CHAPTER I

INTRODUCTION

The genus Streptococcus is the only one of the five in the family Streptococcaceae that contains organisms pathogenic to man (1). Their ability to hemolyze red blood cells to various degrees is one of the most useful schemes for the initial classification of streptococci. Beta-hemolytic streptococci are those that produce a clear zone around the colony on fresh blood agar as a result of the total lysis of red blood cells by hemolysins. Alpha-hemolytic organisms produce a zone of incomplete hemolysis and greenish discoloration of the medium. Many which produce no reaction on blood agar are called non-hemolytic or gamma-hemolytic streptococci.

Pasteur described chains of streptococci in patients with puerperal sepsis, and Koch identified them in pus from wound infections. Pure cultures of the organisms were first obtained in 1883, but a long delay followed before there was full recognition and understanding of the multifaceted manifestations of streptococcal disease. In 1933, Rebecca Lancefield introduced a method for the classification of streptococci into serologic groups, which was based upon the antigenic composition of the carbohydrates of the cell wall. This discovery provided the necessary stimulus for the unraveling of the entire spectrum of streptococcal infections and their nonsuppurative complications (2).

Some streptococci are members of normal human flora while others are associated with various clinical patterns of disease, such as streptococcal pharyngitis, scarlet fever, erysipelas, impetigo and endocarditis. In addition, streptococcal sore throat may be followed by an attack of rheumatic fever while acute glomerulonephritis may be a sequela of sore throat or streptococcal skin disease.

Over 90 % of human streptococcal infections are caused by beta-hemolytic streptococci of Lancefield's group A-Streptococcus pyogenes (3)., and streptolysin O (SLO) is considered to be one of the important toxins that is produced by most strains.

1. Characteristics of Beta-Hemolytic Streptococci Group A

1.1 Morphology

Individual cocci are spherical to ovoid in shape, and about 0.6 to 1.0 μm in diameter. Cell division occurs in one plane, resulting in pairs or chains. The length of the chains vary widely and are conditioned largely by environmental factors. Although usually staining gram positive, organisms may become gram variable or gram negative as the culture ages. (2, 4).

The organisms are non-motile and non-sporing. The majority of group A strains produce antiphagocytic hyaluronic acid capsules, which may be demonstrable during 2 to 4 hours of growth.
Since most of these strains also produce hyaluronidase, capsules connot be demonstrated in older culture. Beneath the capsule is the cell wall, which, in a simplified way, can be visualized as having a three-layer structure.

A. The outermost cell wall layer contains

A - 1 Lipotiechoic acid

A - 2 Fc reacting factor, which is the binding site for the Fc fragment of the heavy chain of the immunoglobulin

A - 3 A number of protein components, namely M, T, R antigens ; M - associated protein, or so-called non-type specific protein, which is non-type-specific substance but closely associated with the M protein ; and serum opacity factor, which was found to be a second type-specific-substance in some types of group A streptococci, namely those that produce poorly antigenic M protein, against which M typing sera are difficult to prepare.

B. The second layer consists of carbohydrate or polysaccharide

C. The third layer is peptidoglycan, which was at first considered solely to be a structure responsible for the rigidity of the cell wall, but may also play a role in the genesis of streptococcal disease and possibly its sequelae.

From the cell wall, hairlike pili (fimbriae) project through the capsule. They consist partly of M protein and are covered with the lipoteichoic acid. The latter is not only important in the attachment of streptococci to epithelial cells but also able to bind with the receptor sites on the surface of human polymorphonuclear leucocytes which are involved in the recognition of group A streptococci and enable attachment in the process of phagocytosis.

Beneath the cell wall is the cytoplasmic membrane which encloses the cytoplasm. The cytoplasm contains a complex of nucleoproteins and proteins, some with enzymatic activity.

Scheme of the cellular composition of group A streptococcus is shown in Figure 1. (4, 5)

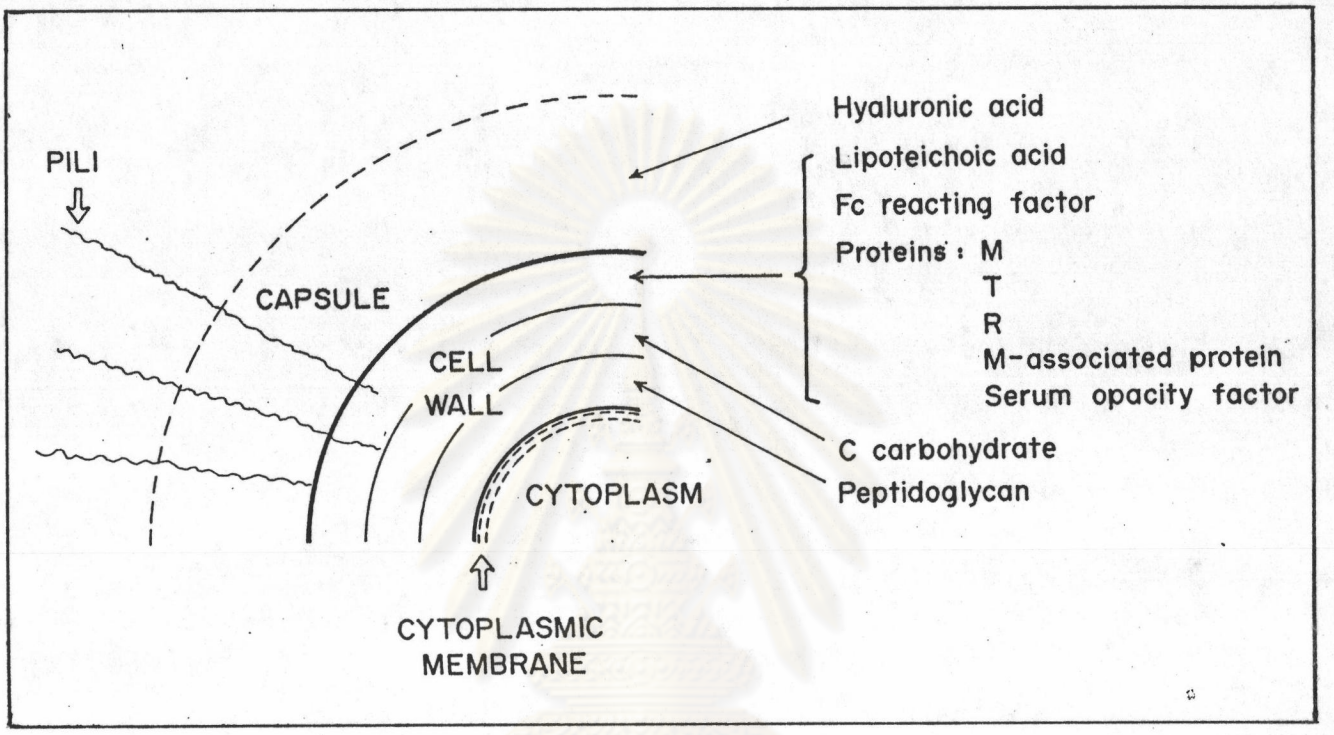

Figure 1 Scheme of the Cellular Composition of Beta-Hemolytic Streptococci Group A

Group A streptococci grow in three main colony forms on blood agar :

A. Mucoid colonies, which are large and watery, with a raised surface ; streptococci growing in this phase regularly produce hyaluronic acid capsules and frequently produce the M protein.

