M+	1st	-	-	160	-	-	80	-	320	-	-	-	-
		2nd	-	-	160	-	80	80	80	320	-	80	-	80
S.Skj.(M) 28 years	12 M+	1st	-	-	160	-	40	80	-	160	-	-	-	-
		2nd	-	-	320	-	80	160	-	640	-	160	320	-

Table 37 continued .(2).

Patient	Infecting type	Serum	Titres of M antibodies of M-types											
			1	2	3M	4	5	6	9	12	14	18	22	49
Hea.(M) 16 years	5 M+	1st	-	-	320	-	-	-	-	-	40	40	40	-
		2nd	-	-	640	640	320	40	-	640	-	80	160	160
Woo.(M) 16 years	5 M+	1st	-	-	-	-	160	320	-	640	-	80	-	-
		2nd	-	-	-	2560	640	320	40	640	80	160	40	40
Cur.(M) 16 years	5 M+	1st	-	-	320	-	-	-	-	-	-	-	-	-
		2nd	-	-	320	-	320	640	80	1280	-	160	-	80
Max.(M) 16 years	5 M+	1st	-	160	160	-	40	80	-	320	-	160	-	-
		2nd	-	320	160	-	160	160	40	640	-	160	-	-
Fra.(M) 16 years	5 M+	1st	-	-	-	-	-	-	-	-	-	-	-	-
		2nd	-	-	-	-	-	-	-	-	-	-	-	-
Dav.(M)	5 M+	1st	-	-	160	-	-	160	-	320	-	-	80	-
		2nd	-	-	640	-	640	160	-	320	-	320	640	-
Hos.(M) 16 years	5 M+	1st	-	-	320	-	160	320	-	640	40	80	-	-
		2nd	80	-	320	-	320	320	160	1280	40	160	160	-
Cha.(M) 16 years	5 M+	1st	-	-	-	-	-	40	-	-	-	-	-	-
		2nd	-	-	80	-	-	40	-	160	-	40	-	-

Table 37. Results of latex agglutination tests on 26 paired sera from patients suffering from upper respiratory tract infection. The latex particles were coated with purified S60 fractions of M-proteins from several serotypes of group A streptococci.

Patient	Infecting type	Serum	Titres of M antibodies of M-types											
			1	2	3M	4	5	6	9	12	14	18	22	49
Eva.(M) 16 years	5 M+	1st	-	-	-	-	-	320	-	320	-	40	40	-
		2nd	-	40	-	-	320	160	-	320	-	40	80	-
Joh.(M) 16 years	5 M+	1st	40	40	320	320	320	-	40	320	-	80	160	160
		2nd	40	40	320	160	320	320	-	320	40	160	160	160
Wic.(M) 16 years	5 M+	1st	-	-	-	-	40	-	-	160	-	40	80	40
		2nd	-	-	160	-	160	160	-	160	-	40	160	40
Fle.(M) 16 years	5 M+	1st	-	40	320	40	40	160	40	320	-	80	160	-
		2nd	-	-	320	640	160	320	160	640	-	80	160	-
McD.(M) 16 years	5 M+	1st	-	-	160	-	40	40	-	80	-	40	40	-
		2nd	-	-	320	-	160	160	-	160	-	40	40	-
Sav.(M) 16 years	5 M+	1st	-	-	640	-	160	320	-	320	-	-	-	-
		2nd	-	-	640	-	160	640	-	320	-	40	-	-
Hay. 16 years	5 M+	1st	-	-	160	-	-	40	-	320	-	-	-	-
		2nd	-	-	320	-	320	-	-	160	40	-	-	-

that the highest titre was seldom for the type which appeared to have been responsible for the most recent infection.

The results for the 26 paired sera are set out in detail in Table 37. It will be observed that of 25 cases in which the presumed infecting type was known, there was a high titre of antibody (>160) for the type in the second serum specimen in all except three; in 16 cases there was a four-fold increase in titre with a final titre of 160 or more; and in 6 cases the initial and final titres were both 160 or more, while 3 cases were with no antibody response. However, equally significant rises in heterologous antibody titres were very common. In only 6 pairs of sera was there no four-fold or greater rise in titre for any type other than the infecting one (where the final titre was >160); in 4 pairs there was one heterologous rise in titre, in 6 pairs there were two, in another 6 pairs there were three and in 3 pairs there were four (Figure 17).

The results on all the first and second sera compared with the results on the control sera are summarized in Figure 18. In each case the number of types with which the sera reacted, at a titre of

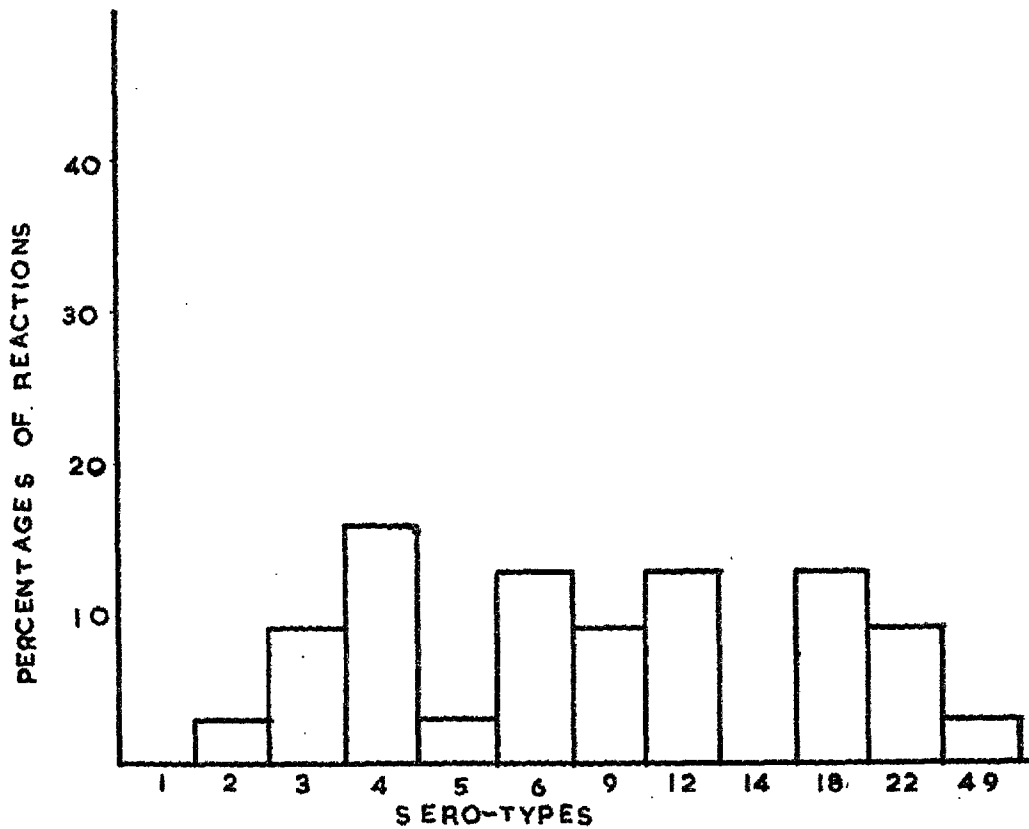


Figure 19: The percentages of reactions at high titres of type-specific antibodies against serotypes other than the infecting serotype in 12 out of 26 second sera of patients.

