DEVELOPMENT OF AN ELISA TEST FOR THE SERODIAGNOSIS OF TYPHOID INFECTIONS

PRINSLOO AZWITEVHELWI NEVHUTALU

Submitted in part fulfilment of the requirements for the degree of

MASTER OF SCIENCE

In the Department of Medical Laboratory Sciences
Faculty of Mathematics and Natural Sciences

UNIVERSITY OF THE NORTH

Supervisor : M. Alberts

I declare that this thesis hereby submitted to the University of the North by me for the degree of Master of Science has not been submitted by me for a degree at another university and that it is my own original work.

Signed: P.A. Nevhutalu

P.A. Nevhutalu

Date: 29/03/83

ACKNOWLEDGEMENTS

This thesis would not have been possible without the help and advice of the following people, to whom I wish to convey my sincere thanks:

PROF. M. ALBERTS	For promoting this thesis, her advice and encouragement.
DR. M. LEWIS and the staff of the Institute of Tropical Diseases in Tzaneen.	For their advice and supply of specimens.
The staff members of Department of Medical Laboratory Sciences University of the North	For their help and advice in this work.
MR. L. MINTER	For reading the manuscript.
PROF. H. W. ALBERTS	For helping and advising me during the preparation of this thesis.
MRS. HAGEN and MRS. MOJAPELO	For typing this manuscript.

Finally I would like to thank my wife, Khanyisa and my family for the support they gave me.

SUMMARY

Typhoid fever is an acute infectious disease which is prevalent in the rural areas in South Africa.

The causative agent, Salmonella typhi is exclusively a human pathogen and belongs to the family Enterobacteriaceae, a genera of gram-negative bacili. The outer capsule of S. typhi contains two types of antigens, the Vi and the O antigens, while the flagella contains the H-antigens. S. typhi enters the small intestine and after multiplication, invades the systematic circulation and releases endotoxins.

The most commonly used laboratory tests in diagnosis of typhoid fever include bacteriological isolation of the organism, Widal test, rapid slide test and the diazo test. These tests have certain disadvantages. A need exists for a quicker and more specific test for use in the diagnosis of typhoid fever.

The application of the enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies in acute typhoid infections is discussed.

As in all serodiagnostic tests, it is important to use an antigen which is specific for the antibody to be determined so as to avoid cross reactions. A satisfactory antigen was prepared using the phenol-water procedure for the extraction of lipopolysaccharides from S. typhi. The contaminating nucleic acids were removed by ultracentrifugation. This preparation showed specificity for S. typhi antibodies when used as an antigen in the ELISA assay.

The antibody-enzyme conjugate which was prepared for use in ELISA was found to be less effective and a commercial preparation was used as an alternative.

A high correlation was found between isolations of S. typhi from body fluids and the ELISA titre. Compared with the Widal test, ELISA was found to be more sensitive.

The ELISA method offers several advantages over other currently used tests since it is reproducible, accurate and can be automated to a considerable degree by means of diluters, dispensers, plate washing machines and plate scanners.

OPSOMMING

Maagkoors is 'n uiters ernstige aansteeklike siekte wat nog algemeen voorkom in landelike gebiede van Suid-Afrika.

Die organisme wat die siekte veroorsaak, Salmonella typhii is 'n uitsluitlike menslike patogeen en behoort tot die Enterobacteriacea, 'n familie van gramnegatiewe basillus-bakterië. Die buitenste wand van S. typhi bevat twee tipes antigene, nl Vi en O terwyl die flagellum H antigene bevat. S. typhi dring in deur die dunderm en na vermeerdering kom dit in die bloedsisteem waar die endotoksienes vrygestel word.

By die diagnose van maagkoors word bakteriologiese isolasie van die organisme asook die Widal-toets, sg."Rapid Slide Test" en die Diazo-toets algemeen in laboratoria toegepas. Al die toetse het sekere nadele en 'n behoefte bestaan na 'n vinniger en meer spesifieke toets vir maagkoors.

Die toepassing van die ensiem-gebonde teenliggaambindende bepaling (enzyme linked immunosorbent assay, ELISA) om antiliggame in hewige maagkoorsbesmettings vas te stel, word bespreek.

Soos maar die geval is in alle serodiagnostiese toetse, is dit hier ook belangrik om 'n antigeen te gebruik wat spesifiek is vir die besondere teenliggaampies en daardeur kruisreaksies te voorkom. Deur die fenol-water tegniek vir die ekstraksie van lipopolisakkarides van S. typhi toe te pas, is 'n bevredigende antigeen berei en die besoedelende nukleïsure is verwyder deur ultrasentrifugering. Toe hierdie preparaat as 'n antigeen in die ELISA bepaling gebruik is, het dit spesifiek vir S. typhi anti-

liggaampies getoon. Die antiliggaamensiem-konjugaat wat voorberei was vir ELISA was egter minder effektief en 'n kommersiële preparaat is as alternatief gebruik.

Daar is goeie korrellasies aangetref tussen isolate van S. typhi afkomstig van liggaamvloeistowwe en die ELISA titer. In vergelyking met die Widal-toets, is ELISA beslis sensitiewer.

Die ELISA metode hou dus aansienlike voordele in bo die ander bestaande toetse omdat dit herhaalbaar is, akkuraat is, en dit homself leen tot outomatisasie deur gebruikmaking van verdunners.

C O N T E N T S

CHAPTER I

INTRODUCTION

HISTORICAL REVIEW

The word 'typhoid' comes from the association that was believed to exist between typhoid fever and typhus. The word 'typhos' is derived from the Greek word 'typhos' which means smoke with which the Greeks believed they could become bemused. It was initially believed that typhus was caused by smoke, vapour of exhalation which came from the ground; invisible to the eye though discernible by the nose.

The history of typhoid fever dates back to the time of Hippocrates who described a fever which probably was typhoid (Netter, 1975). Antonius Musa treated Emperor Augustus with cold baths when he fell ill with a disease similar to typhoid (Huckstep, 1962). Thomas Willis in the mid 15th Century described the onset of typhoid fever and the typical pyrexia which rises during the first week, is maintained during the second week and falls during the third week. (Willis, 1864).

Trousseau wrote a paper on typhoid fever in 1826 on the work of Pierre Bretenneau of Tours who distinguished 'dothienenterite' (typhoid fever) as a separate disease from typhus (Carr, 1892).

Trousseau attempted to separate typhoid fever from all other diseases of the gastrointestinal tract on a pathological basis. He described the typical inflammation of the Peyer's patches and Brunner's glands and gave a description of the postmortem appearance of the gastrointestinal tract.

BIOLOGICAL CHARACTERISTICS OF S.TYPHI

Salmonella typhi belongs to the family Enterobacteriaceae which consists of genera of gram-negative bacilli with a common and characteristic morphology. Members of this family inhabit the intestinal tract of humans and animals. The other common genera include Shigella, Escherichia, Klebsiella and Enterobacter. Their outstanding characteristic morphology is their rigid double cell wall and their three types of antigens (Orskov and Orskov, 1962). The rigidity of the cell wall is determined by the mucopeptide (murein) layer and the lipopolysaccharide. The genus Salmonella has specific characteristics which help to distinguish this genus from other genera of the family.

MORPHOLOGY

Salmonella typhi is a non-sporing bacillus measuring approximately 2-4 µ by 0,5 µ. The organism occurs singly, in pairs, and occasionally, in short chains and moves by means of peritrichous flagella. Most of the strains are fimbriate and non-capsulated. Three types of antigens are present, O or somatic antigens, H or flagellar antigens and the Vi antigens which is associated with the K antigens of the Enterobacteriaceae (Fig. 1.1)

Antigenic characteristics

O antigens

Somatic or O antigens are part of the bacterial cell wall (Fig. 1.1). If the strain loses the 'O antigen' it changes from a smooth to a rough colony which will not agglutinate sera containing O-antibodies. The O antigens

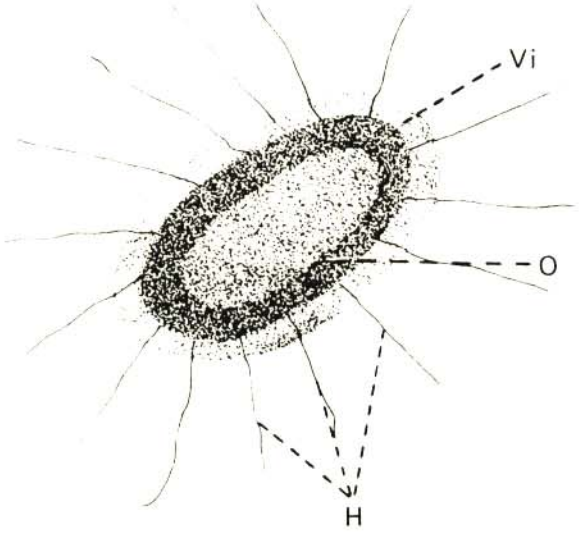
During the late 19th Century, William Budd of Devon, a student of Pierre Louis, showed that typhoid fever was not spread by stench, but was an alimentary disease in which the infective material in faeces contaminated the water, milk and hands of those who tend the sick (Budd, 1873). In 1869 the term Enteric fever was introduced to try and focus the attention on the anatomical seat of lesions (Wilson, 1881).

It was not until Eberth's discovery of typhoid bacilli in the mesenteric lymph nodes that the crucial advance in the aetiology of typhoid fever occurred (Eberth, 1880). Later Pfeiffer isolated Salmonella typhi from stools of patients (Pfeiffer, 1885). The first successful culture of Salmonella typhi was performed by Gaffky (Gaffky, 1884). The diagnosis of typhoid fever up to that time had been purely clinical and the discovery of S. typhi opened new fields in aetiology, diagnosis and prophylaxis.

Although the cause of typhoid fever was unknown and bacteriologists unheard of, the diagnosis was seldom in doubt. Trousseau summarized the knowledge of typhoid fever in the 19th Century with the following : "The disease, just as common and no less murderous than smallpox, measles and scarlet fever, that few people go to the end of the lives without having experienced its attacks, affects an individual only once during his life and is perhaps of a contagious nature." (Trousseau, 1826).

Pierre Louis (1829) a physician in France, described in detail the post-mortem appearance, especially the enlargement and ulceration of Peyer's patches. Although he did not differentiate between typhoid and typhus, he was the first to use the word 'typhoid' (Lilienfeld and Lilienfeld, 1977). Schoehlein (1839) recognised the difference between typhus fever and typhoid fever by giving the name 'Typhus exanthematicus' for typhus fever and 'typhus abdominalis' for typhoid fever. His pioneering work was only credited in Germany and the confusion was not resolved in France (Wilson and Miles, 1964).

It was not until Jenner (1850) published his paper "On the Identity or Non-Identity of Typhoid and Typhus Fevers" that the differences of the two fevers became clear. His evidence was based on clinical and postmortem appearances of 66 fatal cases. Jenner showed how the general symptoms differed in the two diseases,that the rash was never identical and how the lesions of Peyer's patches and mesenteric glands so characteristic of typhoid fever were never present in typhus. In the same article, Jenner also indicated that an attack of one fever gave some protection against a subsequent attack of the same fever, but not against an attack of the other fever (Jenner, 1850).



Antigenic structure of *Salmonella typhi*.

Fig. 1.1: Antigenic structure of Salmonella typhi.

The figure shows three types of antigens; the H or flagellar, the O or somatic and the Vi or capsular antigens (Jawetz et al., 1980).

are complex lipopolysaccharides (LPS) which are released in the host as endotoxin which is responsible for the toxaemia which characterizes typhoid fever. Since lipopolysaccharides are resistant to heat and alcohol, boiled bacteria are suitable for immunisation, agglutination and agglutinin absorption. The O antigens serve as a basis for the division in the Kauffman-White scheme which divides Salmonella into serotypes. The serotypes are denoted by the use of capital Roman letters (A,B, C and D.)

Westphal and Luderitz (1954) made a major contribution to the knowledge of the chemistry of the LPS when they found that the LPS molecule consists of a lipid unit bound convalently to a polysaccharide. The lipid was named lipid A. In addition another lipid moiety was found which is not covalently bound to the polysaccharide. This was called lipid B (Westphal and Luderitx, 1954). Another lipid component, not yet well characterized, seems necessary for the assembly of the monosaccharides into the 'rough core' of the lipopolysaccharide (Rothfield and Horecker, 1964; Rothfield and Takeshita, 1965). Lipid A is a glycolipid with a backbone of a phosphorylated glucosaminyl - (B1 - 6) glucosamine disaccharide unit which is linked ketosidically to a trisaccharide of three 2-keto-3-deoxyoctonic acid (Luderitz et al., 1968; Osborn, 1963; Heath et al., 1966).