B. Matt Colonies, which are opaque and flat, with a tendency to produce a rough surface because in most instances they develop from mucoid colonies as a result of drying, and for this reason

are sometimes called postmucoid colonies ; M protein is frequently produced.

C. Glossy colonies, which are smooth and glistening in appearance, with a much smaller diameter than in the other colony forms, usually 1 - 2 mm only ; the M protein is present only seldom.

It should be noted that the relation between colony form and M protein production is approximative only and should never be considered a definite characteristic because there is considerable variation in these two properties of group A streptococci. (5)

1.2 Physiology and Cultural Characteristics

Although the different species of the genus Streptococcus vary considerably in their nutritional requirements, all streptococci require media rich in nutrients, and the human pathogens, which are most exacting, especially require a variety of growth factors. This is essential in order to achieve a satisfactory growth of germs with an adequate antigenic equipment. The nutritional requirements of the streptococcus are very complex because of the organism's inability to synthesize many of its amino acids, purines, pyrimidines and vitamins.

The basic culture medium must contain meat infusion (a freshly prepared product is preferable to a commercial, dehydrated one), peptone, glucose and salts. Sterilization by heat reduces the quality of the medium in respect to growth. However, heat sterilization is fully satisfactory for most purposes and is generally used. For delicate cultivation purposes, filtration of the medium is superior to sterilization by heating. The addition of blood or tissue fluids

to the medium greatly enhances the growth of streptococci. Such enrichment is especially necessary for cultivating streptococci on solid media (blood-agar plates), but is also recommended for the preparation of highly virulent cultures grown for a short period of time (serum broth).

Hemolytic streptococci utilize polysaccharide as an energy source. Since hemolytic streptococci are facultative anaerobes, their metabolism is fermentative and the resulting metabolic product is lactic acid (2, 5, 6). Growth and hemolysis are aided by 10 % carbondioxide (4).

Similarly as with most other bacteria pathogenic for man, the optimal temperature for the growth of Streptococcus pyogenes is 37^{o}C. The optimal pH is 7.4 to 7.6. Group A streptococci are killed in 30 minutes at 60^{o}C, in contrast to certain streptococci, especially members of group D, that are not killed under these conditions. (2, 4, 5)

1.3 Antigenic Structure

Hemolytic streptococci can be devided into serologic groups (A - V) (4), and certain groups can be sub-divided into types. Several antigenic substances are found :

A. C carbohydrate : This substance is contained in the cell wall of many streptococci and forms the basis of serologic grouping (Lancefield A - V). Extract of C carbohydrate for "grouping" of streptococci may be prepared by extraction of centrifuged culture with hot hydrochloric acid, nitrous acid, or formamide ; by enzymatic

lysis of streptococcal cells (e.g. with pepsin or trypsin) ; or by autoclaving of cell suspensions at 15 lb pressure for 15 minutes (4). The extracts thus obtained are tested against group-specific antisera by capillary tube precipitation reactions. This is the method employed for defining the various serologic groups. The serologic specificity of C carbohydrate (the group-specific antigen) is determined by an amino sugar. For group A streptococci, it is composed of a branched polymer of L-rhamnose and N-acetyl D-glucosamine in a 2 : 1 ratio, the latter being the antigenic determinant. This carbohydrate is linked (possibly by phosphodiester bonds) to the peptidoglycan (or mucopeptide, which consists of N-acetyl D-glucosamine, N-acetyl D-muramic acid, D-glutamic acid, L-lysine, and D-and L-alanine). (2)

B. M protein : This substance is closely associated with the virulence of group A streptococci and occurs chiefly in organisms producing matt or mucoid colonies. Repeated passage on artificial media may lead to the loss of M protein production, which may be restored by rapidly repeated animal passage. M protein not only plays a part in the resistance to phagocytosis in whole blood lacking a specific antibody, but it also appears to facilitate sticking to epithelial surfaces and may, by this mechanism, resist the removal of the organism by bathing secretions. Growing L forms of streptococci also produces M protein as well as hyaluronic acid.

M protein is acid and heat stable but trypsin sensitive. The removal of M protein from the cell does not alter viability.

M protein determines the type specific of group A streptococci, as demonstrated by capillary tube precipitin tests using type-specific antisera and hydrochloric acid extracts. At present, almost 70 M types can be differentiated in group A streptococci (5). Types are assigned in Arabic number. On rare occasions, two different M proteins may be detected in the same strain.

M protein is destroyed during growth by a proteinase if the pH of the medium is allowed to fall below 6.5 (2).

In humans, antibodies to an M protein protect against infection with this specific type of group A streptococcus (4).

C. T substance : This antigen has no relationship to virulence of streptococci. It is resistant to pepsin and trypsin digestion but is acid and heat labile and thus is seperated from the M protein. T typing is done by a slide agglutination test using trypsin-treated whole streptococci (proteolytic digestion, which rapidly destroy M proteins). Some T antigens are restricted to a single M type, while others may be shared by several M types. Antibodies to T antigens are not protective. (2, 4, 5)

Another surface antigen has been called R protein but a typing system employing the R protein is not commonly used. This antigen is destroyed by pepsin but not trypsin (2).

D. Nucleoproteins : Extraction of streptococci with weak alkali yields mixtures of proteins and other substances of little serologic specificity, called P substances, which probably

make up most of the streptococcal cell body.

E. Capsular antigen : Many group A streptococci produce a hyaluronic acid capsule. This is of little antigenic significance but may enhance the virulence of the organism as previously described.

1.4 Toxins and Enzymes

The organism Streptococcus pyogenes group A produce more than twenty extracellular proteins or peptides into their environment, whether it is a culture medium or in host (essentially man) tissues. Some of these extracellular products are identifiable as toxins and enzymes while others are still without known biological activity (Figure 2) (7).