40 or more, and at a titre of 160 or greater, is shown separately. Practically all of the sera in each group gave a titre of 40 for one or more types. Titres of 160 or more for one or more types were given by 81% of first sera, 96% of second sera and 89% of control sera.

Most of the sera whether from patients or controls reacted with more than one M-type antigen. Considering only reactions of 160 or greater, 45% of first sera and 51% of control sera reacted with 3 or more M-type extracts. A considerably greater proportion (87%) of second sera gave 3 or more strong reactions. This is in agreement with the observation (Table 37) that increases of titres to heterologous types occurred with considerable frequency.

Figure 19 shows the relative frequencies of heterologous reactions with M-extracts of different types in 12 of the second specimens of the paired sera whose titres were 160.

2. M antibody titres in sera of patients suffering from rheumatic fever

Sera from 9 patients were tested; 2 of them were paired and the rest unpaired. The ages of the patients ranged from 11-50 years and 5 of them were females and

Table 38. Results of latex agglutination tests on sera from 9 patients suffering from rheumatic fever. The latex particles were coated with purified S60 fractions from twelve different types of group A streptococci.

Patient	Age	Sex	Interval between onset & collection of serum (weeks)	Infecting type	M antibody titres of serotypes											
					1	2	3M	4	5	6	9	12	14	18	22	49
1310	14	M	4½	N.A.	-	-	640	-	40	-	40	-	-	40	40	40
1370	26	M	15½	N.A.	40	-	320	-	320	320	-	320	-	160	-	80
1378	51	F	3	N.A.	80	40	640	40	160	160	80	640	-	80	40	-
° { 13801	15	F	9	N.A.	-	-	640	-	40	80	-	80	-	160	-	-
{ 138011	15	F	23	N.A.	-	40	1280	40	320	160	-	160	-	320	40	-
1393	35	F	3	N.A.	-	-	-	-	40	40	-	-	-	-	40	-
° { 13991	11	M	6	N.A.	-	-	80	-	-	-	40	-	-	-	40	-
{ 139911	11	M	14	N.A.	40	-	80	-	-	-	40	-	-	40	-	-
1404	18	F	2	N.A.	-	-	160	-	40	40	-	40	-	-	-	-
1490	12	F	5	N.A.	-	-	1280	320	-	80	-	160	-	-	-	-
1465	35	M	> 4	N.A.	320	640	640	1280	160	320	320	640	80	80	320	80

° paired sera, N.A. = not available.

4 were males. Streptococcal cultures from these patients were not available. The results are shown in Table 38. Reactions with more than one serotype was the dominating picture. The pair of sera from patient 1380 showed a distinct and high rise in type 5 antibody titre. The other pair of sera from patient 1399, however, showed no such rise of antibody titre to any of the types tested but showed low titres (80 and less) for a few of them. As before, antibodies to types 3M, 6 and 12 were by far the most frequently detected antibodies of group A streptococci showing high titres. In conclusion there were no significant differences between these patients and the previous group of patients suffering from uncomplicated sore throat by comparing the type-specific antibody pattern in both (Tables 36, 37 and 38).

3. M antibody titres in sera of patients suffering from acute glomerulonephritis

Six paired and 1 unpaired sera of patients suffering from acute glomerulonephritis were tested as before. They included 5 paired specimens from Red Lake Indian children (4 females and 1 male) and the remaining 2 from children (male and female) in this country. The serotypes isolated from the Red

Table 40. Results of latex agglutination tests on sera of patients suffering from acute glomerulonephritis. The latex particles were coated with purified S60 fractions of M-proteins from twelve different serotypes of group A streptococci.

Patient	Age	Sex	Interval (wk) between		M antibody titres of serotypes											
			onset & 1st serum	1st serum & 2nd serum	1	2	3M	4	5	6	9	12	14	18	22	49
Rai(1)	6	M	5 *	5½	-	-	160	320	80	160	-	320	-	80	-	640
Rai(11)					-	-	160	640	80	160	-	320	160	40	-	1280
Jou(1)	6	F	4 *	6	-	-	-	-	-	-	-	-	-	-	-	320
Jou(11)					-	-	-	-	-	-	-	-	40	-	-	640
May(1)	4	F	10 *	10½	-	-	160	320	-	80	-	160	-	40	-	320
May(11)					-	-	160	320	320	160	-	160	-	80	-	640
Def.(1)	9	F	11 *	13	-	-	-	1280	320	-	-	-	160	40	40	160
Def.(11)					-	-	160	1280	320	80	-	160	80	320	-	2560
Ben.(1)	11	F	2 yr. *	2 yr & 4/52	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	320
Ben.(11)					80	-	160	640	160	80	320	160	-	80	160	320
1406	10	M	4 †	-	-	-	80	-	-	-	-	40	40	-	-	160
1493(1)	5	F	3 †	9½	40	80	160	160	-	-	-	-	40	40	40	40
1493(11)					640	40	80	1280	40	320	-	160	40	40	40	40

M = males, F = females, (1) = 1st serum, (11) = second serum, N.D. = not done because serum was insufficient. * 1st serum collected before onset, † 1st serum collected after onset.
wk. = weeks.

Table 39. Results of follow up cultures from skins, noses and throats of 5 Red Lake Indian children suffering from acute glomerulonephritis showing the predominating type 49 and the appearance of other types of group A streptococci (Wannamaker, 1967)

Patient	Age	Sex	follow-up period	Streptococci of group A serotypes isolated					
				Skin		Throat		Nose	
				most isolations	occasional isolations	most isolations	occasional isolations	most isolations	occasional isolations
Rai.	6	M	6½ wk.	type 49 "T"	41"M" T3/13/B	49"T"	31"M" & "T" 41"M" T3/13/B	-	41"M" T3/13/B 49 "T"
Jou.	6	F	6 wk.	49"T"	-	-	49"T"	-	49"T"
May.	4	F	10½ wk.	-	49"T"	-	41"M" T3/13/B 12"M" & "T" 49"T"	-	-
Def.	9	F	5 wk.	49"T" 14"T" *	25"M" T8/25/1mpl9	-	25"T" 25"M" T8/25/1mpl9 14"T"	-	-
Ben.†	11	F	4½ wk.	49"T"	-	49"T"	-	-	14"T" * group G group A

* presumably 49"T" (Wannamaker, 1967).

† In 1965 strains of T5/27/44, T3/13/B & Impl9 were isolated (Wannamaker, 1967).

M = male, F = female, "M" = M-protein type, "T" = T type, wk. = weeks.

Lake children were identified as type 49 and glomerulonephritis was noticed to follow skin infection with it (Wannamaker, 1967). Besides type 49 other types were also isolated during the follow-up period which started several weeks before the onset of the disease and in the case of one patient it started 2 years before onset (Table 39). In general type 49 was by far the most prevalent type of group A streptococci isolated from the skins of these particular children who developed glomerulonephritis. Type 49 was also isolated in certain occasions from the throat and nose during the follow-up period.