Lipid B is easily dissociated from the LPS molecule with formalin and ethanol or with alkaline ethanol (Morgan and Patrige, 1941). Lipid B contains palmitic and oleic acids and about 1,5% phosphorus (Luderitz et al., 1966; Luderitz et al., 1968).

The polysaccharide in the LPS consists of two units (Fig. 1.2).

The first unit is the basal core which consists of five sugars, 2-keto-3-deoxyoctonic acid, heptose, glucose, galactose, and N-acetylglucosamine (Westphal and Luderitz, 1954). These sugars are associated with the rough form of Salmonella strains. The second unit is the O-specific side chains, which, together with the first unit forms the smooth form of a Salmonella strain. The side chains are specific for the different groups of Salmonella. In S. typhi, which belongs to group D, the essential sugars in the side chains are galactose, rhamnose, mannose and tyvelose (Hellerqvist et al., 1969). (Fig. 1.3).

As early as 1913, Lingelsheim found a loss of virulence with mutation from the smooth form to the rough form. The rough form is more sensitive to killing by lysosomal enzymes from polymorphonuclear leucocytes than the smooth form and to the bactericidal action of antibody and complement (Nelson and Roantree, 1967; Rowley and Wardlaw, 1958).

H or Flagellar Antigens

The H antigens are located in the flagella of motile organisms (Fig. 1.1) By mild acid treatment Salmonella flagella yield a homogeneous soluble protein, named flagellin, with a molecular weight of 40,000 (Weibull, 1950). The flagellar antigens are inactivated by temperatures above 60°C and by alcohols and acids. These antigens determine the serotype of a particular strain within the somatic groups of Salmonella. In many Salmonella cultures, two alternative types of flagellar antigens are present. One of the antigens

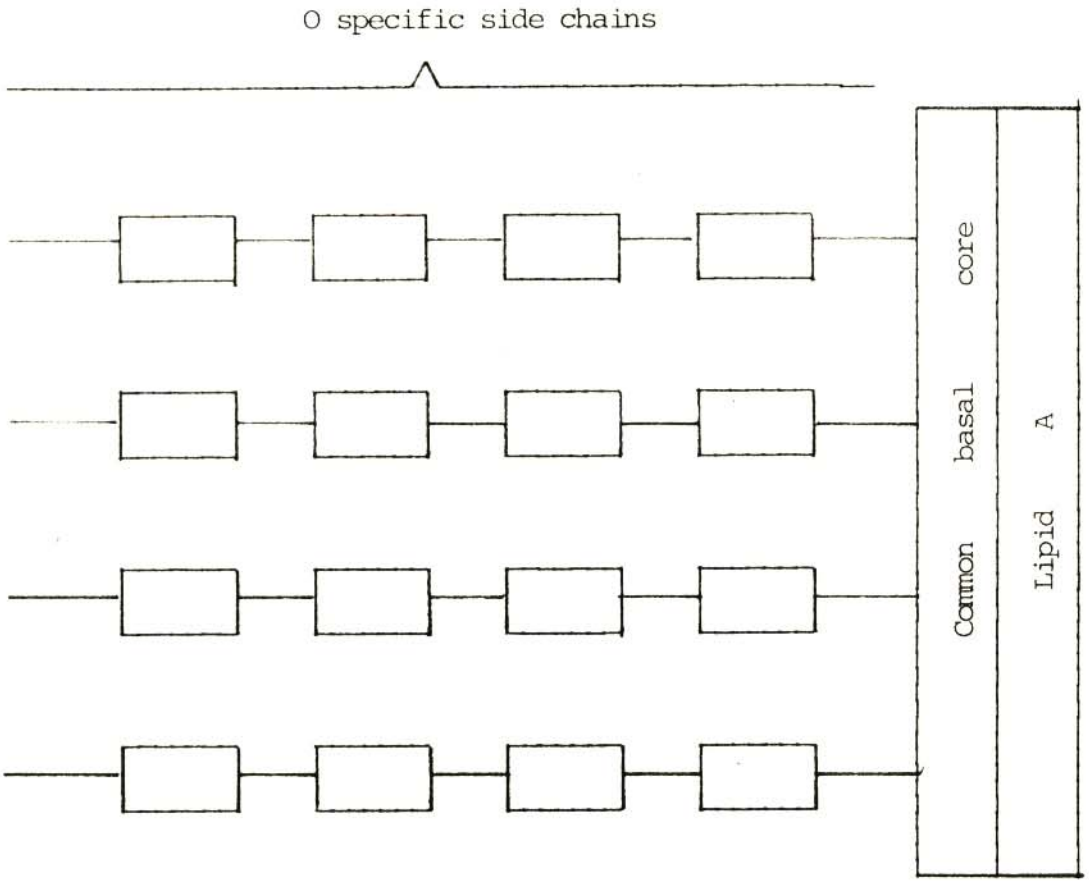


Fig. 1.2 Schematic structural diagram of somatic Salmonella O lipopolysaccharide.

The O lipopolysaccharide molecule consists of a lipid part called Lipid A, a common basal core and O specific side chains which consists of specific sugars. These chains are specific for each group of Salmonella. The common basal core consists of sugars which are characteristics for Salmonellae. (Wicken and Knox, 1980).

is called "specific phase" or phase 1 antigen and the other the "non-specific phase" or phase 2 antigen. Each Salmonella strain has its own specific type of phase 1 and phase 2 H antigens. Salmonella strains expressing phase 1 and phase 2 alternatively are called diphasic, those strains which express only one phase, care called monophasic. Antigens of phase 1, according to the Kauffmann-White scheme are designated by Roman letters and those of phase 2 by Arabic numerals. When the specific H antigens became so numerous that all letters of the alphabet were employed, the newly discovered antigens were designated by using an index number attached to the letter 'Z' e.g. "Z_1 and Z_2" (Edward and Ewing, 1952; Kauffmann, 1966).

Vi Antigens

The Vi antigens were originally discovered in agglutinable cultures of S. typhi with Vi antiserum and were thought to be connected with virulence, while later investigations showed that the Vi antigens are not of any great toxicity (Weil and Saphra, 1953; Felix and Pitt, 1934). Vi antigens are surface antigens and are less resistant to heat than other somatic antigens. The Vi antigens are acidic polysaccharides which consist of partly O-acetylated N-acetyl-D-galactosamine uronic acid (Landy et al., 1955; Baker et al., 1959). Different colonies give strong, weak or no agglutination against Vi antiserum (Orskov and Orskov, 1962). Kauffmann (1966) called the strong agglutinating strains the V form and the weak or no agglutinating strains, the W form. The loss of Vi antigens is associated with colony change from V to W form. Excreters of Salmonella typhi show a high titer of Vi antibodies (Wilson and Miles, 1964).

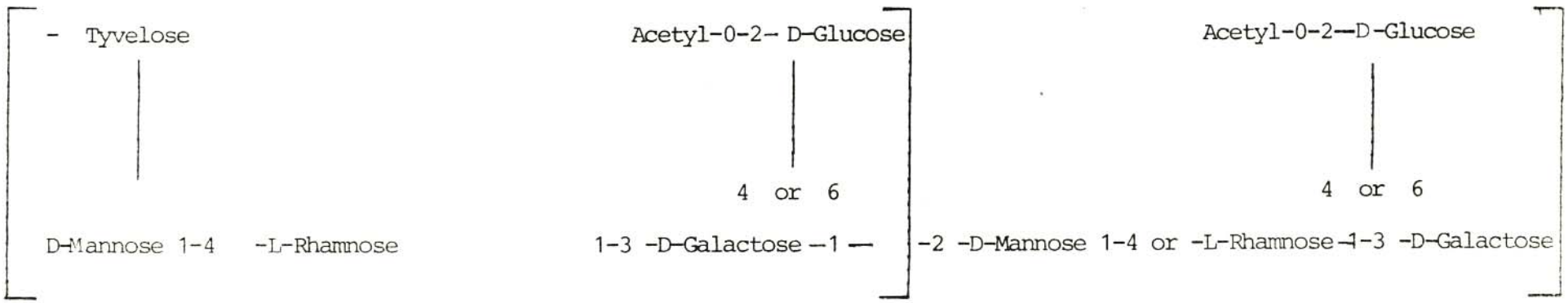


Fig. 1.3 Proposed structure for the 0-specific side - chains of group D1 Salmonella lipopolysaccharide

The structure is based on methylation studies. All sugars are in the pyranose form except L-rhamnose. The tyvelose residue is attached to D-mannose residues in the 3-position, and the mannose residue is in turn linked to the 4-position. The rhamnose residue is linked to the D-galactose residue in the 3-position which is in turn linked to the D-mannose residue through C-2 and building up the repeating unit. The glucose residue is acetylated and linked to the D-galactose residue in the 4- or 6-position. (Hellerqvist et al., 1969).

Biochemical Reactions

The identification of S. typhi is done by culture followed by biochemical tests. Table 1.1 shows the reactions of S. typhi on common media. The biochemical tests employed are shown in Table 1.2 (Sonnenwirth and Jarett, 1980). The ability to ferment carbohydrates is also used in the identification of S. typhi. Typical fermentation patterns of S. typhi are shown in Table 1.3.

Table 1.1 Growth of S. typhi on different media

MEDIA	CHARACTERISTIC REACTION
MacConkey agar	Colourless colonies
SS Agar	Colourless colonies
Bismuth sulphite agar	Black colonies with sheen
Brilliant green agar	No growth
Triple sugar iron agar	Alkaline slant, acid but no gas production, positive H_2S

Table 1.1 shows the characteristic reactions of S. typhi on differential media. From the table, it can be seen that S. typhi shows colourless colonies with MacConkey and SS agars while there is no growth on brilliant green agar. On bismuth sulphate agar S. typhi shows black colonies surrounded by a black or brown zone (Sonnenwirth and Jarett, 1980).

Table 1.2 Characteristic biochemical reactions of S. typhi

SUBSTRATE OR TEST	REACTION
KCN	-ve
Urease	-ve
Indole	-ve
Methyl red	+ve
Voges-Proskauer	-ve
Lysine decarboxylase	+ve
Arginine dihydrolase	+ve
Ornithine decarboxylase	+ve
D-Tartrate	+ve
Trehalose	+ve

Table 1.2 shows some of the biochemical reactions of Salmonella typhi. Salmonella do not ferment lactose or sucrose, do not produce indole and do not show urease activity on Christensen urea agar. In addition, at 37°C they are methyl red positive and Voges-Proskauer negative. Lysine and ornithine are decarboxylated and produce acid in Jordan's tartrate medium. (Ewing, 1974).

Table 1.3 Fermentation patterns of S. typhi on some sugars

SUGAR	REACTION
Glucose	Acid, no gas
Mannitol	Acid, no gas
Xylose	Variable
D-Tartrate	Acid, no gas
Mucate	Variable

When glucose, mannitol or D-tartrate is used, acid is produced but no gas is formed. The fermentation patterns of mucate and xylose are variable but S. typhi shows a high percentage of negative reactions. (Ewing, 1974).

PATHOGENESIS OF TYPHOID FEVER

The causative agent of typhoid fever is Salmonella typhi. The organism is one of the few that is exclusively a human pathogen. The organism enters the body of the new host by ingestion of food and water, which has become contaminated with S. typhi. The contamination is due to human excreta such as faeces and urine.

The conventional concept of pathogenesis is that typhoid bacilli enter the small intestine, invade the mucosa and enter the lymphatics, to be carried to the mesenteric glands where, after a period of multiplication, they invade the systemic circulation via the thoracic duct. The initial early bacteraemia occurs within 24 to 72 hours after ingestion and is rarely detected in natural infections because patients are usually asymptomatic. The bacilli are then ingested by cells of the reticulo-endothelial system where multiplication occurs and viable bacteria are disseminated to all organs, particularly to the liver, gallbladder, spleen and the reticulo-endothelial system. This dissemination occurs within the first seven days of the disease. From the gallbladder a further invasion of the small intestine results and the lymphoid tissue, Peyer's patches and lymphoid follicles are involved in the acute inflammatory reaction which corresponds with the onset of the manifestations of the disease. The second bacteraemia is followed by necrosis, sloughing and the formation of characteristic ulcers in the intestine.

The outcome of the interaction between typhoid bacilli and host depends on a number of factors such as the virulence of the ingested organism, the size

of the infecting dose and the resistance of the patient (Strauss, 1974). The pH of the stomach seems to play a role in the susceptibility to infection since in subjects treated with streptomycin which inhibits the growth of anaerobes responsible for maintaining an acid pH, the susceptibility to typhoid infection increases (Woodward, 1970; Miller et al., 1956). According to Hornick and Woodward (1966) as the pH increases, the number of organisms needed to infect the subject increases. Certain factors known to be associated with typhoid outbreaks such as malnutrition, may be altering the intestinal flora enhance susceptibility to typhoid infection (Hook, 1974).