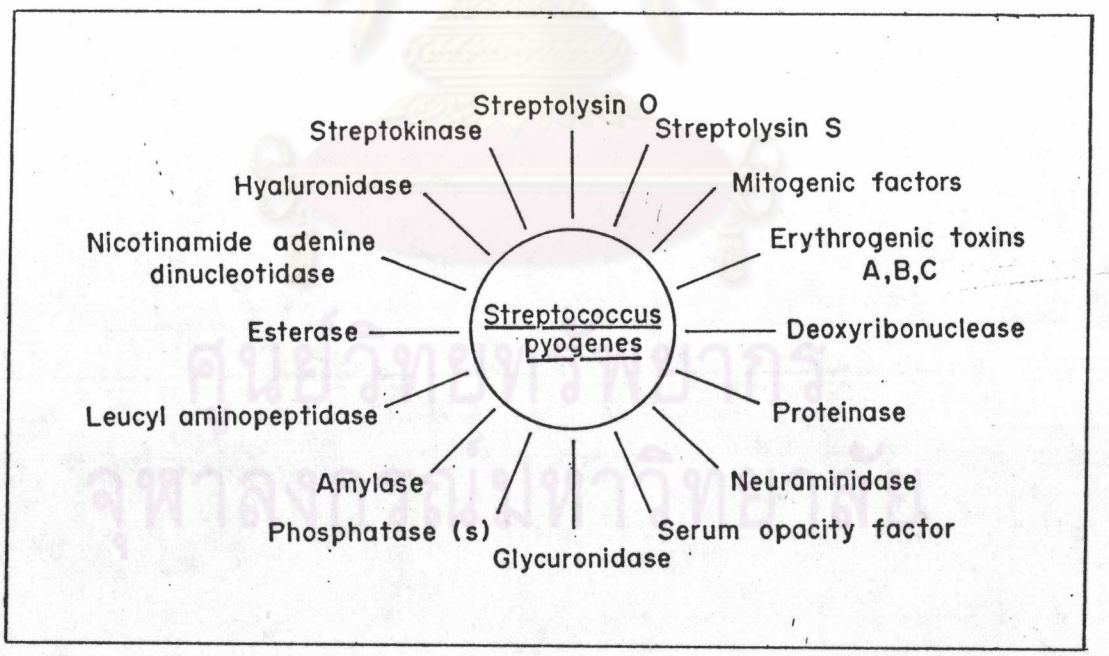

Figure 2 Extracellular Macromolecular Substances Identified in Culture Fluids of Group A Streptococci

Among those which are related with human pathogenesis are (4) :

A. Streptokinase (Fibrinolysin)

It is an enzyme catalyzing the conversion of plasminogen of human serum into plasmin, an active proteolytic enzyme that digests fibrin and other proteins. This process of digestion may be interfered with by non-specific serum inhibitors and by a specific antibodies, antistreptokinase. A skin test with streptokinase-streptodornase is regularly positive in adolescents and adults with normal cell-mediated immunity. It is therefore used as a common test for the latter. Streptokinase has been given intravenously for treatment of pulmonary emboli and venous thromboses.

An assay for antistreptokinase may be another indicator of recent infection. However, because it is technically difficult to perform, it is not routinely used (8).

B. Streptococcal Deoxyribonuclease (DNase) or Streptodornase

It is an enzyme that depolymerizes DNA. The enzymatic activity can be measured by the lowering in viscosity of known DNA solutions.

It exists in four antigenic forms : A, B, C and D. Nucleases A and C have only DNase activity, while B and D also possess RNase activity (2). The most important is the form B (DNase-B) which is produced in a larger amount than the other forms and is the most consistently antigenic form (8).

Purulent exudates owe their viscosity largely to deoxyribonucleoprotein. Mixtures of streptodornase and streptokinase are used in "enzymatic debridement". They help to liquefy exudates and facilitate removal of pus and necrotic tissue; antimicrobial drugs thus gain better access, and infected surfaces recover more quickly.

Antibody titers to DNase-B are of great value in the serodiagnosis of streptococcal infections (normal limit = 250 units) (8), especially after skin infection, where the streptolysin O (SLO) response may be blunted (2, 4). Therefore, the titer of anti-DNase-B is generally recommended to be determined in addition to antistreptolysin O (ASO) - as the second streptococcal antibody.

C. Hyaluronidase

It is an enzyme that splits hyaluronic acid, both in ground substance of animal connective tissues and in the capsule surrounding streptococci. Thus, hyaluronidase aids in spreading infecting microorganisms (spreading factor). Hyaluronidases are antigenic and specific for each bacterial or tissue source. Purified hyaluronidase is employed in medical therapy to facilitate the spreading and absorption of fluids injected into tissues.

Titers of antihyaluronidase rise in the second week after infection with hyaluronidase - producing organisms and fall in three or five weeks. The antihyaluronidase titer is elevated in approximately sixty percent of documented streptococcal respiratory tract infections but is elevated in a much smaller percentage of streptococcal skin infections. It is less helpful than the ASO titer

when used alone, but when the two are used in combination, ninety percent of individuals with a recent streptococcal pharyngitis will have an elevated titer against at least one of the two antigens (8).

D. Erythrogenic Toxin

Other synonyms in the literature are Dick toxin, scarlet fever toxin, scarlatinal toxin and streptococcal pyrogenic exotoxin, which has been proposed by Kim and Watson in 1970 (9).

There are at least three antigenically distinct forms (A, B and C) (2, 7). It is a soluble protein, heat-labile but stable to acid, alkali and pepsin.

It causes the rash that occurs in scarlet fever. Only strains of Streptococcus pyogenes that have been infected with temperate bacteriophage, i.e. are lysogenic, can elaborate this toxin. Strains devoid of the temperate phage genome do not produce toxin (3, 4). A non-toxigenic Streptococcus, after lysogenic conversion, will produce erythrogenic toxin which is antigenic, giving rise to the formation of specific antitoxin that neutralizes the toxin. Persons possessing such antitoxin are immune to the rash though susceptible to streptococcal infection. There exist some minor qualitative differences between the erythrogenic toxins produced by different strains. (4)

Classically, it has been thought that these toxins caused a red reaction in the skin of non-immune individuals (positive Dick test) and no reaction in individuals with immunity (negative Dick test). Antitoxin injected into the skin of a patient with

scarlet fever caused localized blanching due to neutralization of erythrogenic toxin (Schultz-Charlton reaction). Recently, it has been proposed, however, that the rash in some individuals may be more related to hypersensitivity than lack of immunity, and the occurence of rash may depend on an interplay between cellular and humoral factors (2).

E. Hemolysin

Beta-hemolytic streptococci group A elaborate 2 hemolysins (streptolysins)

E - 1 Streptolysin O (SLO)

SLO is an oxygen-labile, immunogenic protein toxin. It will be discussed in detail in other sections.

E - 2 Streptolysin S (SLS)

SLS is an oxygen-stable, non-immunogenic cytolysin. It is composed of polypeptide moiety associated to a carrier, such as serum albumin, RNA, trypan blue, some non-ionic detergents (e.g. Tween 40, 60 and 80 and Triton X - 205), etc. It is only active in the carrier-bound state. As noted by Bernheimer, no one has ever succeeded in isolating in active form the hemolytic moiety by itself, that is, free of a carrier. It may indeed prove impossible to do so because the experience of several investigators suggests the active moiety undergoes rapid decay when the complex is treated in ways designed to remove or destroy only the carrier (10). Ginsburg and co-workers also found that a hemolytic moiety of SLS can be transferred from one carrier to

another under appropriate condition (11). It appears that these variety of unrelated carrier substances can induce the release of active cytolysin by streptococci (7).