The five paired sera from the Indian children together with the sera from the 2 English children were tested by the latex agglutination as before. The results are shown in Table 40. As expected all Red Lake sera gave high titres for type 49 both initially and in convalescence. Reactions for type 14 were more frequent than in sera from this country (Tables 36, 37 and 38). Also type 4 showed more reactions than before. Types 3, 5, 6, 12 and 18 showed the same frequency of reactions as before. Reactions of type 22 were rarer and as usual types 1 and 2 showed the fewest reactions.

Table 41. Results of latex agglutination tests for types 3, 4, 5, 6, 12 & 49 H antibodies compared with results of bactericidal tests for the same antibodies in the sera of some patients and controls.

Patient	Diagnosis	Infecting serotype	Antibodies tested	Latex agglutination titres		Bactericidal activity *	
				1st serum	2nd serum	1st serum	2nd serum
Rai.	GN	type 49	type 49	640	1280	+	+
Def.	"	" 49	" 49	160	2560	+	+
Steve	S.T.	" 12	" 12	160	640	+	+
Wol.	"	" 12	" 12	1280	N.A.	+	N.A.
Hor.	"	" 12	" 12	2560	N.A.	+	N.A.
Ill.	"	" 4	" 4	320	640	+	+
Rai.	GN	" 49	" 4	320	640	+	+
May	"	" 49	" 4	320	320	+	+
Def.	"	" 49	" 4	1280	1280	+	+
Dav.	S.T.	" 5	" 5	<40	640	-	+
Spe.	"	" 5	" 5	160	640	+	+
Dav.	"	" 5	" 3	160	640	+	+
1370	R.F.	unknown	" 3	320	N.A.	+	N.A.
B(cont)	H.	no infection	" 3	320	N.A.	+	N.A.
M(cont)	N.	" "	" 3	160	N.A.	+	N.A.
F(cont)	N.	" "	" 3	320	N.A.	+	N.A.
1490	R.F.	unknown	" 3	640	N.A.	+	N.A.
Hor.	S.T.	type 5	" 6	640	N.A.	+	N.A.
1370	R.F.	unknown	" 6	320	N.A.	+	N.A.
H.E(cont)	N.	no infection	types 3,4,5,6,12 & 49	all types negative	all types negative	all types negative	all types negative

* The bactericidal activity was taken as the lowest dilution of organisms that showed no growth in presence of the homologous antiserum. GN = glomerulonephritis, R.F. = rheumatic fever, S.T. = sore throat, N = normal, N.A. = serum not available.

4. Verification of some results of latex agglutination tests for M antibodies in human sera by the indirect bactericidal test

Paired and unpaired sera chosen at random from all patients and controls tested above were tested by the indirect bactericidal test to verify some of the high antibody titres to some serotypes. Red Lake sera were tested for type 49. For type 12 one paired and one unpaired serum from one of the 26 patients and one of the 12 patients suffering from uncomplicated sore throat were chosen, both patients had an infection with type 12; for type 5 four paired sera from 4 patients originally infected with type 5 (also from the same group of patients) were chosen. Similarly, for type 4 serum from a patient with type 4 infection from the 26 patients was tested, and in addition 4 of the Red Lake sera which showed high antibody titres for type 4 were also tested for this type. For type 3 and type 6 also paired and unpaired sera were tested. There was good agreement between the M antibodies as detected by latex agglutination and the bactericidal activity (Table 41). The bactericidal activity was expressed as the lowest dilution of streptococci that showed no growth in the presence of homologous anti-

serum. Attempts to develop a quantitative technique for the indirect bactericidal test in order to facilitate comparisons in titres with the latex agglutination titres were not successful owing to the laborious technique for performing a two dimensional bactericidal test, the variation in counts from culture to culture of the same strain and several other factors all of which led to the lack of reproducibility of the results. Therefore the bactericidal activity in a qualitative manner was adopted as a guide to whether the titres shown by the latex agglutination test were due to their homologous streptococcal antigen. In that sense there was satisfactory agreement between the two tests especially for types 49, 12, 3, 5 and 6. In the case of type 4 it should be noted that the killing effect was less marked. On the other hand the M antibodies detected in some of the control sera showed a marked bactericidal activity against the homologous organisms. One of these control sera showed no antibody titres for types 3, 4, 5, 6, 12 and 49 by latex agglutination and consequently gave no bactericidal activity against any of the homologous streptococci. This was, therefore, a satisfactory negative control.

PART FOUR

DISCUSSION

As predicted, polystyrene latex particles proved to be useful carriers of streptococcal cellular antigens. Agglutination tests with suspensions of latex particles sensitised with antigen are a convenient, sensitive and reliable means of detecting antibody in sera. The

advantages of the method may be summarised as follows:

(1) Polystyrene latex particles are readily available from commercial sources in suspensions of uniform particle-size (0.81μ), and are ready for immediate use with a minimum of preparation.

(2) The non-biological nature of the latex particles circumvents many of the difficulties inherent in agglutination methods. In bacterial agglutination, the antigenic complexity of the cell surface leads to lack of precision. If bacterial antigens are adsorbed on to erythrocytes there is often trouble with intraspecies cross-reactions and with haemolysis. Latex particles have simple surfaces to which antigens of diverse chemical composition are readily adsorbed without previous modification. In contrast, red blood cells often have to be tanned to modify their

surfaces before antigens can be adsorbed to them. Also, sometimes it may be necessary to restrict extraction of the antigen to a single method in order to obtain a preparation that is adsorbed efficiently to the surface of the tanned red cells. Schmidt & Moore (1965) showed that formamide-extracted streptococcal polysaccharide did not sensitise tanned human O erythrocytes for haemagglutination tests, and obtained efficient sensitisation of the erythrocytes only with the ethanol-precipitated fraction of an extract prepared with Streptomyces albus enzymes which had been treated with di-isopropyl fluorophosphate. Goldstein & Caravano (1967), on the other hand, were able to sensitise red cells with formamide-extracted polysaccharide only after coupling the latter with an ester of stearic acid. Since formamide-extracted polysaccharide is easy to prepare in a serologically pure form, the latex agglutination test with the unmodified extract and the unmodified latex particles is obviously convenient for all practical purposes. Like the polysaccharide, type-specific protein antigens are readily adsorbed to latex particles.

(3) Suspensions of latex particles are stable. Non-specific or spontaneous aggregation of latex particles did not present as a problem all through this work. The latex particles suspensions with adsorbed antigen (latex-antigen suspensions)

were quite stable. Batches of latex-antigen suspensions that were prepared during the course of this work generally remained stable until used up (4-6 weeks). However, to achieve this stability some precautions were necessary. First, bacterial contamination was excluded by the addition of merthiolate to a final concentration of 1:10,000. Second, the glass containers used were chemically clean and sterile; cleanliness was achieved by washing in acid and rinsing in distilled water. Third, the latex-antigen suspensions were made up in phosphate-buffered saline, pH 7.2-7.5 to avoid wide shifts in pH. Our current knowledge is that latex particles are precipitated at pH 9.0 and over. On the other hand M-proteins are precipitated at a pH range of 5.0-6.0 with an isoelectric point of 5.3 (Lancefield & Perlmann, 1952 a). To avoid these two extremes pH 7.2-7.5 was therefore chosen and proved to be quite satisfactory. Latex-antigen suspensions were conveniently stored at refrigerator temperature and were ready for immediate use.