In the small intestine Salmonella typhi pass from the gut into the intestinal epithelium. The bacteria advance through the brush border and the microvilli and apical cytoplasm in close proximity to the organism degenerate. Further advances into the host cell are accompanied by cavity formation in which the organism, the degenerated microvilli and the parts of the host cytoplasm are accommodated. The cavity is transformed into a vacuole which eventually becomes disconnected from the luminal plasmalemma and the overlying brush border is reconstructed (Smith, 1973).

During the incubation period, jejunal biopsy shows an inflammatory reaction, which may be diffuse or granulomatous. The exudate consists of histiocytes, plasma cells, lymphocytes, a few polymorphonuclear cells and eosinophils (Sprinz et al., 1966). As already mentioned the bacilli may pass from the systemic circulation to almost any organ but there are three organs of importance in the attack: the spleen, the intestine and the gallbladder. As a result of infection, the spleen commonly enlarges and palpation of the spleen is a valuable aid in clinical diagnosis.

IMMUNOLOGY OF TYPHOID FEVER

The immunology of typhoid fever will be discussed under the following headings:

(i) serum antibodies, (ii) intestinal antibodies, (iii) cell-mediated immunity.

(i) Serum Antibodies

Salmonella typhi has three major types of antigens, 0 antigens, H antigens and Vi antigens as was discussed previously. The knowledge of the humoral immune response to Salmonella typhi during typhoid infection has been limited to data obtained by bacterial agglutination or indirect agglutination using antigens coated on red blood cells or inert particles. Using these tests no conclusive evidence as to the type of antibody formed has been obtained (Los palluto et al., 1962; Chernokhvostavo et al., 1969; Weidanz et al., 1964; Fukazawa et al., 1970; Gannon et al., 1980; Tsang et al., 1981). Vaccine trials in different areas of the world have shown that serum antibodies to 0- and Vi-antigens are of little importance in reflecting immunity to typhoid fever while antibodies to H antigens are associated with subsequent resistance to infection (Hornick et al., 1970; Who, 1962; Who, 1964; Who, 1966; Ashcroft, 1964; Ashcroft et al., 1967; Wahdan et al., 1975; Levine et al., 1976; Gilman et al., 1977).

The role of serum antibodies to H antigens is thought to be the immobilization of the organisms, thus preventing access to the gut wall for subsequent penetration and invasion. Evidence for this hypothesis has been demonstrated with

The role of endotoxin which is a biologically active lipopolysaccharide released by bacteria after lysis, is not fully understood. Hook (1974) states that endotoxin produces fever, leucopenia, thrombocytopenia and hyperplasia of the reticulo-endothelial system. Intravenous injection of endotoxin causes headache, malaise, chills, myalgia, fever and leucopenia all of which are typical symptoms of typhoid fever (Chordiker and Tomasi, 1963). However endotoxin per se in serum may not be responsible for the sustained fever and clinical manifestations of typhoid fever, although the endotoxin may contribute to focal necrosis and haemorrhage in the intestinal tract by altering catecholamine reactivity (Thomas, 1956.).

Clinical features

The usual incubation period of typhoid fever is about 14 days but may be shorter than seven days or longer than 21 days. The symptons are of a non-specific nature and variable, and include headache, body pains, cough, constipation and pain in the upper right abdomen (Strauss, 1974). Other commonly found symptons are malaise, chills, loss of appetite, polyathralgia, sore throat, stiff neck with tender cervical musculature and epitaxis (Strauss, 1974). The temperature gradually increases for five to seven days and the pulse is usually weak, often dicrotic and relatively slow (Wickos and Mansonbass, 1975). The abdomen can be distended and the spleen enlarged. Rose spots usually appear about the seventh day but may not be visible in the Negroid (Huckstep, 1962), The stool is characteristic in most of the case, being loose and pale.

Vibrio cholerae and indications are that this is also true for typhoid bacilli (Geuntzel and Berry, 1975).

(ii) Intestinal Antibodies

Central to the theory of immobilisation is the presence of antibodies in the gut lumen and this has been shown to be of fundamental importance in preventing invasion and infection by specific pathogens (Walker and Isselbacher, 1977).

The main immunoglobulin in human secretions is IgA which is present in concentrations averaging 30 mg/100ml of fluid in the intestinal secretions (Girard and Kalbermatten, 1970; Heremans and Vaerman, 1971). The source of IgA appears to be the lymphocytes that are present in the lamina propia (Crabke et al., 1965). Antibodies of both IgA and IgM classes are present in high levels in the intestinal secretions of typhoid patients while typhoid carriers have only IgA class antibody (Chau et al., 1981; Walker, 1976). The role of IgA antibodies in intestinal secretions is to prevent adherence of an organism to the mucosal surface (Williams and Gibbons, 1972).

(iii) Cell-mediated Immunity

At an early stage in Salmonella typhi infection, macrophages, lymphocytes and plasma cells are concentrated in the lamina propia of the small intestine (Sprinz et al., 1966). It is not clear whether there is phagocytosis at this stage since S. typhi is capable of surviving and dividing in macrophages (Blander et al., 1966). At subsequent stages of infection, there is necrosis and cell depletion of lymphatic organs indicating that the

organism interferes with the patient's immunological defences (Stuart and Collee, 1967). The capacity of S. typhi to proliferate intracellularly suggests that the immunity depends on the induction of a cell-mediated immune response in which T-cell co-operation with macrophages kills the intracellular organisms (MacKannes, 1971; Collins and Carter, 1974). Studies of the reticulo-endothelial system indicate that phagocytic activity is increased in typhoid fever volunteers (Blander et al., 1966 and Wagner et al., 1963). However the role of cell-mediated immunity in typhoid fever is still not well understood.

LABORATORY DIAGNOSIS OF TYPHOID FEVER

The most commonly used laboratory tests include bacteriological isolation of the organism, Widal test, rapid slide test and the diazo test (Huckstep, 1962). More recently introduced tests are diffusion in gel, enzyme-linked immunosorbent assay and counter immunoelectrophoresis (Lange et al., 1980; Tsang and Chau, 1981).

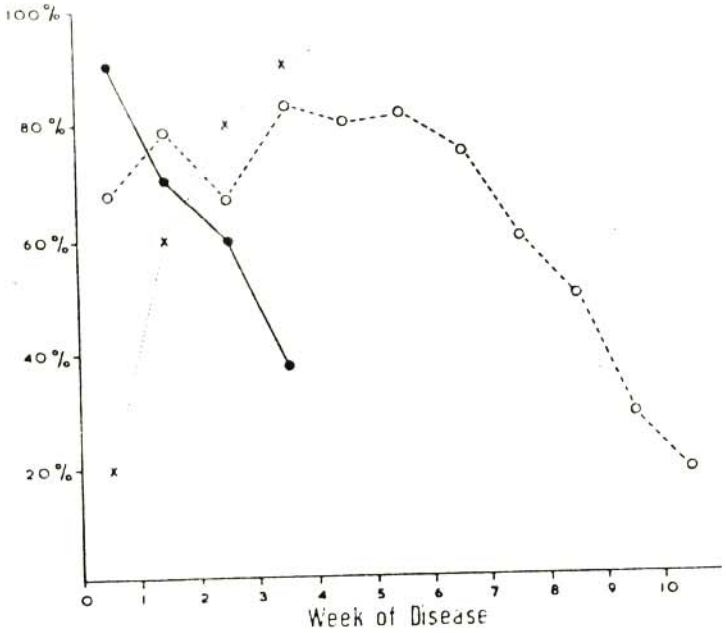
Bacteriological Isolation

The definitive diagnosis of typhoid fever depends on the isolation of S. typhi from blood, stools, urine, rose spots, bone marrow or other body fluids. Improvements in culturing techniques, and the introduction of highly selective media has greatly improved the chances of isolating bacilli.

(a) Blood culture

The organism can usually be isolated from blood in the acute stage of illness. The proportion of positive cultures is usually highest in the first week of the disease but in severe cases maximum blood bactereamia may not be reached until the second or third week (Wilson and Miles, 1964). A positive blood culture is present in about 80% of cases with typhoid fever infection while 30% of the typhoid cases are negative on one or more attempts to isolate S. typhi by blood culture method (Huckstep, 1962; Hornick et al., 1970).

Figure 1.4 Changes in concentration of Antibodies and percentage recovery of S. typhi from blood and faeces.



—●———● Percentage of cases yielding positive cultures from blood.

.....x.....x Percentage of cases with agglutinins in blood.

...o....o...o Percentage of cases yielding positive cultures from faeces.

As indicated in the figure the onset of disease is associated with a bacteraemia. From the first week onward the frequency with which S. typhi can be isolated from blood falls but it stays high in faeces until it falls as from the seventh week. (Wilson and Miles, 1964).

Bone marrow culture is of great value in patients who have taken antibiotics prior to the diagnosis because antibiotics do not easily eradicate organisms in the marrow (Gilman et al., 1975). Although bone marrow culture is suitable for diagnosis, some authors point out that the procedure cannot be used routinely due to financial and technical limitations (Debre et al., 1935, Ling et al., 1940 and Robertson et al., 1968).

(c) Urine

Culture of urine may be positive in 25% to 33% of cases but there seems to be no regular excretion pattern (Huckstep, 1962). Bacteriuria is usually limited to the first few weeks of illness although it can persist in the carrier state, so a positive urine culture does not necessarily indicate active typhoid (Smith, 1973).

(d) Stools

The percentage recovery of S. typhi in stools is high during the first and second week, reaches a maximum during the third week, and then declines, (fig. 1.4). The faeces continue to be positive even after treatment with chloramphenicol. Positive faeces culture after three or more months, may indicate the carrier state (Wilson and Miles, 1964).

(e) Rose spots and other body fluids

Hickstep (1962) has shown that rose spots, if visible, may be of great value in diagnosis of typhoid fever. However rose spots are not visible in blacks and therefore are of no value in Black patients (Wicks et al., 1971).

Culture of bile may also be performed and can be of great value in detection of carriers although the procedure is not very pleasant for patients. Typhoid bacilli have been isolated in serious fluid from breast (Campbell et al., 1980).

It must be emphasized that whatever method of isolation is used, a second culture must be performed because a first negative culture may not exclude an infection.

In many rural hospitals there is a lack of proper bacteriological facilities and specimens have to be transported to distant laboratories for culture. Therefore submission of blood for serological tests, at the expense of a definitive bacteriological diagnosis is often preferred by clinicians in these regions.

Widal test

The Widal test has been in use as an aid in the diagnosis of typhoid fever since the beginning of this century. The test is based on the demonstration of agglutinating antibodies in the serum of an infected patient, against the H (flagellar) antigen and the 0 (somatic) antigen present on Salmonella typhi. Several factors tend to obscure the serological picture of typhoid fever. The most important of these factors is that the antigens which stimulate antibody synthesis are shared by other Salmonella serotypes which belong to group D. Thus antibodies to one member of group D organisms will cross react with other members of the group, (Schroeder, 1968). The production of antibodies may also be stimulated by antigens of dead organisms as in T.A.B. vac-

cination. Therefore positive agglutination reaction may indicate contact with any Salmonella organism dead or alive (Warren and Hornick, 1979). Traditional views that agglutinin titres only become positive towards the end of the second week of illness do not appear to be true since many investigators have reported high agglutinin titres during the first week of illness. This suggests that in endemic areas where there is frequent exposure to S. typhi and other antigenically related Salmonellae, the immune response is not a primary one (Wicks et al., 1974; Senewiratne and Senewiratne, 1977; Levine et al., 1978).

In about ten percent of cases no antibodies against the S. typhi antigens can be demonstrated. (Sommerville et al., 1981). These negative cases could be ascribed to a poor immune response due to malnutrition, or to the organism being situated in an area, where an immune response cannot be elicited effectively (Chandra, 1981). False positive results are produced by conditions such as rheumatoid arthritis, rheumatic fever, ulcerative colitis, nephrotic syndrome and multiple myeloma (Senewiratne and Senewiratne, 1977).

In spite of its shortcomings the Widal test is a good alternative to the bacteriological diagnosis and can be used in endemic areas (Senewiratne and Senewiratne, 1977; Sommerville et al., 1981). Prior administration of antibiotics, which may suppress production of antibodies, should be avoided (Bai et al., 1980).

Quick agglutinating test

The principle of the quick agglutinating test is the same as that of the Widal test and measures the presence of antibodies in patients' serum against

O and H antigens. However no dilutions are made; agglutination of antigens with serum is an indication of infection. This test is potentially useful, particularly where laboratory facilities are not available (Wicks et al., 1974).

Diazo test

The Diazo test was introduced by Ehrlich (1883). It involves the mixing of patient's urine with diazo reagents. If the colour of the froth is red or pink then the reaction is positive. The Diazo test is positive during the first week and stays positive until the second week of illness (Osler, 1912). The test is non-specific and shows a positive reaction with malaria or any other acute fever with high temperatures (Huckstep 1962; Wicks et al., 1974).