SLS produces hemolysis by direct cell-to-cell contact and thus is nondiffusible except when transported by carriers (2). It is a very potent cytolytic agent active on eukaryotic as well as prokaryotic cells (bacterial protoplasts and spheroplasts) (7). It is responsible for the surface beta-hemolysis surrounding colonies on blood agar plates, and those occasional strains lacking SLS may appear non-hemolytic on surface growth (2, 4, 7). SLS appears to be endowed with antitumor activity (7).

SLS is thermolabile (5). The molecular weight is probably considerably less than 20,000, which may account for its lack of antigenicity (2).

1.5 Pathogenesis and Clinical Findings

A variety of distinct disease processes are associated with streptococcal infection. The clinical pattern varies depending on the biological properties of the infecting organisms, the nature of the host response, and the portals of entry of the infection. Infection can arbitrarily devided as follows :

A. Streptococcal Sore Throat

The most common and most typical infection caused by Streptococcus pyogenes is an acute sore throat called "tonsillitis" if the tonsils are maximally involved or "pharyngitis" if there is little or no tonsillar tissue in the fauces. Virulent

group A streptococci adhere to the pharyngeal epithelium by means of lipotiechoic acid covering surface pili. In the infant and small child, the sore throat occurs as a subacute nasopharyngitis with a thin serous discharge and little fever but will have a tendency of extending the infection to the middle ear, the mastoid and the meninges. The cervical lymphnodes are usually enlarged. The illness may persist for weeks. In older children and adults, the disease is more acute and is characterized by intense nasopharyngitis, tonsillitis, and intense redness and edema of the mucous membranes, with purulent exudate ; enlarged, tender cervical lymphnodes ; and (usually) a high fever. Twenty percent of the infections are asymptomatic (4).

If the infecting streptococcus is capable of producing a considerable amount of an erythrogenic toxin and the host has no antitoxic immunity, the sore throat may be accompanied by a generalized punctate erythema or rash and this syndrome is called "scarlet fever" (3, 4). The association of a scarlatinal rash (erythema which blanches on pressure, initially involving the trunk and neck, spreading to the extremities) is almost diagnostic of streptococcal infection. Desquamation may occur during convalescence. Antitoxin to the erythrogenic toxin prevents the rash but does not interfere with the streptococcal infection (4). However, individuals may have several episodes of scarlet fever, as there are at least three different erythrogenic toxins (2).

Local extension of the streptococcal infection from the throat may result in such complications as "peritonsillar abscess, sinusitis, otitis media, mastoiditis or

meningitis" (3).

Streptococcal infection of the upper respiratory tract does not usually involve the lungs (4).

Specific antibodies to the M protein, the virulent antigen of the infecting streptococcus, develop slowly after the sore throat or other streptococcal illness and persist for a long time so that repeat attacks by the same streptococcus serotype are unlikely ; but this specific antibody does not protect against infection by other serotypes (3, 4).

B. Skin Infection

Streptococcus pyogenes may cause two types of skin infection (2 - 4) :

B - 1 Erysipelas : This is a spreading inflammation of the dermis seen most commonly on the face and neck and may be associated with antecedent streptococcal infection of the nose, throat or ear. The lesion is characterized by erythema, edema, and induration, which usually has a distinct advancing border. Some individuals are prone to recurrences, usually in the same site and may be related to an allergy to the streptococcal toxins.

B - 2 Impetigo or Streptococcal Pyoderma : This is a local infection of superficial layers, especially in children. It consists of superficial blisters that break down or of eroded areas whose denuded surface is covered with pus or crusts. It usually occurs on exposed parts of the body (legs, arms, face). It spreads by continuity and is highly communicable in school and

pre-school children, especially in hot, humid climates. More widespread infection occurs in eczematous or wounded skin or in burns and may progress to "cellulitis". It is often attributable to types 49, 57, and 59 - 61 and may precede "glomerulonephritis" but does not often lead to "rheumatic fever". A majority of these skin infections could be prevented by improved skin hygeine.

C. Puerperal Sepsis or Child-Bed Fever :

This is traditionally associated with infection by Streptococcus pyogenes, although there are other causal agents. Streptococci may be normal vaginal flora or are introduced during delivery, occasionally by the attending physician or nurse. Besides local inflammation of uterine tissues, infection may spread to the adnexa ("pelvic cellulitis" or "peritonitis") or may become generalized ("septicemia"). It is characterized by chills, fever, facial flushing, abdominal distention with pelvic tenderness, and serosanguineous vaginal discharge. (2, 3)

Puerperal sepsis may be prevented, for the most part, by strict attention to aseptic techniques.

D. Poststreptococcal Diseases

Following an acute group A streptococcal infection, there is a latent period of 1 - 4 weeks, after which "rheumatic fever" or "acute glomerulonephritis" occasionally develops. The latent period suggests that these poststreptococcal diseases are not attributable to the direct effect to disseminated bacteria but represent instead a hypersensitivity response that follows streptococcal insult to the affected organs. "Rheumatic fever" is more commonly

preceded by infection of upper respiratory tract ; "acute glomerulonephritis", by infection of the skin (2 - 4, 12).

D - 1 Rheumatic Fever : Rheumatic fever is the most serious sequela to hemolytic streptococcal infection because it results in damage to heart muscle and valves. In some developing countries, rheumatic heart disease still ranks high as a cause of death in young adults.

Typical symptoms and signs of rheumatic fever include fever, malaise, a migratory nonsuppurative polyarthritis, and evidence of inflammation of all parts of the heart (endocardium, myocardium, pericardium). The carditis characteristically leads to thickened and deformed valves and to small perivascular granulomas in the myocardium (Aschoff bodies) that are finally replaced by scar tissue (4).

The clinical experience of T. Duckett Jones led to the establishment of the Jones criteria for the diagnosis of acute rheumatic fever, as shown in Table 1 (2).

Table 1 Modified Jones Criteria for the Diagnosis of Rheumatic Fever

Major Manifestations* :	Carditis
	Arthritis
	Chorea
	Erythema marginatum
	Subcutaneous nodules
Minor Manifestations :	Fever
	Arthralgia
	Elevated sedimentation rate or C - reactive protein
	Electrocardiographic changes
	History of previous rheumatic fever or rheumatic heart disease

Plus evidence of preceding streptococcal infection (scarlet fever, culture-proven group A streptococcal pharyngitis, or elevated streptococcal antibodies test).

*Two major or one major and two minor manifestations with evidence of previous streptococcal infection indicate a high probability of rheumatic fever.

Feinstein has also described rheumatic fever as an "arbitrarity designated portion of the spectrum of inflammatory complications that may follow group A streptococcal infections...manifested by the appearance, either alone or in various combinations, of arthritis, carditis, chorea, erythema marginatum or subcutaneous nodules." This constellation of symptoms usually occurs within two or three weeks after the onset of streptococcal infection, although chorea and erythema marginatum may be seen as late as six months after infection (2).