(4) Latex agglutination tests appear to be very sensitive. Titres of 1280-5120 were frequently obtained when sera were tested with suspensions sensitised both with group-specific polysaccharide and with M-protein antigen extracts. Although no direct comparisons were made there is reason to believe that sensitivity is of the same order as that

obtained in haemagglutination tests.

(5) Since the surface of the latex particles is inert, it is to be expected that the specificity of reactions obtained would be limited only by the purity of the antigenic fractions adsorbed to them. With the streptococcal group antigens, which can be obtained in a reasonable state of purity with little trouble, latex agglutination tests appear to be quite specific, and the cross-reactions observed with rabbit hyperimmune sera were referable to known chemical relationships between the antigens. With the M-protein fractions, on the other hand, it was difficult to be sure when cross-reactions were due to the relative impurity of the extracts and when they represented real interrelations between antigens. Such cross-reactions were observed occasionally with the rabbit antisera used for M-typing and thought to be monospecific on the basis of precipitation reactions; they were very much more common in the sera of convalescent patients. Their possible significance will be discussed later.

The first application of the latex agglutination in this work was the use of latex suspensions with adsorbed group A specific polysaccharide for the detection of group A specific antibodies in hyperimmune rabbit sera and in patients' sera. The results obtained confirmed

the possibility of adopting this test for routine use. Other methods that have been developed recently are of no advantage over the latex agglutination test; the disadvantages of the haemagglutination techniques (Schmidt & Moore, 1965; Goldstein & Caravano, 1967) were fully discussed above. However, the other technique that would closely compete with this test is the cell wall agglutination technique (Karakawa et al, 1965). Like the latex agglutination test, this is a highly sensitive and a highly specific test, but it is laborious and more complicated. It employs a modified Coombs test for the detection of group A antibodies in human sera, viz. indirectly. Also the preparation of suitable batches of cell wall suspensions, the centrifugation of cell walls and serum dilution mixtures in the cold, the reading of the end-points of the titrations by slide-agglutination, all make the test less handy for routine purposes and general surveys in spite of its high sensitivity.

In addition to group A polysaccharide, groups A-var, B, C, G and L polysaccharides have all been successfully adsorbed to latex particles and gave reactions with their homologous antisera. The finding that group A-var polysaccharide reacts to a high titre in group A antiserum is in agreement with previous findings by McCarty & Lancefield (1955), and also by Schmidt & Moore (1965), Karakawa et al (1965) and Karakawa, Lackland & Krause (1966a). In the

results obtained in this work, group A polysaccharide reacted to an insignificant titre of 10 in group A-var antiserum by the use of the latex agglutination test. Also, in cell wall agglutination test, Karakawa et al (1965) obtained a titre of 160 with group A cell walls and a group A-var antiserum which had a homologous titre of 2560. The original work of McCarty & Lancefield (1955) also showed this reaction of group A polysaccharide in group A-var antiserum to occur in a minor degree. The cross-reactions involving groups A, A-var, C and G agree with those reported by Cummins & Slade (1961) amongst groups A, C and G. Krause & McCarty (1962) showed the similarities in antigenic determinants of groups A-var, and C, viz. rhamnose oligosaccharides. Furthermore, Krause (1963) reported the presence of rhamnose and galactosamine in the polysaccharide of group G streptococci, and Curtis & Krause (1964) found that the antigenic determinant in group G was rhamnose monosaccharide. Rhamnose and galactosamine are also present in group C whose antigenic determinant is galactosamine. Another reaction noted was given by group A polysaccharide in group O antiserum. Our knowledge of group O and the details of its antigenic structure is far from satisfactory, so an explanation of this reaction of low titre cannot be pursued on the same immunochemical basis followed above.

However, the nature of this particular reaction was that of a weak agglutination (1+ reaction) and could be due to a minor relationship.

The human sera tested for the presence of streptococcal group antibodies belong to two main categories, viz. patients thought to be suffering from streptococcal infection and sequelae and control subjects with no such infection. The diagnosis of streptococcal infection and sequelae was based on the clinical picture confirmed by either isolation of group A streptococci from the patient or the demonstration of elevated ASO titres in the serum or both. The control subjects were chosen from individuals who had no such clinical or laboratory criteria and, therefore, were expected to yield lower antibody titres. Thus about 90% of the former category showed demonstrable antibodies against the group-specific antigen, compared with less than 40% of the latter. This difference argues in favour of group-specific antibodies being a response to either a past or a present group A streptococcal infection.

A further comparison of the titres of antibody to group A polysaccharide and of ASO in individual sera showed that, when the ASO titre was raised, significant amounts of group antibody were usually present. On the other hand, a considerable number of other sera contained group antibody

without the ASO titre being raised. This could be explained in one of three ways:

(1) The "group A antibody" was not related to a streptococcal infection. This is unlikely, in view of the specificity of the test with rabbit antisera, and the relative infrequency of group antibody in the sera of patients not currently suspected of suffering from streptococcal infection.

(2) That the group antibody test is very much more sensitive than the ASO test for detecting streptococcal infection.

(3) That the antibody response to group polysaccharide and to streptolysin O have different time relationships.

Since ASO is formed early in disease the most likely possibility is that antibody response to group polysaccharide might take place later and might persist longer. The appearance of group antibody at detectable low levels seem to take place more than one week after onset of infection. This is well demonstrated by patient 1381 and patient 1399 (Table 17 facing page 109). On the other hand after reaching the peak these antibodies seem to last at least for six months without showing any decline in titre as is normally seen with ASO (Table 17). It should be remembered that hyperimmunisation of rabbits with group A cells gave rise to antibodies at low levels only a week after the initial dose of the vaccine (Table 15 facing page 102) and reached

the peak in about the third week. High levels of antibodies in these rabbits were detected even after three months rest from vaccination.

Possibilities (2) and (3) will be discussed together. It is generally believed that ASO is detectable in significant amounts in the sera of some 70% of patients suffering from streptococcal disease. However sensitive the test for group antibody was, therefore, it is unlikely that all the cases with this antibody but without a raised ASO titre were recent streptococcal infections. The patients from whom the bulk of one single sera were obtained were suspected of suffering from the sequelae of streptococcal infection and were generally fairly late in the course of whatever disease they had. Examination of the available case-histories suggested that many had suffered from recurrent upper respiratory tract disease. It was possible, therefore, that their streptococcal illness might often have ante-dated the onset of the current episode of illness.