Counter immunoelectrophoresis

Counter immunoelectrophoresis was introduced by Gapta and Rao (1979). In this method the antigen is put into a well on the cathodal side and serum in wells on the anodal side. A current is applied and the presence of a precipitation line indicates a positive reaction. Ninety eight percent of cases were positive when compared with culture isolations. Although data are still lacking, this test may prove to be of great value in the future (Tsang and Chau, 1981).

In view of the unreliable results obtained when employing the Widal test and the inconveniencies of using the culture method, it was decided to in-

vestigate the application of enzyme-linked immunosorbent assay (ELISA) in the diagnosis of typhoid fever. For ELISA as in other serologic tests, it is important to use an antigen which is specific for the antibody to be studied so as to avoid cross-reactions.

The experimental work covered in the following four chapters is divided into the following sections:

(2) Preparation of a specific antigen from S. typhi.

(3) Development of the antibodies for use in ELISA.

(4) Preparation of the enzyme-antibody conjugate and the standardisation of ELISA.

(5) The application of ELISA in the diagnosis of typhoid fever.

CHAPTER 2

ENZYME-LINKED IMMUNOSORBENT ASSAY FOR TYPHOID FEVER

Introduction

The enzyme linked immunosorbent assay (ELISA) introduced by Avrameas and Uriel (1966) and by Nakane and Pierce (1966) was initially used for the cytochemical localisation of cellular antigens in histochemistry.

The introduction of ELISA for the determination and quantitation of soluble antigens was pioneered by Engvall and Perlmann (1971) and by Schuurs (1971). The technique revolutionised the clinical diagnostic world, because it offered an attractive alternative to radioimmunoassay (RIA). Radioimmunoassay is highly sensitive and permits precise quantification of antibodies and antigens but has certain disadvantages (Skelley et al., 1973). The isotopes used in RIA have a short shelf life, and complex expensive equipment is necessary for their determination. The radioactive reagents used present a health hazard to the staff handling the tests. (Felber, 1978; Herner, 1978; Voller et al., 1976; Engvall and Pesce, 1975; Conradie and Mbhele, 1981). ELISA fulfils the requirements of objectivity, simplicity and sensitivity previously achieved only by means of RIA. ELISA employs stable reagents, requires less expensive equipment and is more suitable for automation (Ruitenberg and Brosi, 1975). A major advantage of ELISA over RIA is that it uses an enzyme as a marker instead of an isotope. (Engvall and Perlmann, 1971; Van Weeman and Schuurs, 1971). Engvall and Ruoslahti (1979) once wrote "ELISA is fun; a colorimetric assay gives a colour gourmet pleasure in daily work".

PRINCIPLE

ELISA uses the specific reactivity of antibody with an antigen to reveal the presence of antibodies in sera or other body fluids or to identify antigens. ELISA can be formulated in different ways.

1. Competitive ELISA (Fig. 2.1) Here the antibody to be measured competes with the labelled antibody for the reacting site on the antigen absorbed on a solid phase. This type of system has been applied for the measurement of drugs, hormones and lipids (Pal, 1978; Kominami et al., 1980; Holmqvist, 1980).

2. Non-competitive ELISA. This system can be performed in two different ways - the direct assay system and the indirect assay system. The direct assay system is shown in Fig. 2.2. This procedure has been applied for the measurement of human anti-endotoxin, immunoglobulins, viruses, antibodies, ferritin, serodiagnosis of infectious diseases and sexually transmitted diseases (Ruitenberg et al., 1976; Gaffin et al., 1982; Carlsson and Lindberg, 1978; Conradie and Mbhele, 1980; Engvall and Perlmann, 1972; Viljanen et al., 1982).

3. Indirect System of ELISA is shown in Fig. 2.3. This assay differs from the direct assay by employing a single enzyme labelled anti-species globulin or even enzyme labeled staphylococcal protein A. This system has been applied for the measurement of E. coli, enterotoxin and virus (Yolken and Stopa, 1980; Yolken et al., 1978).

The experimental work of this thesis sets out to develop, standardise and determine the optimal conditions for a direct ELISA system in the sero-

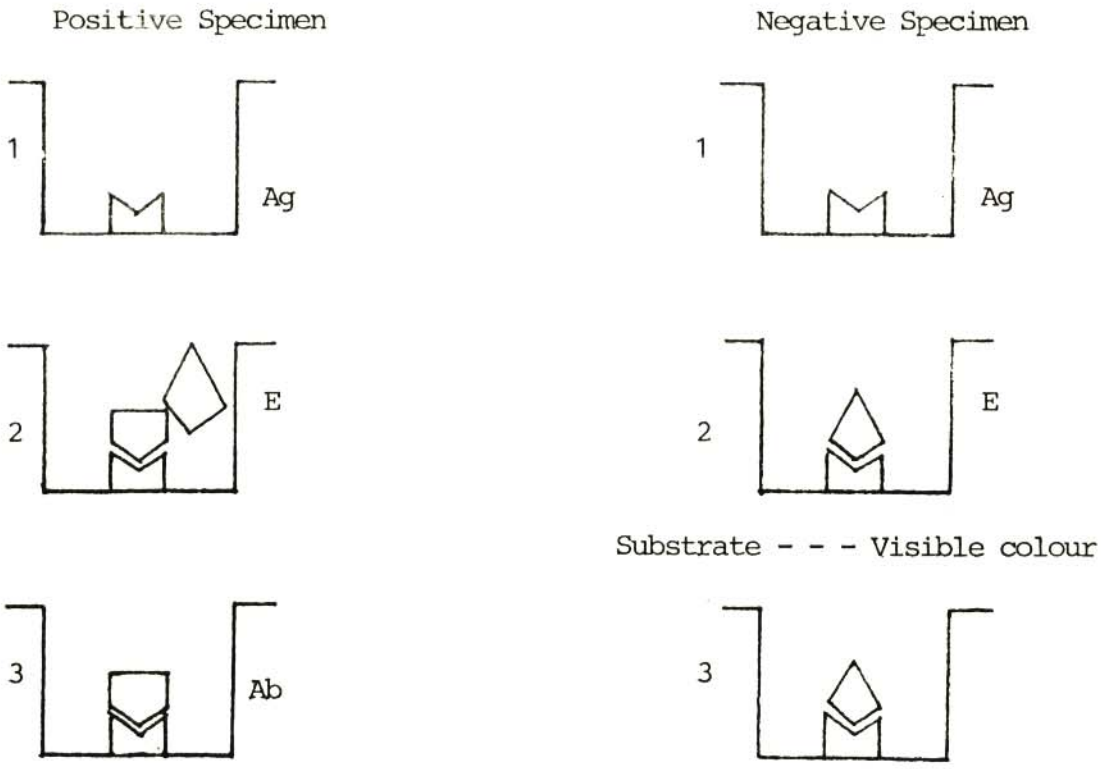


Fig. 2.1: Competitive ELISA for measuring antibody

1. Microtitre plates are coated by antigen against antibody to be measured.
2. The test specimen is added and if any antibodies are present they bind to the antigen.Enzyme labelled antibody E is added and will react with antigen sites that are not occupied by the antibody from the test specimen.
3. Unbound enzyme-linked antibody is washed and the substrate is added to quantify the amount of enzyme-antibody. The amount of substrate product is inversely proportional to the amount of antibody in the test specimen.

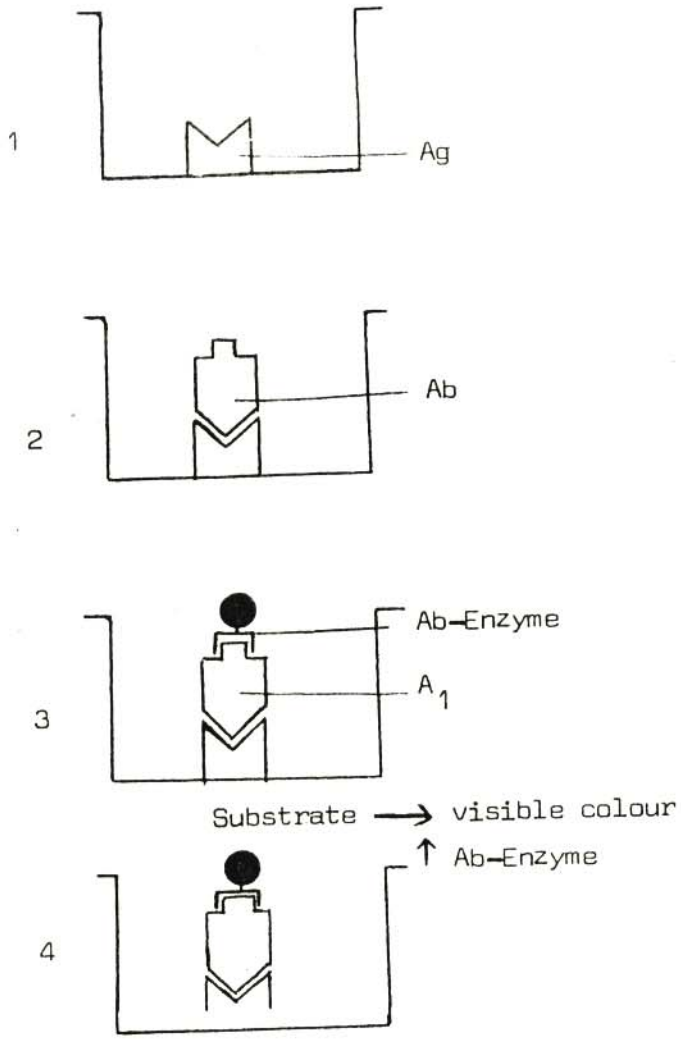


Fig. 2.2: Direct enzyme-linked immunosorbent assay for antibody measurement.

1. Microtitre plates are coated by antigen against antibody to be measured.
2. The test material is added, and antibody against the antigen will bind.
3. Anti-antibody labelled with an enzyme is added, and this complex will react with the antibody that is bound to the antigen.
4. A substrate is added and the enzyme bound to the well will convert the substrate to a visible form. The colour intensity is proportional to the amount of antibody in the test material.

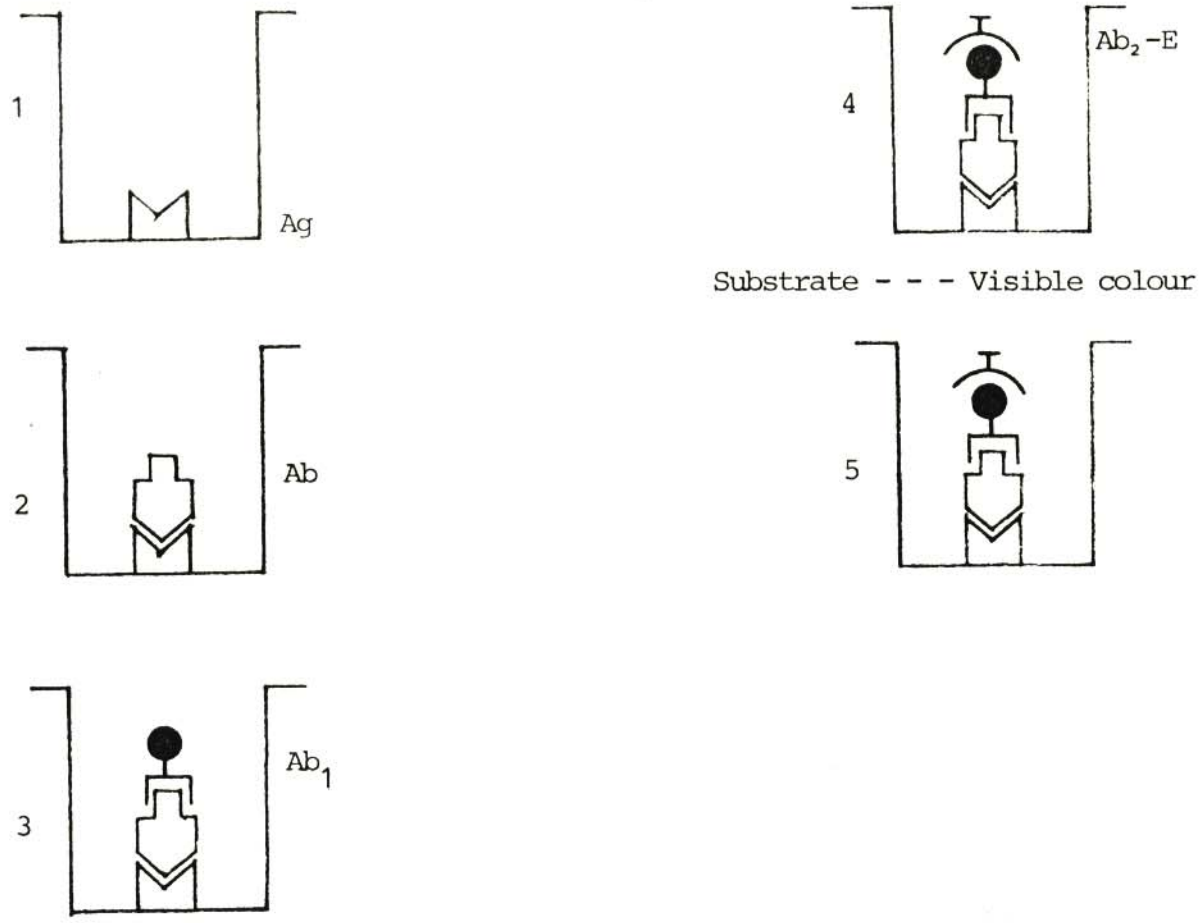


Fig. 2.3 Indirect ELISA for antibody measurement

1. Antigen against the antibody to be measured is fixed to the well of a microtitre plate. 2. The test material is added, and antibody against the antigen will bind to the antigen. 3. Unlabelled anti-antibody from a different source is added, and will bind with the antibody adhered to the antigen. 4. Enzyme labeled antibody directed against anti-antibody is added. 5. A substrate is added, and the enzyme will convert the substrate to a visible form. The colour intensity is proportional to the amount of antibody in the test material.