In addition to the clinical criteria, the documentation of recent streptococcal infection by culture or serology is of utmost importance. Since the causative streptococcal infection may have resolved or may have been asymptomatic, it is necessary to detect an increase in antibody titer to at least one of several streptococcal antigens, e.g. SLO and/or DNase-B, hyaluronidase, streptokinase. These antibodies are usually at higher levels and persist for longer periods in rheumatic disease than happens in uncomplicated streptococcal infections (2, 3).

The pathogenesis of rheumatic fever is poorly understood. Various theories have been proposed, including antigenic cross-reactivity between streptococcal antigens and heart tissue, direct toxicity due to streptococcal exotoxins, actual invasion of the heart by streptococci, or localization of antigens within damaged muscle or valvular tissue. Needless to say, the streptococcus produces many potentially damaging exotoxins, and the components of its cell wall have been shown to produce inflammation

in mammalian tissues. The true pathogenesis of acute rheumatic fever may never be elucidated because of the lack of a suitable experimental model (2).

No particular streptococcus serotypes are incriminated as happens with glomerulonephritis and it is for this reason that repeat throat infections with different serotypes occur and cause relapses of rheumatic fever (3). The firsr attack of rheumatic fever usually produces only slight cardiac damage which, however, increases with each subsequent attack. It is therefore important to protect such patients from recurrent group A hemolytic streptococcal infections by prophylactic penicillin administration (2, 4).

D - 2 Acute Glomerulonephritis : This is a complication of group A streptococcal pharyngitis or skin infection. There are several distinctive features about this streptococcus-mediated disease : (a) it follows infection with a limited number of streptococcal 'nephritogenic' serotypes, as shown in Table 2 (14) ; (b) the primary infection usually affects the skin and (c) second attacks are very rare. (3)

Table 2 Association of Certain Serotypes with Acute Glomerulonephritis

M type	Pharyngitis associated	Pyoderma associated
1	+ + +	
2	0	+ + +
3	+ +	±
4	+ + +	±
12	+ + + +	±
25	+ +	±
49	+ +	+ + + +
55	0	+ + +
57	0	+ +

+ + + + = Strong evidence of association

± or 0 = Questionable or no evidence

Acute glomerulonephritis is most often seen in children. It may be initiated by antigen-antibody complexes on the glomerular basement membrane. The most important antigen is probobly in the streptococcal protoplast membrane. In acute cases there is blood and protein in the urine, edema, high blood pressure, and nitrogen retention ; serum complement levels are low. A few patients die, some develop chronic glomerulonephritis with ultimate kidney failure ; the majority recover completely (4).

In order to establish a streptococcal etiology, it is necessary to document previous or concurrent

streptococcal infection or immune response to streptococcal products. A great majority of patients will show a serologic response either to SLO or to DNase-B. Interestingly, fewer persons with poststreptococcal glomerulonephritis show anti-DNase-B elevations (75 %) than ASO elevations (90 %) after streptococcal pharyngitis. This situation is reversed in glomerulonephritis following group A streptococcal pyoderma, in which 60 % show elevated anti-DNase-B titers in contrast to 25 % with ASO titer elevations (8). Thus, it is important that anti-DNase-B titers be sought in patients with streptococcal pyoderma, as the antibody response to SLO is poor following skin infection (2, 12).

1.6 Diagnostic Laboratory Tests (4)

Specimens to be obtained depend upon the nature of the streptococcal infection. A throat swab, pus, or blood is obtained for culture. Serum is obtained for antibody determinations, particularly ASO titer.

A. Stained Smears

Smears from pus often show single cocci or pairs rather than definite chains. Cocci are sometimes gram-negative. If smears of pus show streptococci but cultures fail to grow, anaerobic organisms must be suspected. Smears of throat swabs are rarely contributory because viridans streptococci are always present.

Smears from broth cultures of throat swabs 2 - 3 hr old can be stained with fluorescent group A - specific antibody for the most rapid identification of group A streptococci

in clinical disease or carriers.

B. Culture

For rapid identification, all specimens suspected of containing streptococci are cultured on blood agar plates so as to observe the degree and kind of hemolysis including colonial appearance.

The hemolytic group A streptococci (e.g., in sepsis) will grow in blood cultures within hours or a few days.

Serologic grouping and typing by means of precipitin tests should be performed whenever possible for the definitive classification.

Group A streptococci may be presumptively identified by determining sensitivity to bacitracin disk.

C. Serologic Tests

A rise in antibody titer to many antigens can be estimated, including ASO (particularly in respiratory tract infection), anti-DNase-B and antihyaluronidase (particularly in skin infections), and others.

2. Streptolysin O (SLO)

2.1 Definition

Streptolysin O may be defined as a toxic immunogenic extracellular protein produced by most strains of Lancefield group A and many strains of group C and G streptococci, particularly those

causing human infections, both in vitro and in vivo (7, 8, 15 - 18). The toxin is cytolytic or cytotoxic on eukaryotic cells including erythrocytes from mammals and other species (hemolysis), whereas prokaryotic cells (bacterial protoplasts or spheroplasts) are unsensitive (7). This heat-labile cytolysin can be reversibly oxidized and reduced : its biological activities, e.g., hemolysis, lethal toxicity and cardiotoxic effect, are lost by oxidation and restored upon reduction by reducing agents such as thiols or hydrosulfite (7, 19, 20).

2.2 Historical Background

The hemolytic activity, in the filtrates of certain streptococcal cultures, toward red blood cells was first discovered by Marmorek in 1895 (21). The substance responsible for this hemolytic activity was long believed to be very unstable. In 1926, Neill and Mallory found that although the hemolytic activity disappeared rather rapidly on standing in the presence of air or oxygen, it could be restored again by treatment with reducing substances such as sodium hydrosulfite (22). This observation has been confirmed by other workers (19, 20, 27). Then, Todd and Weld demonstrated that at least two distinct hemolysins were synthesized by most strains of streptococci ; one became inactive on exposure to oxygen, named streptolysin O (SLO) ; the other was oxygen-stable and extractable from microorganisms grown in serum, named streptolysin S (SLS) (23 - 25). The recognition of two different streptococcal hemolytic agents helped to clarify the mechanism of lysis in blood agar plates. Although most beta-hemolytic streptococcal strains synthsize both lysins, surface colonies bring about hemolysis primarily through the

mediation of SLS, since the SLO becomes oxidized, while deep colonies are hemolytically active through both (19). Not only the reversible inactivation on exposure to oxygen (22), but also the inhibition of its hemolytic effect (26) including the other toxic effects (lethality, cardiotoxicity and cytolysis on other eukaryotic cells) (27) by minute amounts of cholesterol and related sterols are essential features of SLO.