We had hoped to learn more of the time relations of the response to streptococcal group antibody from the examination of paired sera, but it proved difficult to obtain large numbers of pairs from patients with known streptococcal illness in which the first sample was collected in the first few days of illness. In the 26

pairs of sera where this appeared to be the case, it was found that the initial titres were generally high, and that further rises were few and insignificant. Most of these sera came from one of two sources: (1) young men in a single closed community in which streptococcal infection is known to be constantly endemic (2) Red Lake American Indian children who have repeated skin infections (Anthony, Kaplan, Chapman, Quie & Wannamaker, 1967). It is very likely that in both cases the high level of group antibody referred to previous infections. This conclusion is suggested by the finding that in many of the same pairs of sera there was evidence of a significant rise of type-specific antibody to the current infecting strain (type 5 in the community of young men, and type 49 at Red Lake).

Further study of group antibody in the sera of patients with known streptococcal infections is needed to establish the time relations of the antibody response. It is clear, however, that the presence of antibody is related to streptococcal infection, and the test for it may therefore be of value in epidemiological studies. This is particularly the case in countries where a high proportion of sera contain significant amounts of ASO, but few streptococci have been isolated, such as the Sudan (Dunbar & Erwa, 1967) and Nigeria (Rotta, 1967b). It is not yet certain whether

these results are due to the presence of antibodies referable to streptococcal disease, or to non-specific inhibitors of streptolysin O.

What has been said about the response to group A specific antibodies in human sera is also true for group A-var antibody response. Infection with group A-variant has not been reported in the general population. Yet its antibodies are detectable in the vast majority of patients suffering from group A infection and even in rabbits immunised with group A cells (Table 15). Degradation of the bacterial cell follows soon after phagocytosis, leading to loss of the antigenic determinant of group A polysaccharide (N-acetylglucosamine) and presumably exposing the oligosaccharide moiety which is the antigenic determinant of group A-var. Ayoub & Wannamaker (1967) attributed this intraphagocytic effect to the action of a multiplicity of factors including digestion with the intra-phagocytic enzyme β -N-acetylglucosaminidase. In this work the majority of patients had antibodies to both antigens, suggesting that the degradation of N-acetylglucosamine is not complete under natural conditions and that enough of it will escape digestion and thus give rise to group A specific antibodies. On the other hand a small minority of the patients behaved differently; 2 patients had responded by forming group A

antibodies only and 2 by forming group A-var antibodies only (Table 20 facing page 111). Explanation of these results on the whole is difficult but again the suggestion may be made that for unknown biological reasons degradation of group A specific polysaccharide in the former two patients was negligible while it was greatly enhanced in the latter two patients.

In the work involving the type-specific M-proteins of group A streptococci it has been shown that they appear in at least two serologically active fractions. One of these two fractions viz. the S60 fraction was found to be more specific than the other fraction viz. the P60 fraction. This implies either that amongst the several antigenic parts of the M-protein of each serotype one part is specific to the particular serotype with minimum cross-reactions, if any, with heterologous types, or that the M-protein in the S60 fraction is relatively free from contamination with the less specific proteins. However, specificity of reactions by precipitation and latex agglutination is not the only characteristic of the S60 fractions but also their ability to remove bactericidal activity of hyperimmune rabbit sera should be put into account when relating them to the M-proteins. These two criteria have favoured the S60 fractions over the P60 fractions as reliable representatives of M-proteins for accurate identification of serotypes.

The complexity of protein antigens extracted in hot acid has been demonstrated by Wilson & Wiley (1963) by a study on serotype 17 streptococci. In these organisms the authors have demonstrated one fraction of the protein extract that had both specific serological reaction and antibactericidal activity associated with M antigen.

The reactions between latex suspensions sensitised with S60 fractions and rabbit hyperimmune sera were in the main type-specific, but a few cross-reactions were seen. Although in general the M antigens of streptococci are believed to have separate antigenic specificities the conclusion was reached on the basis of relatively insensitive tests such as the precipitin reaction. In the literature there are descriptions of several individual cross-reactions, and this number has increased lately. Examples include the cross-reactions between types 1 and 3 (Hirst & Lancefield, 1939), types 3 and 12 (Fox & Wittner, 1967), and between certain "non-typable" streptococci and type 33 (Wiley & Bruno, 1967). The relation between type 14 and 51 (Wiley, 1960) appears to be of a rather different character, since the streptococcus is in this case capable of producing two serologically distinct M-proteins. Moreover, Maxted & Potter (1967) have demonstrated the presence of type 12 M-protein in some strains of group G streptococci isolated

from patients. The possession of type antigens of protein nature by group C streptococci is known, but whether they bear any relationship to group A M-proteins is not yet known. On the whole more information is needed to clarify the antigenic relationships amongst the different M-proteins of group A streptococci and between them and protein antigens of other groups of streptococci particularly groups C and G. This may prove of some value in our studies of protection against streptococcal infections.

The study of cross-reactions related to M-proteins of various serotypes is handicapped by many factors, amongst which is the non-availability of a simple method of sufficient sensitivity. The bactericidal test is difficult to perform as a routine test, but it is sensitive enough to establish the identity of M-protein. It is much preferred to the mouse protection test, which is less sensitive and presents several difficulties as mentioned in Part One (page 49). Latex agglutination with S60 fractions seems to be a promising method. It is highly sensitive, as demonstrated by the comparative studies presented above on absorbed and unabsorbed typing sera from hyperimmunised rabbits. In comparison with the precipitation reactions its sensitivity is overwhelming. Furthermore, the antibodies detected by the latex agglutination test are in

general correlated to the presence of bactericidal activity of sera, and absorption of the sera with S60 fractions resulted in specific removal of bactericidal activity.

Cross-reactions have been met with in latex agglutination tests with S60 fractions of types 12, 14, 18 and 49. Type 12 S60 fraction reacted in type 15 antiserum; type 14 S60 fraction reacted in types 49 and 51 antisera; type 49 in types 14 and 51 antisera, while type 18 S60 fraction reacted in types 17, 19, 23 and 30 antisera (Table 33 facing page 126). In the case of types 14 and 49 it has been suggested in cross-absorption tests involving bactericidal tests that bactericidal antibodies of type 49 were partially removed by type 14 S60 fraction, thus giving more evidence of antigenic relationship between the two types.

The application of the latex agglutination test with S60 fractions for the detection of type-specific antibodies in patients' sera gave results that were encouraging but in some ways difficult to interpret. Antibody was detectable in high titre, and in a number of cases it was possible to demonstrate a significant rise in titre for the infecting streptococcus in paired sera. That this was indeed M antibody was confirmed by the bactericidal test, which gave results that were comparable with the latex agglutination titres.

Most human sera appeared to contain type-specific antibody to several different M-types in agreement with results reported by Markowitz (1963) and Stoyanova (1966) who both used an indirect bactericidal test. In this respect there was little difference between the "acute-phase" sera of patients with respiratory tract infection and sera from persons not suspected of having streptococcal infection; but second specimens of sera from patients convalescent after streptococcal infection had on average high titres to more types of streptococci. An examination of the results obtained with individual pairs of sera revealed many cases in which there was a significant rise in titre for one or more heterologous types. This was difficult to explain, particularly since the cross-reactions did not appear to occur in any definite pattern. Several possibilities exist.