PREPARATION AND ISOLATION OF S. TYPHI ANTIGENS

INTRODUCTION

The first investigators to describe the extraction of lipopolysaccharide antigens from bacteria using a trichloroacetic acid extraction procedure were Biovin et al., (1933). However, their extract contained nucleic acids from the bacterial cells. Palmer and Gerlough (1940) introduced 90% phenol for the dissociation of proteins from polysaccharides, and this procedure was later modified by Westphal et al., (1952) for the preparation of antigens from Salmonella typhi. The basic principle of the method is that proteins and lipoproteins are dissolved in the phenol phase while the upper water phase will contain the water soluble and liberated lipopolysaccharides together with nucleic acids. To remove the nucleic acids from lipopolysaccharides Davies (1958) used ultracentrifugation to sediment the lipopolysaccharides while other compounds are left in the supernatant.

Chen et al., (1973) introduced an extraction procedure using chloroform and methanol for the extraction of antigens from mutant strains of Salmonella.

H antigens which are proteins on the flagella, are extracted by ammonium sulphate precipitation and fractional sedimentation during centrifugation (Weibull, 1950; Koffler and Smith, 1971).

The work reported in this chapter was undertaken to prepare antigens for the use in in the diagnosis of typhoid fever using ELISA.

MATERIALS AND METHODS

Cell cultures

Materials

Salmonella typhi were obtained from the South African Institute of Medical Research in Pietersburg. Tryptic soy broth was purchased from Difco. Phosphate Buffered Saline (PBS) pH 7.4 and 90% (w/v) aqueous phenol solution.

Dialysis tubing was purchased from Sigma Chemical Co. USA. Ribonuclease type XII-A was obtained from Sigma.

Methods

Tryptic soy broth was prepared according to the manufacturers recommended instructions and autoclaved at 15 bar and 121°C for 20 minutes. Cultures were performed in 40 one-litre bottles and after 24 hours the bacteria were centrifuged at 3000 rpm using a swing-out rotor and collected. The bacteria were washed three times in buffered saline pH 7.4. To kill the bacteria a volume of cold acetone ten times that of the bacterial suspension was used. The bacteria were then dried on a filter paper and weighed. A yield of 5,13g dried bacteria was obtained from 40 one-litre culture bottles. The bacteria were freeze-dried into a fluffy white powder.

Extraction

Extraction of the lipopolysaccharides (LPS) was done according to the method of Westphal and Jann (1965) as modified by Nowotny (1979).

Five g of lyophilised bacteria was suspended in 120 ml distilled water and the mixture was stirred in a wide-necked Erlenmeyer flask kept in a water-bath at 70°C. One hundred and twenty ml of 90% (w/v) aqueous solution of phenol previously heated to 70°C was added and stirring was continued for 20 minutes, and then the contents were cooled to 10°C by

placing the flask in ice. The mixture was centrifuged at 3000 rpm for 30 minutes in a swing-out head of a refrigerated centrifuge at 16°C resulting in the formation of three layers, a water layer, a phenol layer and an insoluble residue. The water layer was collected and the extraction procedure repeated twice. The water supernatants were collected, combined and centrifuged at 500g for 30 minutes and dialysed against distilled water at 4°C for seven days with changes of water every day.

The extracts were concentrated to 20 ml using a Rotavapor at 37°C. Five ml of the concentrate was treated with 1 mg ribonuclease for 5 hours at 37°C, to digest the nucleic acids. After digestion the extract was freeze-dried and stored until use.

The remaining fifteen ml from above was centrifugated at 104,000g for 6 hours. The sediment was resuspended in half the original volume of distilled water and recentrifuged. The final sediment of lipopolysaccharides was suspended in 2 ml distilled water and freeze-dried. After freeze drying, the extracted and purified lipopolysaccharides were weighed and a yield of 132 mg was obtained.

Two mg of the ultracentrifuged lyopholised extract was dissolved in 2 ml water and tested by agarose gel electrophoresis for protein contamination.

Preparation of Veronal-buffer extract

The procedure involves the use of veronal buffer with vigorous stirring until the cells are broken and antigens released into the solution. The

antigens prepared by this method are crude and consist of proteins, LPS, RNA and DNA materials (Tsang and Chau, 1981).

Materials

0,1m Veronal buffer pH 8.4.

Method

Preparation was carried out according to the method by Tsang and Chau (1981). One gram of acetone-killed bacteria was suspended in 20ml of veronal buffer and the suspension was stirred gently for 24 hours at 37°C. Cell debris was removed by centrifugation at 300g for 30-45 minutes and the supernatant containing the antigens was freeze-dried and stored at 4°C.

1ml/mg of the antigen was tested for protein by the Folin-Lowry method using BSA as a standard (Plummer, 1978).

Preparation of Lysate-Crude-extract

Preparation was done according to the method of Tsang and Chau (1981).

One g of acetone dried cells in 30ml of distilled water was sonicated under cold conditions for 15 minutes using an intermediate tip. The lysate was spun at 4 500g for 30 minutes to remove the cell nuclei. The supernatant was freeze-dried and stored to be used as an antigen. 1ml/mg of the antigen solution was tested for the presence of proteins by Folin-Lowry method. (Plummer, 1978).

Results

1. Protein contamination of LPS preparation

To determine if any proteins were present, electrophoresis was used. After the gel was stained and dried it was inspected visually. No band was visible. The gel was later scanned at 550nm using a scanner supplied by Gelman. The scan was compared to a normal serum scan as a reference. The scan is shown in Fig. 2.4. It can be seen from the graph that no proteins were present in the LPS.

2. Protein contamination of Veronal-buffer extract and Lysate extract.

Table 2.1 shows the results of protein determination by the Folin-Lowry method.

Table 2.1: Protein contamination of prepared antigens

	Protein concentration
Veronal-buffer extract	20-80 µg/ml
Lysate extract	40-80 µg/ml

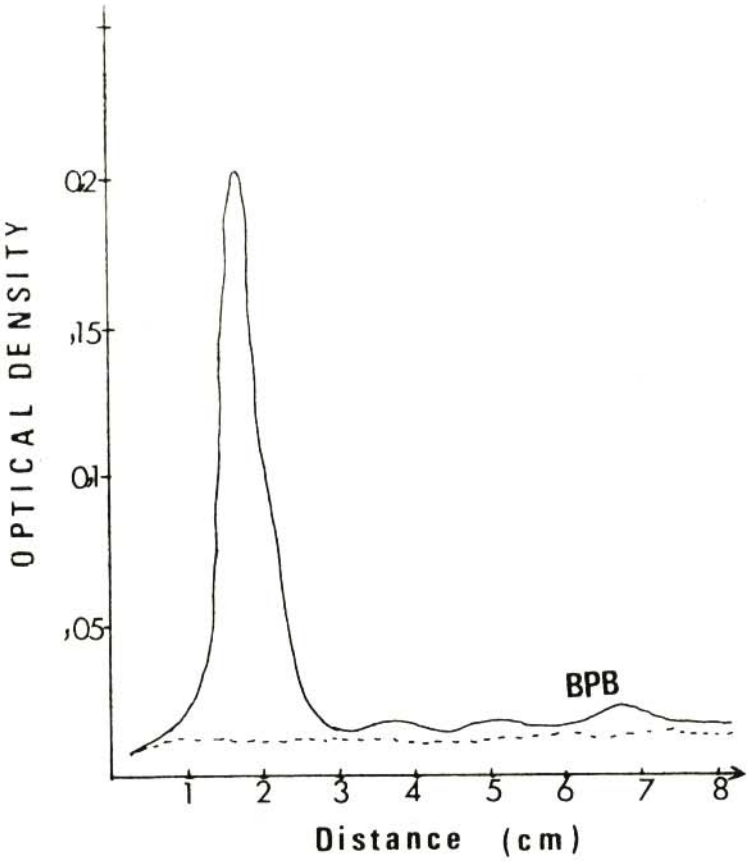


Fig. 2.4 : Scanning pattern of LPS and normal human serum.

———————— Indicates normal human serum sample

---------- Indicates LPS sample

Electrophoresis was performed at 20 Vcm^{-1} regulated by a DC voltage meter for 45 minutes. The gel was fixed and stained with amido black and scanned at 550 nm. BPB indicates the position of the bromophenol blue tracking dye. The arrow indicates the direction of migration.

DISCUSSION

The extraction of a pure antigen for use in ELISA assay is of importance for accurate and reproducible results. Salmonella typhi O antigen used in the Widal test is a crude extract which is not suitable for use in ELISA and moreover the extract have been found to give variable results in the Widal test. (De Villiers et al., 1965), It was therefore necessary to have a purer preparation of the LPS antigens for the use in the ELISA assay. Antigens from S. typhi can be prepared in different ways including extraction with trichloroacetic acid, sodium hydroxide, acetic acid and phenol-water(Luderitz et al., 1966). As was mentioned in the introduction, polysaccharide side chains contain the sugars which give specificity to Salmonella sero-groups and in order to avoid cross reactions, contaminating sugars must be removed.

The method found to yield the purest preparation was that of phenol-water extraction (Davies, 1958). The phenol-water method is unique since it preferentially extracts the LPS while the nucleic acids found with LPS are easily removed by high speed centrifugation. In addition, the phenol-water procedure does not have purification steps using alkaline or acid conditions which may destroy some of the important-structural features in the specific polysaccharides.

The results show that the phenol-water extraction method yielded LPS free from proteins. The other two antigenic preparations, veronal buffer extract and lysate extract were found to contain some contaminating proteins, as well as DNA and RNA. Similar results were obtained by Tsang and Chau (1981).

CHAPTER 3

PREPARATION OF ANTIBODIES AGAINST HUMAN IgG AND IgM

Introduction

The procedure for the preparation of antibodies against human IgM and IgG involves the following two steps:

1) Separation and purification of human IgG and IgM.
2) Production of antibodies against the human antibodies and their purification.

Separation and Purification

The principle involved in the separation of antibodies from serum proteins involves precipitation of proteins by ammonium sulphate and separation of the different gamma globulins using ion exchange chromatography and gel filtration. The precipitation procedure described by Kendall (1937) for gamma globulin isolation gives a preparation containing all immunoglobulins together with some proteins.

Ion exchange chromatography involves the electrostatic binding of proteins onto a cellulose resin suspended in buffer and packed into a column. Elution may be effected, either by changing the pH of the buffer passing through the column thus affecting the charge on the protein molecules or by increasing the molarity of the buffer thus providing more salt ions to compete with the proteins for the charged groups on the resin. By a gradual increase in salt concentration of the buffer, proteins are eluted in order

of increasing number of charged groups bound to the resin.

When a mixture of immunoglobulins is added to a DEAE Sephacel column under suitable conditions, most of the serum proteins will bind to the resin. When buffers of low molarity and a pH greater than 6.5 is used, IgG is not bound and passes through with the first buffer volume. The first buffer eluate is read at 280nm to detect the IgG fraction. The other adsorbed serum globulins are released by use of gradient elution. IgM being eluted later than IgA. It must be stated that the resolution of only IgG is satisfactory but not for the other immunoglobulins.

Gel filtration which is an exclusion chromatography procedure was used for the IgM preparation. This procedure uses gels to fractionate components of a mixture on the basis of molecular size. Protein molecules larger than the bead pores will pass unhindered through the column and emerge with the void volume. IgM which is the largest of the serum globulin (m.w. 900 000) appears first in the effluent and is detected by reading the absorbance at 280nm.