2.3 Some Characteristics of Streptolysin O

SLO is considered an important pathogenic factor of beta-hemolytic group A streptococci (28). It is the prototype of oxygen-labile bacterial cytolytic protein toxins, named "sulfhydryl-activated toxins" (27) or "thiol-activated cytolysins" (29), which represent a category encompassing at least fifteen exotoxins (Table 3) (27, 30) elaborated by gram positive bacteria of the genus Streptococcus, Bacillus, Clostridium and Listeria (16, 27, 29, 30). These toxins share a number of common properties : they are water-soluble toxins (31) ; they consist of single polypeptide chains in the MW range 50,000 to 80,000 (28, 31) ; they are antigenically related as shown by cross neutralization and immunoprecipitation (7) ; their cytolytic and other biological effects are lost by oxidation and restored by thiols or sulfhydryl (SH - compounds) and other reducing agents (7, 28, 31) ; they are inactivated by cholesterol and certain related sterols (7) ; they interact with surface-exposed membrane cholesterol and exert cytolytic-cytotoxic effects on a broad spectrum of mammalian cells (16, 28, 31). The mechanism of action of these toxins is very likely identical or at least closely similar (7). However, Kehoe and his colleagues found that there was

no homology detected between the cloned SLO determinant and DNA isolated from bacteria expressing other thiol-activated cytolysins related to SLO (16).

Table 3 Gram-Positive, Bacterial Species Producing the Immunologically and Chemically Related Sulfhydryl Dependent ('Oxygen-Labile') Cytolytic Toxins

Family	Genus	Species	Toxin
Streptococcaceae	Streptococcus	S. pyogenes	Streptolysin O
		S. pneumoniae	Pneumolysin
Bacillaceae	Bacillus	B. cereus	Cereolysin
		B. thuringiensis	Thuringiolysin O
		B. alvei	Alveolysin
		B. laterosporus	Laterosporolysin
	Clostridium	Cl. bifermentans (sordellii)	Bifermentolysin
		Cl. botulinum	Botutinolysin
		Cl. histolyticum	ϵ-Toxin (Histolyticolysin)
		Cl. novyi type A (oedematiens)	γ-Toxin (Oedematolysin O)
		Cl. perfringens	θ-Toxin (Perfringolysin O)
		Cl. septicum	Septicolysin O
		Cl. tetani	Tetanolysin
		Cl. chauvoei	Chauveolysin
Lactobacillaceae	Listeria	L. monocytogenes	Listeriolysin

The molecular weight of SLO, assumed by Alouf (7), falls very likely within the value 67,000 ± 5,000. He also commented that the discrepancies among the values reported by different investigators may not only stem from the methods in the measurement of the MW but could also be due to other factors. Possible nicking by the streptococcal proteinase which may cleave a peptide from the native toxin could explain the lower values reported. In contrast, the high values could be due to small peptides from the medium or from other streptococcal protein (s) which may co-purify with the toxin. Other characteristics of SLO are shown in Table 4 (7)

Table 4 Some Characteristics of Streptolysin O

Molecular weight (SDS-Page)			67,000 ± 5,000
Isoelectric point			7.8
Hemolytic units (HU) per mg protein*			400,000
LD_{50} (i.v.) in μg of protein :			
20 g mouse			0.2 μg
250 g guinea pig			6 "
2 kg rabbit			3 "
Minimum lethal dose for 9 day old embryonated hen egg			2×10^{-2} μg
Amino acid composition (Best whole numbers of residues)			
Aspartic acid	76	Isoleucine	24
Threonine	33	Leucine	23
Serine	38	Tyrosine	15
Glutamic acid	48	Phenylalanine	16
Proline	19	Histidine	7
Glycine	27	Lysine	57
Alanine	42	Arginine	18
Valine	42	Half-cystine	ND
Methionine	ND**	Tryptophane	ND

* Assay system on rabbit erythrocytes as described by Alouf et al. (32)

** ND = Not determined

SLO and the related 'oxygen-labile' cytolysins can be reversibly oxidized and reduced. The cytolytic and other biological activities of SLO are lost upon exposure to atmospheric oxygen or by treatment with mild oxidizing agents, such as hydrogenperoxide, iodine, and rapidly restored by adding SH-compounds or other reducing agents. These properties led to the assumption by Herbert and Todd (33) that SLO is a protein containing at least one disulfide bond in the oxidized (biologically inactive) state which can be reduced into free -SH groups in the biologically active state, as shown in figure 3 (7). Thus, oxidized SLO is much less toxic than the reduced activated form (7).

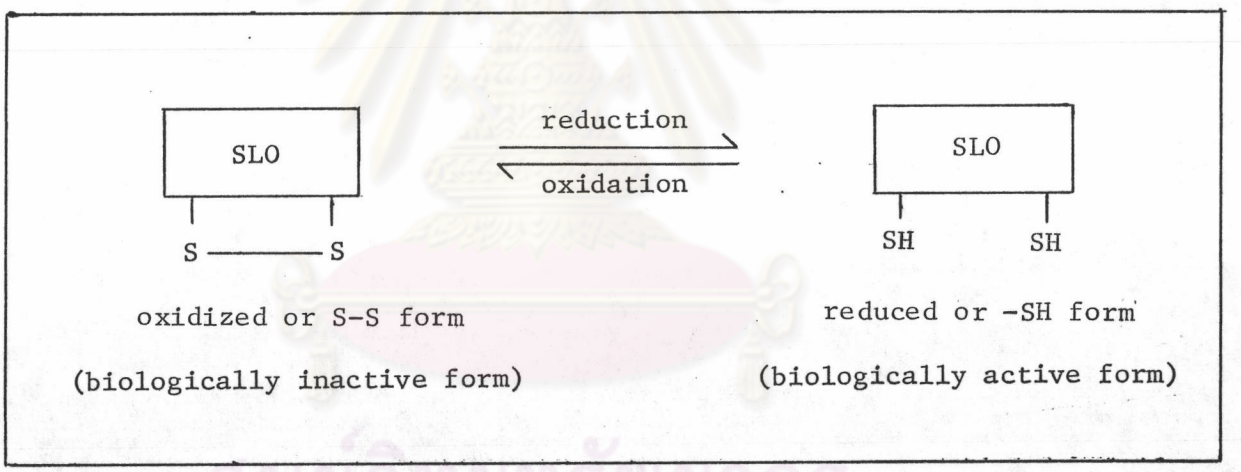

Figure 3 Oxidation-Reduction System of Streptolysin O

It is of interest to note the fact that there is no significant difference in the serum titer attained whether the animals were immunized with SLO in the oxidized or reduced state ; however, this fact may be explained by reduction in vivo (19, 20).

When the sulfhydryl groups of SLO are oxidized, the capacity of SLO to hemolyze erythrocytes is lost, but not that of binding capacity for specific antibodies (34-36). This fact leads to a new hemolytic method for determination of ASO in whole blood, proposed by Rici et al. (34).