- (1) The cross-reactions may be due to impurities in the S60 fractions. The presence of other protein materials less type-specific than the M-protein, even in very small amounts, may sensitise the surface of the latex particles sufficiently to give rise to relatively strong cross-reactions.
- (2) The reactions might represent true cross-reactions between M-proteins that are revealed for the first time because of the great sensitivity of the latex agglutination test.

(3) They may be some form of anamnestic reaction. If so, their pattern would be more closely related to the previous experience of the patient than to the antigenic structure of the organisms or the impurities in a particular extract. Although anamnestic reactions are frequently mentioned in the bacteriological literature, there are few well documented accounts of their occurrence.

Correlation of the results obtained from testing sera from a group of patients infected with type 5 streptococci showed a variety of heterotypic responses. Had these results been due to non-specific material in the extracts, or to cross-reactions between the M5 antigens and other antigens, a more definite pattern would have been expected. The idea that a second streptococcal infection might stimulate the formation of antibody for a type responsible for a previous infection is, however, difficult to accept.

It is clear, however, that the latex agglutination test with S60 fractions cannot be used as a means of detecting type-specific antibody in the sera of patients for whom the infecting type is not known until some way has been found of distinguishing between homotypic and heterotypic reactions. Nevertheless, the test might be of value in deciding whether a respiratory infection is due to a streptococcus that has been isolated from the patient, or is a virus infection in a carrier of group A streptococci.

REFERENCES

- Abdulla, E.M., & Schwab, J.H. (1965a) Proc. Soc. exp. Biol. Med., 118, 359.
- Abdulla, E.M., & Schwab, J.H. (1965b) Fed. Proc., 24, 186.
- Abdulla, E.M., & Schwab, J.H. (1966) J. Bact., 91, 374.
- Anthony, B.F., Kaplan, E.L., Chapman, S.S., Quie, P.G., & Wannamaker L. (1967) Lancet, 11, 787.
- Araujo, P., & Krause, R.M. (1963) J. exp. Med., 118, 1059.
- Ayoub, E.M., & Wannamaker, L.W. (1963) J. Immunol., 90, 793.
- Ayoub, E.M., & Wannamaker, L.W. (1967) Fed. Proc., 26, 581.
- Barkulis, S.S., & Jones, M.F. (1957) J. Bact., 74, 207.
- Bernheimer, A.W. (1948) Bact. Rev., 12, 195.
- Bernheimer, A.W., & Catoni, G.L. (1945) J. exp. Med., 81, 295.
- Bernheimer, A.W., Lazarides, P.D., & Wilson, A.T. (1957) J. exp. Med., 106, 27.
- Bernheimer, A.W., & Rodbart, M. (1948) J. exp. Med., 88, 149.
- Boyden, S.V. (1951) J. exp. Med., 93, 107.
- Bozicevich, J., Bunim, J.J., Freund, J., & Ward, S.B. (1958) Proc. Soc. exp. Biol. Med., 97, 180.
- Bray, J.P., Bass, J.A., Dodge, W.F., & Travis, L.B. (1966) Bact. Proc. 1966, 59.
- Brighton, W.D. (1967) Personal communication.
- Carlisle, H., & Saslaw, S. (1958) J. Lab. Clin. Med., 51, 793.

- Carlson, A.S., Kellner, A., & Bernheimer, A.W. (1956)
J. exp. Med., 104, 577.
- Carlson, A.S., Kellner, A., & Bernheimer, A.W. (1957)
J. exp. Med., 106, 15.
- Cayeux, P., & Wahl, R. (1964) Zentbl. Bakt. I. Ref., 196, 658.
- Christensen, L.R. (1945) J. Gen. Physiol., 28, 363.
- Cinader, B., & Pillemer, L. (1950) J. exp. Med., 92, 219.
- Coffey, J.M. (1938) J. Immunol., 35, 121.
- Cole, R.H., & Hahn, J.J. (1962) Science, 135, 722.
- Colman, G., & Williams, R.E.O. (1965) J. Gen. Microbiol.,
41, 375.
- Cromartie, W.J., & Craddock, J.G. (1966) Science, 154, 285.
- Crowley, N. (1950) J. Gen. Microbiol., 4, 156.
- Crowley, N. (1954) J. Gen. Microbiol., 10, 411.
- Crowley, N. (1955) J. Gen. Microbiol., 13, 218.
- Crowley, N., & Jevons, H.P. (1955) J. Gen. Microbiol., 13, 226.
- Cummins, C.S., & Harris, H. (1956) J. Gen. Microbiol., 14, 583.
- Cummins, C.S., & Slade, H.D. (1961) Bact. Proc. 1961, 94
- Curtis, S.N., & Krause, R.M. (1964) J. exp. Med., 119, 997.
- Deibel, R.H. (1964) Bact. Rev., 28, 330.
- Denny, F.W., Jr., Perry, W.D., & Wannamaker, L.W. (1957)
J. Clin. Invest., 36, 1092.
- Dische, Z. (1955) in Methods of Biochemical Analysis, vol. II,
edited by D. Click, Interscientific Publishers, Inc.,
New York. P.313.

- Dische, Z., & Shettles, L.B. (1948) *J. Biol. Chem.*, 175, 595.
- Duboczy, B.O., & White, F.C. (1966) *Am. Rev. Resp. Dis.*,
94, 914.
- Dunbar, J.M., & Erwa, H.H. (1967) *Bull. Wld. Hlth. Org.*,
37, 492.
- Dunlap, M.B., & Harvey, H.S. (1967) *Am. J. Dis. Child.*,
114, 229.
- Edebo, L. (1960) *J. Biochem. Microbiol. Tech. Eng.*, 2, 453.
- Edebo, L. (1961) *Acta Path. Microbiol. Scand.*, 52, 300.
- Ekstedt, R.D., & Stollerman, G.H. (1960a) *J. exp. Med.*,
112, 671.
- Ekstedt, R.D., & Stollerman, G.H. (1960b) *ibid*, 112, 687.
- Elliot, S.D. (1945) *J. exp. Med.*, 81, 573.
- Elliot, S.D. (1950) *ibid*, 92, 201.
- Elliot, S.D., & Dole, V.P. (1947) *ibid*, 85, 305.
- Foley, M.J., & Wood, W.B. (1959) *ibid*, 110, 617.
- Fox, E.N. (1964) *J. Immunol.*, 93, 826.
- Fox, E.N., & Krampitz, L.O. (1956) *J. Bact.*, 71, 456.
- Fox, E.N., & Wittner, M.K. (1965) *Proc. Nat. Acad. Sci.*,
54, 1118.
- Fox, E.N., & Wittner, M.K. (1966) *J. Immunol.*, 97, 86.
- Fox, E.N., & Wittner, M.K. (1967) *Fed. Proc.*, 26, 581.
- Freimer, E.H., Krause, R.M., & McCarty, M. (1959)
J. exp. Med., 110, 853.