MATERIALS AND METHODS

Preparation of immunoglobulins

500ml of human plasma (supplied by SAIMR, Pietersburg) was precipitated according to the following procedure.

500ml plasma was added to a 2 litre beaker. Under constant stirring, 300ml of saturated $(NH_4)_2SO_4$ was added and the pH adjusted to 7.4 with 1N NaOH.

The contents of the beaker were spun for 30 minutes at 1000g in a IEF centrifuge using a swing-out rotor. The sediment was collected and 300ml of saturated $(NH_4)_2SO_4$ was again added while the mixture was stirred. The pH was adjusted as above and the mixture spun again as above. The sediment was dissolved in 500ml distilled water and divided in 10 x 50ml aliquots for dialysis. The aliquots were dialysed at 4°C for 5 days against 0,2M phosphate buffer pH 8.0 with a change of the outer fluid once every day. After dialysis gel filtration and ion exchange were performed.

ION exchange:

Materials

DEAE sephacel (Pharmacia)

Initial buffer : 0,02mol/1 phosphate buffer pH = 7,2

Limit buffer : 0,5mol/1 phosphate buffer pH = 7,2

Methods

Packing the column

The glass column 60 x 1.6cm and the plungers were assembled according to the manufacturer's instructions (LKB 2137 Chromatography column). Two hundred ml of a thin suspension of degassed DEAE Sephacel was prepared using the initial buffer. This was introduced into the column from the top using a glass funnel. After packing, the gel was equilibrated with initial buffer by means of a peristaltic pump at a rate of 3 ml per

minute. The equilibration point was checked by measuring the pH of the effluent.

Sample application

A sample of protein was applied to the column and eluted with the initial buffer. The eluate was collected in test tubes, using a fraction collector at a rate of 50 ml/hour. Protein concentration was continuously monitored with a dual path monitor at 280 nm and recorded on Rec-2 recorder.

After the first peak was recorded, 0,5m phosphate buffer pH = 9.2 was applied using a density gradient mixer.

The samples were collected, freeze dried and used for production of antibodies in the rabbits.

Gel filtration

Materials

Sephacryl S-300 (Pharmacia)
0,1M phosphate buffer pH 8.0 containing 0,02% NaN_3.
The same chromatographic equipments as for ion exchange were used in this work.

Methods

Preswollen Sephacryl S-300 was packed to a height of 50cm and equilibrated with 0,1M phosphate buffer pH 8.0 overnight.

Sample application

Twenty ml of $(NH_4)_2SO_4$ fractionated serum was applied to the top of the column. 0,1M phosphate buffer was used to elute the column. The eluated fractions were continuously monitored by a Uvi-cord connected to a recorder. The flow rate was 50ml/hour.

Human antiserum

Antihuman IgG and antihuman IgM were obtained by immunising two rabbits. One of the rabbits was immunised with an intra-muscular injection of 1mg/ml of IgG in Freunds complete adjuvant (FCA) (Difco Laboratories). The second rabbit was injected intramuscularly with 1mg/ml of IgM in FCA. Booster injections were given to the two rabbits weekly for two months.

Rabbit serum was collected after the 4th booster injection by cardiac puncture after the rabbits were anaesthetized with Saffan (Glaxo).

The serum collected was subjected to ammonium sulphate precipitation and later dialysed for five days at 4°C against 0,2M phosphate buffer with a change of the outer fluid every day. After dialysis the precipitate was freeze-dried and stored until use.

2. RESULTS

2.1 Separation of immunoglobulin with ion exchange chromatography

Separation of serum by DEAE-sephacel chromatography into a series of fractions is shown in Fig. 3.1. The fractions were collected and quantitated by Tripartigen plates specific for each immunoglobulin class. The first peak of protein elution (peak A) contained IgG only. This was found to be a pure separation of IgG with no other contaminants.

Peak B came after the application of the gradient and consisted of IgA with some IgG as contaminant. Peak C contained IgM and some traces of IgG. Peak D contained only IgM and was the last to emerge from the column.

Peak E which emerged before peak C was also found to contain IgM, however this peak was not tested for the presence of IgG.

2.2 Separation of immunoglobulins with gel filtration

Serum separated by Sephacryl S-300 yielded three major peaks of proteins in the effluent. The peaks are shown in Fig 3.2. The fractions were collected and tested by Tripartigen plates specific for each immunoglobulin class.

The first peak, A, contained only IgM. The second peak, B, contained IgA and the third peak, C, contained IgG only. The concentrations of the different immunoglobulins is shown to be the highest for IgG followed by IgA and lastly by IgM.

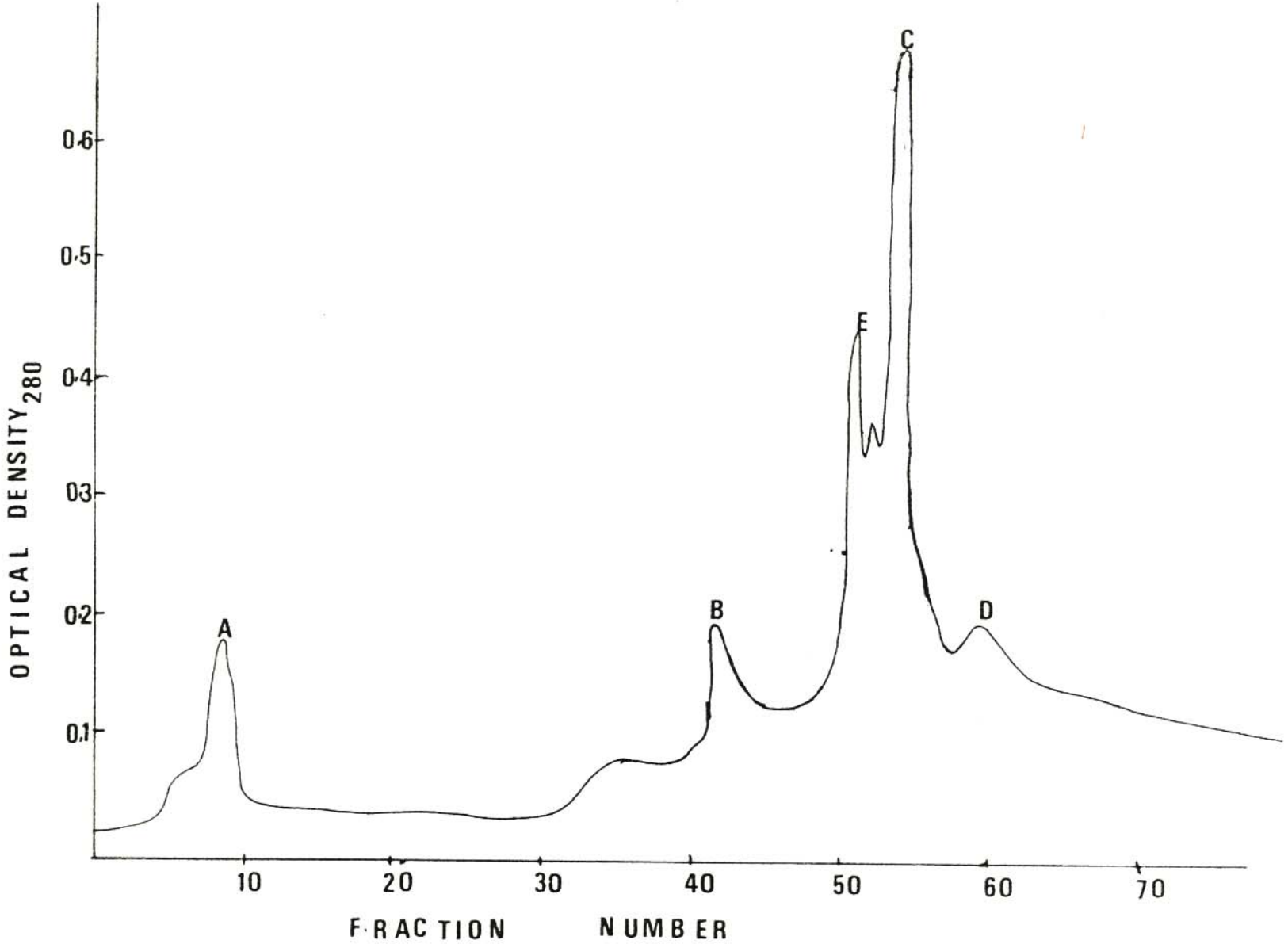


Fig. 3.1: Distribution of Immunoglobulins after separation on DEAE Sephacel

500ml of normal human serum were precipitated by $(NH_4)_2SO_4$ and later dialysed against 0,2M phosphate buffer pH 8.0 and applied to a 60 x 1,6cm column containing DEAE Sephacel equilibrated with the same buffer. Elution was performed with the phosphate buffer in a gradient from 0,02M to 0,6M at pH 8.0. Immunoglobulin distribution was determined with the quantitative assay of each component using Tripartigen plates.

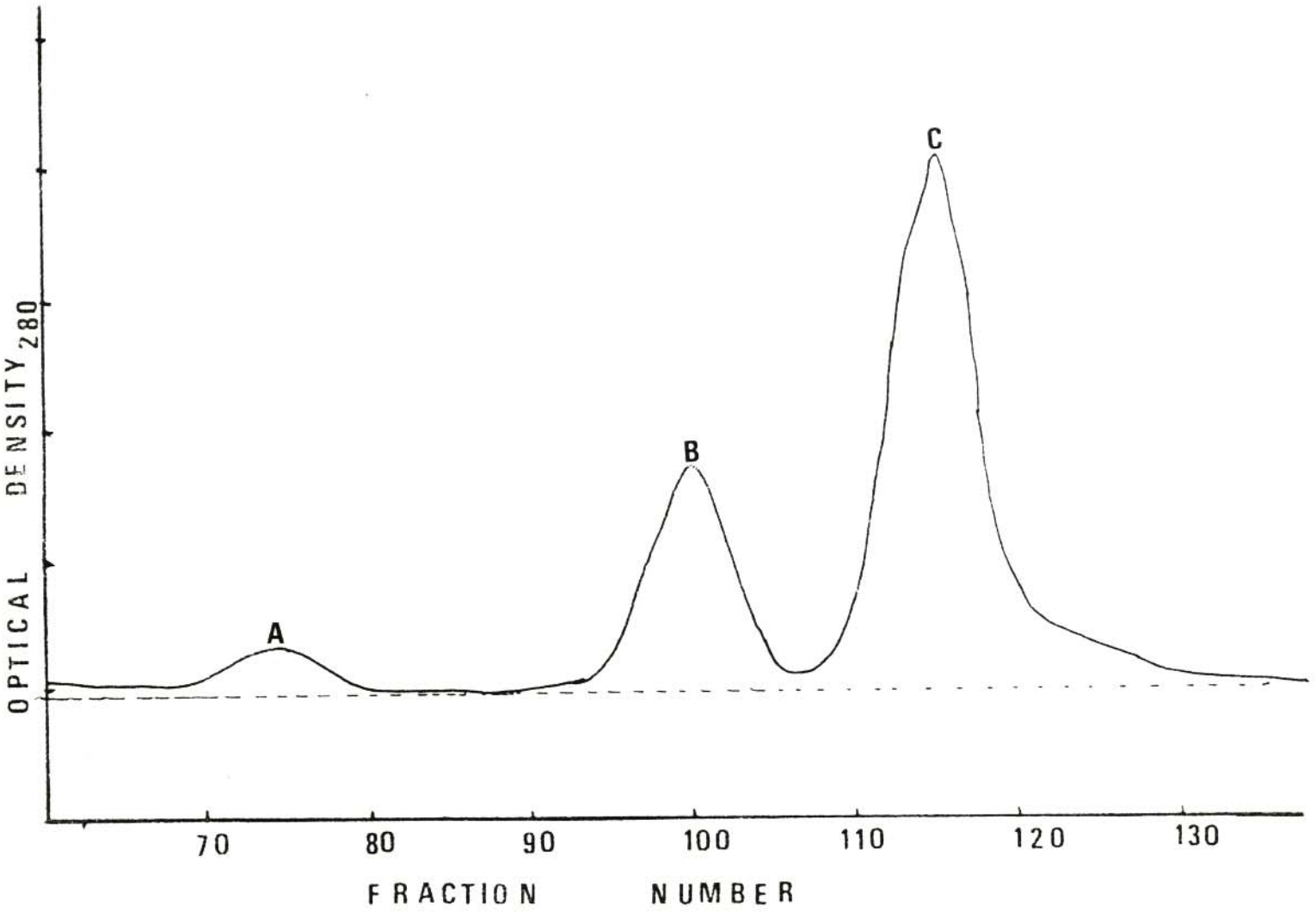


Fig. 3.2 Immunoglobulin distribution using Sephacryl S-300 chromatography

Twenty ml of normal human serum was precipitated by $(NH_4)_2SO_4$ and dialysed against 0,1M phosphate buffer pH 8.0 and applied to a column (1.6 x 60)cm equilibrated with 0,1M phosphate buffer pH 8.0. Elution was carried at a rate of 50ml/hour with 0,1M phosphate buffer and fractions were monitored and collected. Different fractions corresponding to different peaks were tested by tripartigen plates to show the type of the immunoglobulin present.