SLO is able to invoke membrane damage by binding to cholesterol-containing target membranes and assembling into supramolecular curved rod structures to form rings and arcs which penetrate into the apolar domain of the bilayer. The convex sides of these rods are a hydrophobic, lipid-binding domain, whereas the concave sides appear to be hydrophilic. Embedment of ring-shaped and arc-shaped SLO oligomers generates large transmembrane channels of 25 to 30 nm internal diameter and allows SLO to be classified as a member of the channel-forming proteins (28). However, after the isolation of SLO oligomers in extensively delipidated form in detergent solution, it became apparent that cholesterol, although representing the primary toxin acceptor, could not itself substantially contribute to the observed rod structures. The rods are stable structures that resist prolonged exposure to trypsin and chymotrypsin. They can be reincorporated into cholesterol-free phosphatidylcholine liposomes to generate lesions identical to those observed on erythrocytes lysed by native SLO. Thus, although the cholesterol plays a key role in the initial binding of SLO to the membrane, it does not directly participate in the formation of the membrane-penetrating toxin channels. The membranes damaged by SLO haboured extremely large functional pores through which hemoglobin would directly escape, and this would explain why SLO-dependent hemolysis

does not follow the characteristic of osmotic lysis, as found for complement and staphylococcal α-toxin induced hemolysis (28, 31).

2.4 Production of Streptolysin O

Culture supernate fluids are the starting material for toxin production as SLO is an extracellular toxin (37) released during the exponential and stationary phases of bacterial growth (7). Cellular location of SLO before release appears essentially periplasmic, the region between cell wall and cell membrane (38). Early work on SLO was often carried out with culture filtrates prepared from organisms grown in very complex media, such as Todd Hewitt broth (26), which was not appropriate for purification purposes because of the presence of large molecular weight peptides. However, several investigators showed that SLO can be synthesized in a chemically defined medium (39-41). Excellent yields have also been obtained in media prepared by dialysis of peptones casein hydrolysate (Trypticase) supplemented with yeast extract (7).

In general, it appears that increased growth of the microorganisms is accompanied pari passu by increased SLO yields (18). However, Slade and Knox, as well as Fuvessy, et al., obtained evidence that some added reducing agents result in enhanced SLO formation without a concomitant growth increase (42, 43).

Fuvessy, et al. had studied the role of reducing agents in the formation of SLO and reported that SLO formation was markedly increased by the combination of cysteine and ascorbic acid, and slightly by cysteine as well as the combination of cystine and ascorbic acid. The other reducing agents were ineffective in this

respect. Specific growth rate of the strain was not influenced by the reducing compounds. (43)

Boszormenyi, et al. found that a glucose concentration of about 1.0 % and neutral pH, maintained during the entire cultivation period were necessary for optimal SLO production. If the glucose concentration was above or below 1.0 %, SLO production was reduced to a greater extent than the resulting total cell count. (44) There is general agreement that optimum concentrations of glucose are essential for the satisfactory synthesis of SLO, and that the resulting acids, particularly lactic (6) must be neutralized so as to maintain the cultural pH between 6.8 - 7.4, in order to avoid toxin destruction by acidity and (or) by proteolysis by the streptococcal proteinase which is released into the medium when pH drops below 6.7 (7, 45).

The peak of SLO occurs between 6 - 12 hr after inoculation, and in many studies the titers dropped rapidly following this time (7).

Although SLO is usually carried out by batchwise cultures, continuous cultivation of the microorganisms may result in good yields (19).

Most strains of group A and many strains of group C and G streptococci appear to produce a SLO which is identical biologically and immunochemically. Individual strains vary considerably in their quantitative ability to produce SLO in suitable media. (19)

The best yields in optimal culture conditions have been obtained with the following strains : C 203 S (20, 39, 46 - 48),

Richard type 3, Kalback's S 84 type 3, strain 814 type 4. (7) The production of SLS by these strains may be avoided if culture media are free from SLS-inducers, e.g. serum components, RNA, detergents. This is the case for the dialized medium (49). The SLS^- mutant C 203 U derived from C 203 S strain (50) has also been successfully employed for SLO production and purification (51, 52).

Dassy,et al. indicated that biosynthesis and release of this cytolytic toxin did not require specific stimulatory or toxinogenic factors, such as those presented in certain protein hydrolysates of complex medium, as was the case for many bacterial toxins (41).

2.5 Titration of Streptolysin O

The quantitative determination of SLO in either crude or purified material can be done with two distinct methods :

A. Estimation of the Hemolytic Activity of SLO

This estimation expresses as hemolytic units per ml of toxin solution (53). The hemolytic unit is arbitrarily defined as the smallest amount (highest dilution) of toxin previously activated (54) that will liberate half the hemoglobin (50 % lysis) from a suspension of washed erythrocytes (usually sheep or rabbit) under fixed conditions of time, temperature, ionic environment and red blood cell concentration. As shown in Table 5 (7) the hemolytic unit employed is unfortunately not identical in all instances, the parameters of the assay system being different depending on laboratories. However, the figures found in the literature are at least roughly comparable (10).

Table 5 Some Parameters of Assay System at 37°C of Streptolysin O from Various Laboratories

Reaction mixture volume (ml)	Erythrocyte suspension (volume and %)	Buffer	Incubation time (min)	References
2	1 ml 2.25 % RRBC	PBS pH 6.5	30	(53)
1.6	0.4 ml 4 % RRBC	PBS + BSA pH 6.7	60	(55)
1.5	0.5 ml 2.25 % RRBC	PBS + BSA pH 6.5	45	(56)
1.5	0.5 ml 5 % SRBC	PBS + BSA pH 6.5	45	(57)
2	1 ml 2 % RRBC	PBS + BSA pH 6.4	45	(58)
4	2 ml 0.7 % RRBC	PBS pH 6.5	30	(59)
2	1 ml 0.7 % RRBC	PBS + BSA pH 7	30	(60)
2	1 ml 1 % SRBC	PBS pH 6.8	30	(61)
5.5	0.5 ml 3 % SRBC	PBS pH 6.5	75	(38)
2	2 ml 2 % RRBC	Saline pH ?	45	(34)

Abbreviations : RRBC = rabbit red blood cells
SRBC = sheep red blood cells
PBS = phosphate buffered saline
BSA = bovine serum albumin

B. Estimation of Antistreptolysin - Combining Capacity of SLO

The combining unit of toxin is defined as that amount of SLO which in the presence of 1 international unit of ASO lyses under the experimental condition described of 50 % of the standard cell suspension.

1 combining unit of SLO is equivalent on average to 50 hemolytic units in the rabbit cells assay system or 25 hemolytic units in the sheep cells assay system.

It can be explained by the fact that ASO combines with SLO in its oxidized form as well as in its reduced form and different lots of SLO differ not only in their total amounts but also in the proportion of reduced to the oxidized form (62). Thus, different lots of SLO which have almost the same combining capacity may have different hemolytic activity.