- Fuller, A. (1938) Brit. J. exp. Path., 19, 130.
- Goldstein, I., & Caravano, R. (1967) Proc. Soc. exp. Biol. Med., 124, 1209.
- Goldstein, I., Halpern, B., & Robert, L. (1967) Nature, 213, 44.
- Gooder, H. (1961) J. Gen. Microbiol., 25, 347.
- Gooder, H., & Maxted, W.R. (1958) Nature, 182, 808.
- Gooder, H., & Maxted, W.R. (1961) Soc. Gen. Microbiol., Symposium No. XI, p.151.
- Gooder, H., & Williams, R.E.O. (1959) Assoc. Clin. Path. Broadsheet No. 25.
- Grabar, P. & Williams, C.A. (1953) Biochim. Biophys. Acta, 10, 193.
- Griffith, F. (1926) J. Hyg. Camb., 25, 385.
- Griffith, F. (1934) ibid, 34, 542.
- Halbert, S.P., Bircher, R., & Dahle, E. (1961) J. exp. Med., 113, 759.
- Hälen, J. (1963) Acta Path. Microbiol. Scand., 57, 301.
- Hambly, J.S. (1958) J. Gen. Microbiol., 18, 285.
- Hayashi, J.A., & Walsh, G. (1961) J. Bact., 82, 736.
- Herbert, D., & Todd, E.W. (1944) Brit. J. exp. Path., 25, 242.
- Hill, M.J., James, A.M., & Maxted, W.R. (1963) Biochim. Biophys. Acta, 66, 264.
- Hill, M.J., & Wannamaker, L.W. (1968) J. Hyg. Camb., 66, 37.
- Hirst, G.K., & Lancefield, R.C. (1939) J. exp. Med., 62, 425.

- Holmes, M.C. (1958) Aust. J. exp. Biol. Med. Sci., 36, 395.
- Johnson, G.D. (1955) J. Clin. Path., 8, 296.
- Kabat, E.A., & Meyer, M.M. (1961) in Kabat & Meyer's Experimental Immunochemistry, Published by Charles C. Thomas, Springfield, U.S.A. P.480.
- Kantor, F.S., & Cole, R.M. (1960) J. exp. Med., 112, 77.
- Kaplan, M.H. (1958) J. exp. Med., 107, 341.
- Kaplan, M.H. (1963) J. Immunol., 90, 595.
- Kaplan, M.H. (1964) in The Streptococcus, Rheumatic Fever & Glomerulonephritis, Edited by J. Uhr, Published by Williams & Wilkins Co., Baltimore, U.S.A. P.169.
- Kaplan, M.H., & Meyeserian, M. (1962) Lancet, 1, 706.
- Kaplan, M.H., & Suchy, M.L. (1964) J. exp. Med., 119, 643.
- Kaplan, M.H., & Svec, K.H. (1964) ibid, 119, 651.
- Karakawa, W.W., & Krause, R.M. (1966) ibid, 124, 155.
- Karakawa, W.W., Lackland, H., & Krause, R.M. (1966a) J. Immunol., 96, 204.
- Karakawa, W.W., Lackland, H., & Krause, R.M. (1966b) J. Immunol., 97, 797.
- Karakawa, W.W., Osterland, C.K., & Krause, R.M. (1965) J. exp. Med., 122, 195.
- Karakawa, W.W., Rotta, J., & Krause, R.M. (1965) Proc. Soc. exp. Biol. Med., 118, 198.
- Kellner, A., Bernheimer, A.W., Carlson, A.S., & Freeman, E.B. (1956) J. exp. Med., 104, 361.

- Kellner, A., & Robertson, T. (1954) ibid, 99, 495.
- Krause, R.M. (1957) ibid, 106, 365.
- Krause, R.M. (1963) *Bact. Rev.*, 27, 369.
- Krause, R.M., & McCarty, M. (1961) *J. exp. Med.*, 114, 127.
- Krause, R.M., & McCarty, M. (1962) ibid, 116, 131.
- Krumwiede, E. (1954) ibid, 100, 629.
- Kuttner, A.G., & Lenert, T.F. (1944) *J. Clin. Invest.*, 23, 151.
- Lancefield, R.C. (1928a) *J. exp. Med.*, 47, 91.
- Lancefield, R.C. (1928b) ibid, 47, 469.
- Lancefield, R.C. (1928c) ibid, 47, 481.
- Lancefield, R.C. (1933) ibid, 57, 595.
- Lancefield, R.C. (1940) ibid, 71, 521.
- Lancefield, R.C. (1941) Harvey Lectures, series 36, p.251.
- Lancefield, R.C. (1943) *J. exp. Med.*, 78, 465.
- Lancefield, R.C. (1957) ibid, 106, 525.
- Lancefield, R.C. (1958) ibid, 108, 329.
- Lancefield, R.C. (1959) ibid, 110, 271.
- Lancefield, R.C. (1962) *J. Immunol.*, 89, 307.
- Lancefield, R.C., & Dole, V.P. (1946) *J. exp. Med.*, 84, 449.
- Lancefield, R.C., & Perlmann, G.E. (1952a) ibid, 96, 71.
- Lancefield, R.C., & Perlmann, G.E. (1952b) ibid, 96, 83.
- Lawrence, H.S. (1952) *J. Immunol.*, 68, 159.
- Lazarides, P.D., & Bernheimer, A.W. (1957) *J. Bact.*, 74, 412.
- Lian, T.S., & Pierce, W.A. (1961) *Proc. Soc. exp. Biol. Med.*,
106, 207.

Lyampert, I.M., Vvedenskaya, O.I., & Danilova, T.A. (1966)

Immunology, 11, 313.

Lyons, C., & Ward, H.K. (1935) J. exp. Med., 61, 531.

Markham, R. (1942) Biochem. J., 36, 790.

Markowitz, M. (1963) J. Clin. Invest., 42, 409.

Maxted, W.R. (1948) Lancet, 11, 255.

Maxted, W.R. (1949) J. Gen. Microbiol., 3, 1.

Maxted, W.R. (1956) Brit. J. exp. Path., 37, 415.

Maxted, W.R. (1957) J. Gen. Microbiol., 16, 584.

Maxted, W.R., & Potter, E.V. (1967) ibid, 49, 119.

McCarty, M. (1948) J. exp. Med., 88, 181.

McCarty, M. (1952) ibid, 96, 569.

McCarty, M. (1956) ibid, 104, 629.

McCarty, M. (1958) ibid, 108, 311.

McCarty, M. (1959) ibid, 109, 361.

McCarty, M., & Lancefield, R.C. (1955) ibid, 102, 11

McClean, D. (1941) J. Path. Bact., 53, 13.

McLean, S.T. (1953) J. Gen. Microbiol., 9, 110.

Miller, F. (1961) Proc. Soc. exp. Biol. Med., 108, 539.

Milstone, H. (1941) J. Immunol., 42, 109.

Molina, A.M., & Saletti, M. (1961) Ann. Sclavo, 3, 755.

Morison, J.E. (1940) J. Path. Bact., 51, 401.

Murray, I.G., Pepys, J., & Brighton, W.D. (1967)

Month. Bull. Minist. Health, 26, 96.

Nakhla, L.S., & Glynn, L.E. (1967) Immunology, 13, 209.