DISCUSSION

The results of the separation of immunoglobulin show that a purer preparation of IgG is obtained with ion exchange whereas gel filtration gives a purer separation of IgM. If both methods are employed for the preparation of different classes of immunoglobulin, then a superior preparation is obtained.

The rabbit antiserum was only purified by precipitation thus giving all the different classes of immunoglobulins in the mixture. This was done to avoid the loss of binding affinity inherent in different classes of antibodies. IgM for instance has 10 binding sites, 5 of which are of low affinity, and 5 of high affinity. This is the main reason why anti-human antibodies were not separated into different classes.

CHAPTER 4

SECTION I

CONJUGATION OF ENZYME TO ANTIBODY

INTRODUCTION

The enzyme is used as a marker in enzyme immunoassays in a manner similar to a radioisotope in radioimmunoassays. The sensitivity of enzyme immunoassays depends to a great extent on the preparation of enzyme-antibody conjugate possessing high enzymatic and immunological activity.

A variety of enzymes are used as markers in the immunoassays and in choosing an enzyme the following criteria are used (Nakane, 1979).

1. The enzyme must be readily available in a highly purified form.
2. The enzyme must have a high specific activity and turnover rate.
3. The enzyme must be relatively stable at working temperatures.
4. The enzyme must not exhibit substantial loss of activity after coupling.
5. The enzyme must have a non-mutagenic substrate which is readily available.
6. The end product of the enzymatic action must be easy to measure.
7. The end-product of the enzymatic action must be highly soluble (Yolken, 1982; Avrameas et al., 1978; Nakane, 1979).

The conjugation of an enzyme to antibodies involves the use of a cross-linking agent, which reacts with the active groups, e.g. E-amino groups, carboxy

groups, hydroxy groups, sulphydryl groups and phenol groups on proteins (Means and Feeney, 1971). These functional groups may be part of, or contribute to the active site of the enzyme. The coupling of the antibody to the enzyme should be in such a way that the immunological activity of the antibody and the activity of the enzyme is not affected (Rubenstein, 1978).

Two types of coupling reactions have been described. The first involves a one step procedure and the second is a two step procedure (Avrameas, 1969); Avrameas and Ternynck, 1971).

In one step reactions, the cross-linking reagent, the antibodies and the enzyme are mixed together and allowed to react. In the two step procedure the enzyme is first treated with the cross-linking agent and then the antibody added. The major advantage of the one step procedure is that the conjugate can be prepared easily and rapidly and also that it retains a high degree of immunological and enzymatic activity (Boorsma and Streefkerk, 1976). Although this method is found to be effective it involves two extensive time consuming dialysis steps (Parkinson et al., 1982). Another disadvantage is the difficulty of controlling the reaction and this may lead to selective polymerisation of either enzyme or antibody (Avrameas et al., 1978). Conjugates prepared by this method are basically heterogeneous.

The major advantage of the two step procedure is the homogeneity of the enzyme-antibody conjugate. The activity of the enzyme is still high 50 - 70% (Boorsma and Streefkerk, 1976). However the procedure is time consuming because of the purification steps which involve the removal of the unreacted cross-linking agent and excess antibodies and enzyme.

In the coupling of enzymes to antibodies the most commonly used cross-linking agents are sodium m-periodate and glutaraldehyde.

Sodium meta-periodate has been used for coupling peroxidase to the antibody (Nakane, 1979). The technique is based on the reaction whereby $NaIO_4$ generates an active aldehyde group on the carbohydrate moiety. The oxidised peroxidase is then coupled to immunoglobulin by formation of a Schiff's base as shown in Fig. 4.1. The efficiency of the coupling is dependent on the amount of $NaIO_4$ used and the time taken for oxidation (Nakane and Kawaoi, 1974). The stability of the Schiff's base is brought about by the addition of sodium borohydride ($NaBH_4$).

Glutaraldehyde has been used for the linkage of alkaline phosphatase to immunoglobulin. The mechanism for this reaction is not known but a possible mechanism has been suggested by Avrameas et al., (1978) and is shown in Fig. 4.2 where glutaraldehyde reacts with the E-amino group of lysine present in the enzyme.

In this section the results of the preparation of enzyme-antibody conjugates using both the one step and two step procedure and the two cross-linking reagents are discussed. The enzyme-antibody conjugates are assessed using enzyme-linked immunoassays (ELISA).

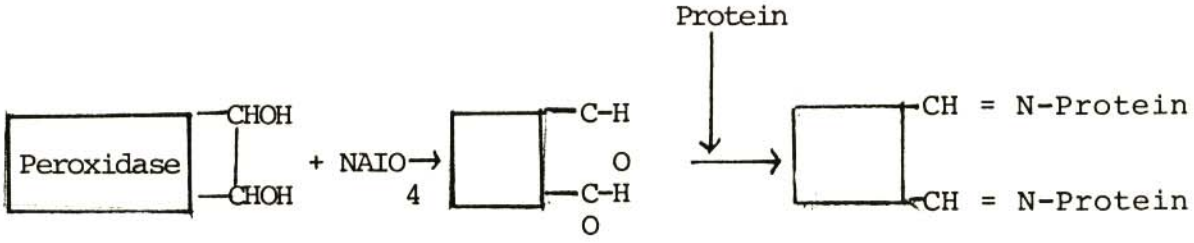


Fig. 4.1 : Mechanism of peroxidase-antibody conjugation by meta-periodate

Sodium meta-periodate oxidises the carbohydrate moiety of the enzyme to an aldehyde group. The aldehyde group is then available to react with the protein. The oxidised peroxidase is coupled to the protein by the formation of Schiff's base (Nakane and Kawaoi, 1974).

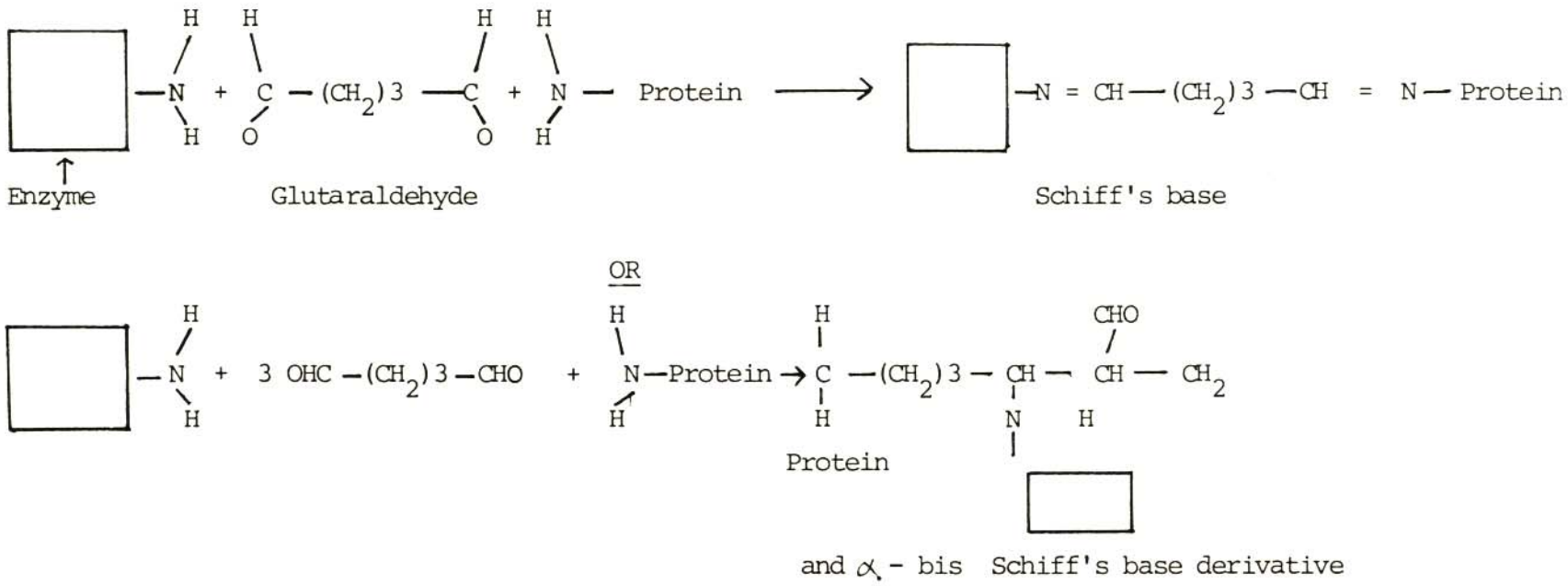


Fig. 4.2 : A possible mechanism for glutaraldehyde coupling to proteins and enzymes

Glutaraldehyde reacts with lysine on protein. The aldol condensation produces an unsatured aldehyde and this double bond reacts with the amino group to form a Schiff's base (Richard and Knowles, 1968).

MATERIALS

Alkaline phosphatase type VII was obtained from Sigma Chemical Co., and Miles Reserach Products.

Horseradish peroxidase (HRPO) type VI was purchased from Sigma Chemical Co., St. Louis, MO., USA or Miles Research Products, Cape Town with RZ 7,3.0.

Sephacryl S-300

0,1M Sodium meta-periodate ($NAIO_4$)

1mM Sodium acetate buffer pH 4.4

10mM Sodium Carbonate buffer pH 9.5

0,2M Sodium Carbonate buffer pH 9.5

Sodium Borohydride ($NaBH_4$) 4mg/ml.

Phosphate Buffered Saline (PBS) pH 7.2

Phosphate Buffered Saline Tween (PBS)
Containing the following:

0,05% w/v Tween 20

0,02% w/v Sodium azide (NaN_3)

0,05% w/v Bovine Serum Albumin (BSA)

Chromogenic Substrate for HRPO

18,4mM Borax

0,035M Succinate

0,037M O-dianisidine

4mM Hydrogen peroxide

0,1M Phosphate buffer pH 6.8

1% w/v Glutaraldehyde

1M Lysine solution pH 7.0

Sephadex G-200 and Sephadex G-25

Equilibration buffer pH 8.0

1,0M Sodium chloride

0,1M Tris-hydrochloric

1mM Thiomersal

Diethanolamine buffer

1,015M diethanolamine

0,02% Sodium azide

0,01% Hydrated Magnesium chloride ($MgCl_2.6H_2O$)

0,05M Carbonate bicarbonate buffer pH 9.5

Alkaline phosphatase substrate

4-Nitrophenyl disodium orthophosphate was prepared in diethanolamine buffer.

1. METHODS

COUPLING OF HORSERADISH PEROXIDASE TO ANTI-IgG.

4.0mg of Horseradish peroxidase (HRPO) was dissolved in 1ml distilled water and 0,2ml of freshly prepared $NaIO_4$ was added and the mixture allowed to stand for approximately twenty minutes at room temperature. The mixture was dialysed against 1mM sodium acetate buffer at pH 4.4 in a 5 litre beaker overnight at 4°C. Eight milligram of lyophilised anti-IgG was dissolved in 1ml of 10mM $Na_2 CO_3$ buffer pH 9.5 and dialysed against the same buffer at 4°C. The pH of the dialysed HRPO was raised from pH 4.0 to pH 9.5 by the addition of 20ml of 0,2M Na_2CO_3 buffer pH 9.5. The dialysed anti-IgG was added to the enzyme and allowed to stand for 2 hours at room temperature with stirring. After 2 hours, 0,1ml of freshly prepared 4mg/ml $NaBH_4$ was added and the reaction mixture was allowed to stand for 2 hours at 4°C.

The mixture was chromatographed on Sephacryl S-300. The 60 x 1.6cm column was packed with Sephacryl S-300 equilibrated in PBS pH 7.2. Fractions were passed through a monitor, collected by means of a fraction collector and recorded.

The fractions were tested as follows:

96 well-microtitre plates were coated with 5µg/ml of IgG overnight at 4°C and then washed three times with PBS Tween. Two hundred microlitres of the different fractions were added to each well and the plates incubated at 37°C for 3 hours. The plates were washed three times, after which 200µl of the chromogenic substrate was added to each well. The plate was incubated for 45 minutes in the dark at 37°C and the reaction stopped by the addition

of 50µl 2N HCL to each well. The colour intensity was measured at 492nm in a Multiscan.

2.3 THE CONJUGATION OF ALKALINE PHOSPHATASE TO ANTI-IgG

One Step Procedure for the conjugation of alkaline phosphatase using glutaraldehyde

This was performed according to the method of Avrameas (1969) and Avrameas and Ternynck (1971).