As is known, an absence of hemolysis is rather difficult to determine accurately with the naked eye. This can be explained by the fact that, when percentages of hemolysis are plotted against the concentration of SLO, a sigmoid curve is formed. Thus at both ends of the curve, the increments of the amount of hemolysis are small for rather large increases of the concentrations of the SLO. However, at the region of 50 % hemolysis, a small increase of SLO will lead to a great increase of hemolysis. Therefore, the 50 % end point can be determined more accurately than the no hemolysis end point (62).

It was found that when a small amount of SLO was added to various concentrations of red blood cells, the amount of hemolysis varied inversely with the amount of red cells. There was more hemolysis in tubes with less red cells than in those with more cells (62). Thus, red blood cells should be standardized by spectrophotometer before being added in the titration system so as to yield accurate results.

It has been demonstrated that hemolytic activity of a reagent varies with the total volume of the lytic system (63). Therefore, it seems advisable to retain the original proportions of the reagents and the total volume of the reaction mixture (62).

2.6 Antistreptolysin O (ASO)

Streptococcal infections of the pharynx and skin are rather common and easily documented by culture. However, such infection may not be clinically apparent, and the patient may not seek medical attention until after the onset of either acute rheumatic fever or post-streptococcal glomerulonephritis. In these situations, demonstration of a serologic response to streptococcal antigen provides evidence of antecedent streptococcal infection and the antistreptolysin O (ASO) titer determination is considered to be one of the most suitable tests (64).

Owing to the ubiquity of group A streptococci among the population, the frequency of streptococcal infections and their repeated occurrence in the same individuals, a certain quantity of ASO called the normal level, persists in human sera. The level usually considered normal is up to 200 units (8, 48, 65). There is some variation in the values of normal levels depending on age, season, climate and geographical location (48, 64, 66). The newborn has the ASO titers similar to those of the mother, but this falls significantly by the age of six months. Streptococcal infections under the age of two years are uncommon, and persons in this age group usually have ASO titers less than 50 units. A child in the school age group, 5 - 12 years of age, is repeatedly exposed to

group A streptococci and often has titers up to 200 units without having experienced a recent clinically apparent infection. In adults, the upper normal titer is in the range of 125 units. (8, 66 - 68)

In human streptococcal respiratory tract disease, a rise in ASO is usually detectable by the end of the first week of illness and the peak level is reached between the third and fifth weeks. The level then decreases, returning to its initial level in the second or third month. On an average, higher levels of ASO occur in the non-suppurative complications of streptococcal infections, e.g. rheumatic fever and acute glomerulonephritis, than in uncomplicated streptococcal infections. (8, 48, 69). It is therefore advisable to examine two serum samples, collected preferably at the begining of the acute phase of the disease and about two weeks later (48). An increase in the ASO titer higher than the titration error of the method, estimated at 30 % (48, 68, 70), or if there is a two-dilution rise in titer (66) indicates recent infection. Although paired serum samples are the best way to demonstrate a recent infection, unfortunately many times only a single sample is submitted to the laboratory ; or if two samples are sent they may both have been collected during the convalescent stage of the infection, in which peak titers are present. Therefore, interpretation of single titer or constant titers must be made with an understanding of the variations seen in "normals" of different ages (8, 15, 66). These stated "normal" values have been obtained by measuring titers in persons lacking evidence of recent group A streptococcal infections and by arbitrarily setting the upper normal limit to include 80 % of the

group tested (71).

It has been estimated that while throat infection with group A streptococcus leads in about 80 % of cases to an increase of the ASO level, skin infection is followed by a rise of this antibody rather rarely, even if glomerulonephritis occurs. (8, 48, 68, 72).

A high ASO titer does not necessarily mean that the patient is suffering from rheumatic fever or acute glomerulonephritis, but it does indicate that a focus of streptococcal infection is present. The deciding factors in diagnosis are the clinical symptoms and other relevant laboratory findings (15).

The ASO titer is the single best test to document antecedent streptococcal infection, elevated titers being present in 80 % of patients with recent pharyngitis. To obtain a 90 % probability of documenting such infection, it is recommended that titers with two different tests be measured. Perhaps an additional 5 % are identified if a third different test is performed. The antihyaluronidase and anti-DNase-B are the two most frequently used secondary procedures ; however, the anti-DNase-B would appear preferable because of its utility in patients with streptococcal pyoderma (8, 12), as prevously mentioned. Though the use of other antistreptococcal antibody determinations increases the percentage of positive results, the ASO titer dertermination is still mainly used both in routine works and in research studies.

Nowadays, there are several techniques used for determination of ASO in human serum, such as the Rantz-Randall macro technique (73) ; the microtitration procedure of Klein et al. (74) ; agglutination

tests utilizing latex or erythrocytes as carrier of SLO (75 - 78) ; the Blue-ASO test, based on the principle of passive agglutination of sensitized bacterial carrier cells (79) ; bentonite antistreptolysin O Test (80) ; new hemolytic method for determination of ASO in whole blood (34) ; the automated method (81) ; etc. The one which is the most generally useful is the procedure standardized by Todd (22) and subsequently modified by Rantz and Randall (73). This classical hemolytic procedure is based on the ability of free reduced SLO, not neutralized by ASO antibodies, to lyse erythrocytes, which serve as an indicator. The end point is the highest dilution of serum having no hemolysis and is expressed in Todd units (36, 46, 68, 70).

The problem in Thailand is that the reagents and antigens have to be imported, making the cost of this test high. Many attempts were made to eliminate this problem. In 1970 Vejjajiva, et al., proposed the details of a method for preparing SLO (69) so as to be used in routine work in the Immunology Unit, Department of Microbiology, Chulalongkorn Hospital. It was quite easy to carry out in average laboratory at a low cost. Vejjajiva also reported the satisfactory results obtained by the use of that preparation (69). At present, it is found that SLO prepared by Vejjajiva's method is not constant in potency. There are several factors which may be involved, such as the strain of the microorgnism, cultural media, cultural conditions, etc. Among these, the most interesting factor is the cultural conditions because there have been very few studies dealing with this factor. Therefore, the cultural conditions

have been so far poorly documented.

Streptococcus pyogenes (group A) strain C 203 S, which is one of the powerful hemolytic strains recommended by several investigators (20, 39, 46 - 48), was chosen to be studied and Todd Hewitt broth (Difco Laboratories, Detroit, Michigan, U.S.A.), recommended by Vejjajiva (69) was an appropriate cultural medium for this study because of its constant in ingredient.

The purpose of this study is to find out various optimum conditions for SLO production by the chosen strain in Todd Hewitt broth. Those cultural conditions are

A. The incubation period
B. The age of starter
C. The size of starter
D. The incubation temperature
E. The initial pH
F. The effect of carbondioxide and agitation

It is hoped that the results obtained will be useful for the improvement of SLO production in average laboratory.