Nishimura, T., Kumagai, N., & Kabayashi, T. (1966)

Jap. Hrt. J., 7, 244.

Ogburn, C.A., Harris, T.N., & Harris, S. (1958) J. Immunol.,
81, 396.

Ohanian, S.H., & Schwab, J.H. (1967) J. exp. Med., 125, 1137.

Ouchterloney, Ö. (1953) Acta Path. Microbiol. Scand., 32, 231.

Pakula, R. (1951) J. Gen. Microbiol., 5, 640.

Parker, M.T. (1967) Bull. Wld. Hlth. Org., 37, 513.

Pike, R.M. (1948) J. Infect. Dis., 83, 1.

Potter, E.V., Stollerman, G.H., & Siegel, A.C. (1962)

J. Clin. Invest., 41, 301.

Quinn, R.W., & Lowry, P.N. (1963) J. Infect. Dis., 113, 33.

Quinn, R.W., & Lowry, P.N. (1967) J. Bact., 94, 274.

Rantz, L.A., & Randall, E. (1945) Proc. Soc. exp. Biol. Med.,
59, 22.

Rantz, L.A., & Randall, E. (1955) Stanford Med. Bull., 13, 290.

Report (1947) J. exp. Med., 85, 441.

Rogers, H.J. (1945) Biochem. J., 39, 435.

Rosendal, K. (1956) Acta Path. Microbiol. Scand., 39, 127.

Rothbard, S. (1945) J. exp. Med., 82, 93.

Rothbard, S., & Watson, R.F. (1948) J. exp. Med., 87, 521.

Rothbard, S., Watson, R.F., Swift, H.F., & Wilson, A.T.

(1948) Arch. Intern. Med., 82, 229.

Rotta, J. (1967a) Folia Microbiol., 12, 255.

Rotta, J. (1967b) Personal communication.

- Salton, M.R.J. (1952) *Biochim. Biophys. Acta*, 2, 334.
- Salton, M.R.J. (1953) *ibid*, 10, 512.
- Salton, M.R.J., & Horne, R.W. (1951) *ibid*, 7, 177.
- Scheidegger, (1955) *Intern. Arch. Allergy*, 7, 177.
- Schmidt, W.C. (1952) *J. exp. Med.*, 95, 105.
- Schmidt, W.C. (1957) *J. Immunology*, 78, 178.
- Schmidt, W.C. (1960) *J. Infect. Dis.*, 106, 250.
- Schmidt, W.C., & Moore, D.J. (1965) *J. exp. Med.*, 121, 793.
- Schwab, J.H., & Cromartie, W.J. (1960) *J. exp. Med.*, 111, 295.
- Schwab, J.H., Cromartie, W.J., & Roberson, B. (1959)
J. exp. Med., 109, 43.
- Schwab, J.H., Gooder, H., & Maxted, W.R. (1962) *Brit. J.
 exp. Path.*, 43, 181.
- Sharp, J.T., Hijmans, W., & Dienes, L. (1957) *J. exp. Med.*,
105, 153.
- Singer, J.M., & Plotz, C.M. (1956) *Am. J. Med.*, 21, 888.
- Slade, H.D., & Stamp, W.C. (1956) *J. Bact.*, 71, 624.
- Slade, H.D., & Stamp, W.C. (1962) *J. Bact.*, 84, 345.
- Stamp, T.C., & Hendry, E.B. (1937) *Lancet*, 1, 257.
- Stewart, F.S. (1961) *Nature*, 190, 464.
- Stock, A.H., Uriel, J., & Grabar, P. (1961) *Nature*, 192, 434.
- Stollerman, G.H. (1967) in: Laboratory Diagnostic Procedures
 in the Rheumatic Diseases. Edited by A.S. Cohen,
 published by J. & A. Churchill Ltd., London. P.168.

- Stollerman, G.H., & Ekstedt, R.D. (1957) *J. exp. Med.*, 106, 345.
- Stollerman, G.H., Siegel, A.C., & Johnson, E.E. (1959) *J. exp. Med.*, 110, 887.
- Stoyanova, M. (1966) *Works of the Res. Instit. Epidem. Microbiol., Bulgaria*, XI, 89.
- Swift, H.F., Wilson, A.T., & Lancefield, R.C. (1943) *J. exp. Med.*, 78, 127.
- Tan, E.M., & Kaplan, M.H. (1962) *J. Infect. Dis.*, 110, 55.
- Tepper, B.S., Hayashi, J.A., & Barkulis, S.S. (1960) *J. Bact.*, 79, 33.
- Tillet, W.S., & Garner, R.L. (1933) *J. exp. Med.*, 58, 485.
- Tillet, W.S., Sherry, S., & Christensen, L. (1948) *Proc. Soc. exp. Biol. Med.*, 68, 184.
- Todd, E.W. (1927a) *Brit. J. exp. Path.*, 8, 1.
- Todd, E.W. (1927b) *ibid*, 8, 361.
- Todd, E.W. (1934) *J. Path. Bact.*, 39, 299.
- Todd, E.W. (1938) *ibid*, 47, 423.
- Todd, E.W. (1939) *J. Hyg. Camb.*, 39, 1.
- Todd, E.W. (1942) *Brit. J. exp. Path.*, 23, 136.
- Vosti, K.L., & Johnson, R.H. (1967) *J. Clin. Invest.*, 46, 1127.
- Vosti, K.L., & Rantz, L.A. (1964) *J. Immunology*, 92, 183.
- Wannamaker, L.W. (1958) *J. exp. Med.*, 107, 797.
- Wannamaker, L.W. (1962) *Fed. Proc.*, 21, 231.
- Wannamaker, L.W. (1967) Personal communication.

Wannamaker, L.W., & Ayoub, E.M. (1960) *Circulation*, 21, 598.

Wannamaker, L.W., Denny, F.W., Perry, W.D., Siegel, A.C.,

& Rammelkamp, C.H. (1953) *Am. J. Dis. Child.*, 86, 347.

Weissmann, C., Keiser, H., & Bernheimer, A.W. (1963)

J. exp. Med., 118, 205.

Williams, R.E.O. (1954) *Month. Bull. Minist. Health*, 13, 171.

Williams, R.E.O. (1957) *ibid*, 16, 163.

Williams, R.E.O. (1958) *Bull. Wld. Hlth. Org.*, 19, 153.

Williams, R.E.O., & Maxted, W.R. (1953) *Atti del VI Con.*

Inter. Microbiol., 1, 46.

Wilson, A.T. (1945) *J. exp. Med.*, 81, 593.

Wilson, A.T. (1957) *ibid*, 105, 463.

Wilson, A.T., & Wiley, G.G. (1963) *J. exp. Med.*, 118, 527.

Wilson, G.S., & Miles, A.A. (1964) Topley & Wilson's

Principles of Bacteriology & Immunity, Vol. I, 5th

edition, Published by E. Arnold Ltd., London. P.693.

Zabriskie, J.B. (1964) *J. exp. Med.*, 119, 761.

Zabriskie, J.B., & Freimer, E.H. (1966) *J. exp. Med.*, 124, 661.