0,5ml of a suspension of alkaline phosphatase type VII was centrifuged at 3000rpm to remove the ammonium sulphate. The supernatant was discarded and the precipitate resuspended in 0,1ml of 0,1M phosphate buffer pH 6.8. 5mg of anti-IgG suspended in 0,1ml of 0,1M phosphate buffer pH 6.8 was mixed with the enzyme solution and the mixture dialysed against 0,1M phosphate buffer pH 6.8 overnight at 4°C. 0,05ml of 1% (w/v) glutaraldehyde in distilled water was added dropwise with continuous shaking and the mixture was left for 3 hours at room temperature. 0,1ml of the 1M lysine solution was added to inactivate the remainder of the glutaraldehyde alkaline phosphatase and left for 2 hours at room temperature. The mixture was dialysed overnight at 4°C against 0,1M phosphate buffer pH 7.0 and the mixture centrifuged for 20 minutes at 40 000g. The precipitate was discarded and the supernatant chromatographed on Sephadex G-200. The column was eluted at a rate of 50ml/hour and fractions tested by ELISA as previously described, using 4-Nitrophenyl disodium orthophosphate as a substrate for the enzyme, and the colour intensity measured at 405nm.

Two step Procedure for coupling of Alkaline Phosphatase to anti-IgG.

This was done according to the method of Avrameas and Ternynck (1971).

2ml of alkaline phosphatase was centrifuged at 500rpm and the precipitate resuspended in 1ml of 1% glutaraldehyde solution in phosphate buffer. The preparation was left overnight at room temperature and the mixture filtered through a Sephadex G-25 column equilibrated with 0,15M NaC1. Protein absorbance was monitored with a UVIcord. Each fraction was tested for the presence of enzyme by mixing a drop of the fraction with a chromogenic substrate for alkaline phosphatase. Fractions containing the enzyme were concentrated using Minicon concentrators. To the enzyme solution 5mg of anti-IgG was added and the mixture left at 4°C for 24 hours. 0,1ml of 1M lysine solution was added and the mixture dialysed overnight against PBS pH 7.0 at 4°C. The mixture was filtered through a Sephacryl S-300 equilibrated with 0,1m Tris-HC1 buffer pH 8.0 and eluted by the same buffer at a rate of 30ml/hour. Optical density was continuously monitored and recorded using the same equipments as above. The pooled fractions were tested for enzyme activity by the ELISA technique as described in the preceeding section. The enzyme-antibody conjugate was filtered through a sterile Millipore membrane (0,22µm). An equal volume of glycerol was added for preservation and the mixture kept at 4°C until used.

RESULTS

Horseradish peroxidase-antibody conjugate

The results obtained when the conjugate of anti-human IgG and the horseradish peroxidase (HRPO) was chromatographed on a column of Sephacryl S-300 are shown in Fig. 4.3. Sephacryl S-200 is a unique gel filtration matrix with a rigid superfine bead form, allowing high resolution of proteins. This particular gel fractionates proteins in the molecular weight range 10 000 - $1,5 \times 10^6$. The enzyme-antibody product has a m.w. of about 400 000.

The elution pattern shows three peaks. The first peak, A, consists of immunoglobulin conjugated to HRPO. The second peak, B, consists of unconjugated IgG and the last peak, C, consists of unconjugated HRPO. No polymerised immunoglobulins were detected.

Enzymatic activity of the chromatographed fraction

Table 4.1 Enzymatic activity of the pooled fractions as assayed by ELISA

Fractions	Peak	Absorbance at 495 nM
1-24		0,001-0,045
25-31	A	0,623-0,901
32-42	B	0,097-0,226
43-50	C	0,073-0,185

The fractions in peak A gave high enzymatic activity as compared to the other peaks. The second peak, B, seems to contain some conjugated material since an absorbance of 0,226 was obtained at 495nm.

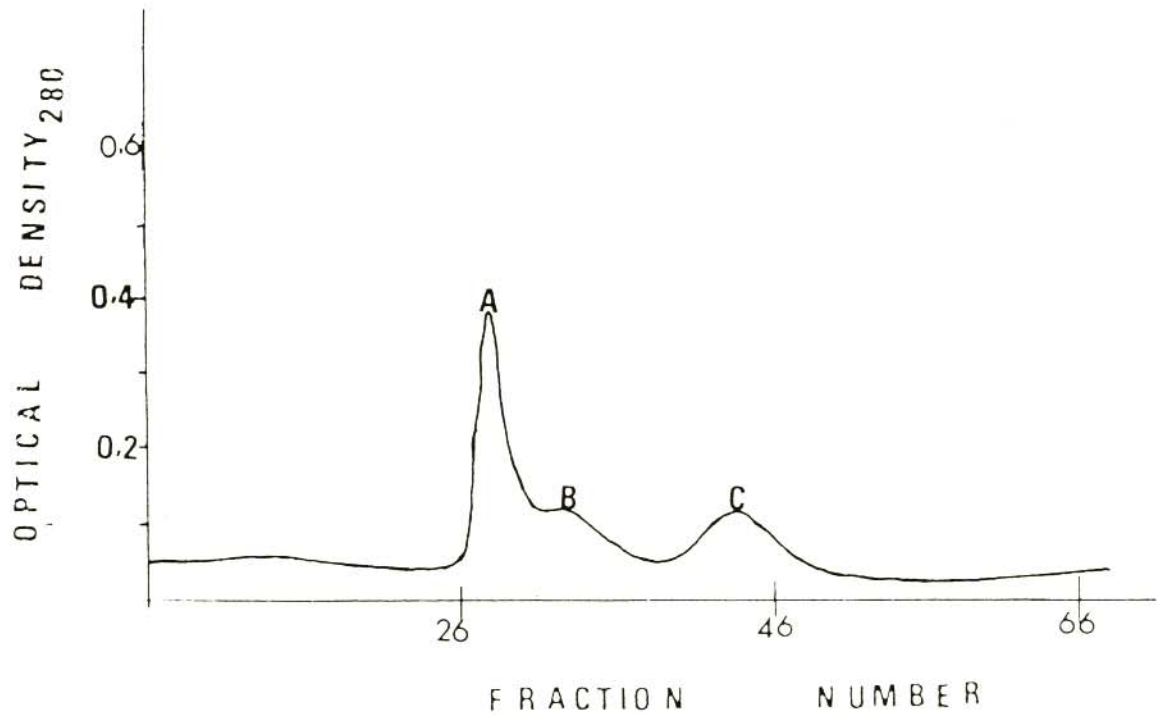


Fig. 4.3 : Sephacryl Chromatogram of Horseradish peroxidase with IgG .

Sephacryl chromatogram obtained with a conjugation mixture consisting of 4mM HRPO and 8mg lyophilished rabbit anti-IgG. 60cm column: buffer phosphate buffered saline pH 7.2; flow rate 50ml/hour; 4ml fractions.

The chromatogram shows 4 peaks. Peak A was the first peak and when tested by ELISA was found to be conjugate peroxidase. Peak B was found to contain no enzyme but only antibodies. Peak C was found to consist of unconjugated enzyme only.

One step procedure for alkaline phosphatase conjugation with immunoglobulin

Fig. 4.4 shows the chromatogram obtained when alkaline phosphatase was conjugated with rabbit antihuman IgG. The elution pattern of the one-step conjugate shows 4 peaks. The first peak, A, consists of polymerised immunoglobulin, the second peak, B, consists of immunoglobulin conjugated to the enzyme, while the third peak, C, consists of unconjugated immunoglobulins. The 4th peak, D, consists of unconjugated enzyme dimers. Only small amounts of unconjugated molecules are present.

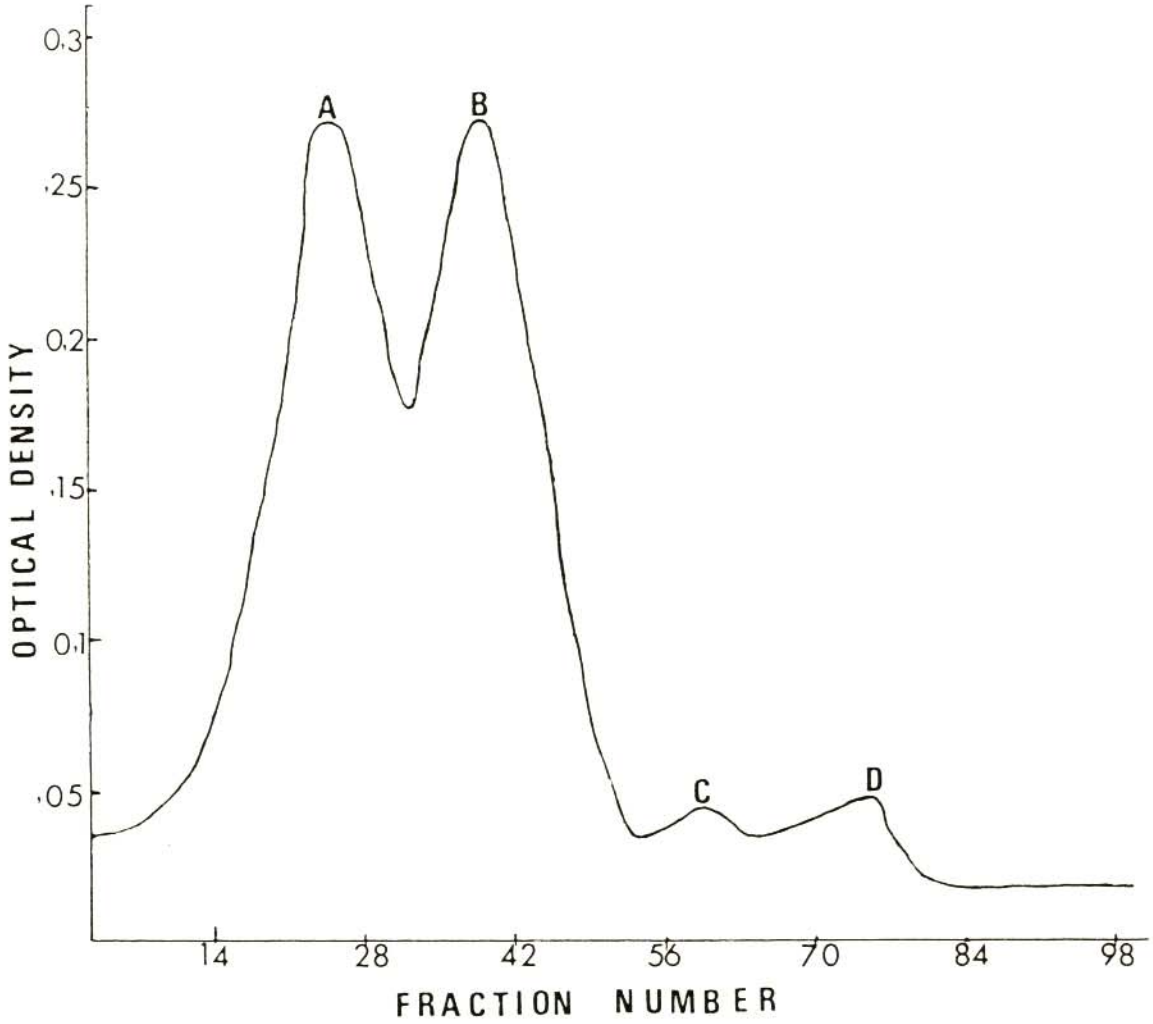


Fig. 4.4: One step procedure for alkaline phosphatase conjugation to anti-IgG.

Column chromatography on Sephadex G-200 of a conjugate mixture consisting of antihuman IgG and alkaline phosphatase preparation using the one step procedure. A 60x 1,6cm column was used and equilibrated with the equilibration buffer (see material) and eluted at 50ml/hour.

Enzymatic activity of the fractions

The results of the ELISA performed on the different fractions are shown in Table 4.2. The enzymatic activity of the anti-IgG enzyme conjugate was high for the fractions in peak B. The other fractions did not show any enzymatic activity, which indicates that a satisfactory separation is obtained with this method.

Table 4.2 Enzymatic activity of pooled fractions

Fraction Number	Peak	Absorbance at 405nm
14-32	A	0,007-0,103
33-52	B	0,312-0,789
53-63	C	0,018-0,067
64-80	D	0,021-0,013

Two-step procedure for alkaline phosphatase conjugation

The elution pattern of the reaction mixture of the two-step conjugation procedure is shown in Fig. 4.5. The results show four peaks. The first peak, A, represents antibody conjugated to enzyme. The second peak, B, is a small peak which consists of unconjugated antihuman IgG. Peak C consists of dimers of alkaline phosphatase and the last peak, D, consists of monomers of the enzyme. Polymerised immunoglogulin was not detected. A small amount of unconjugated immunoglobulin is present in this preparation. A high concentration of unconjugated enzyme was obtained in peak C and D.

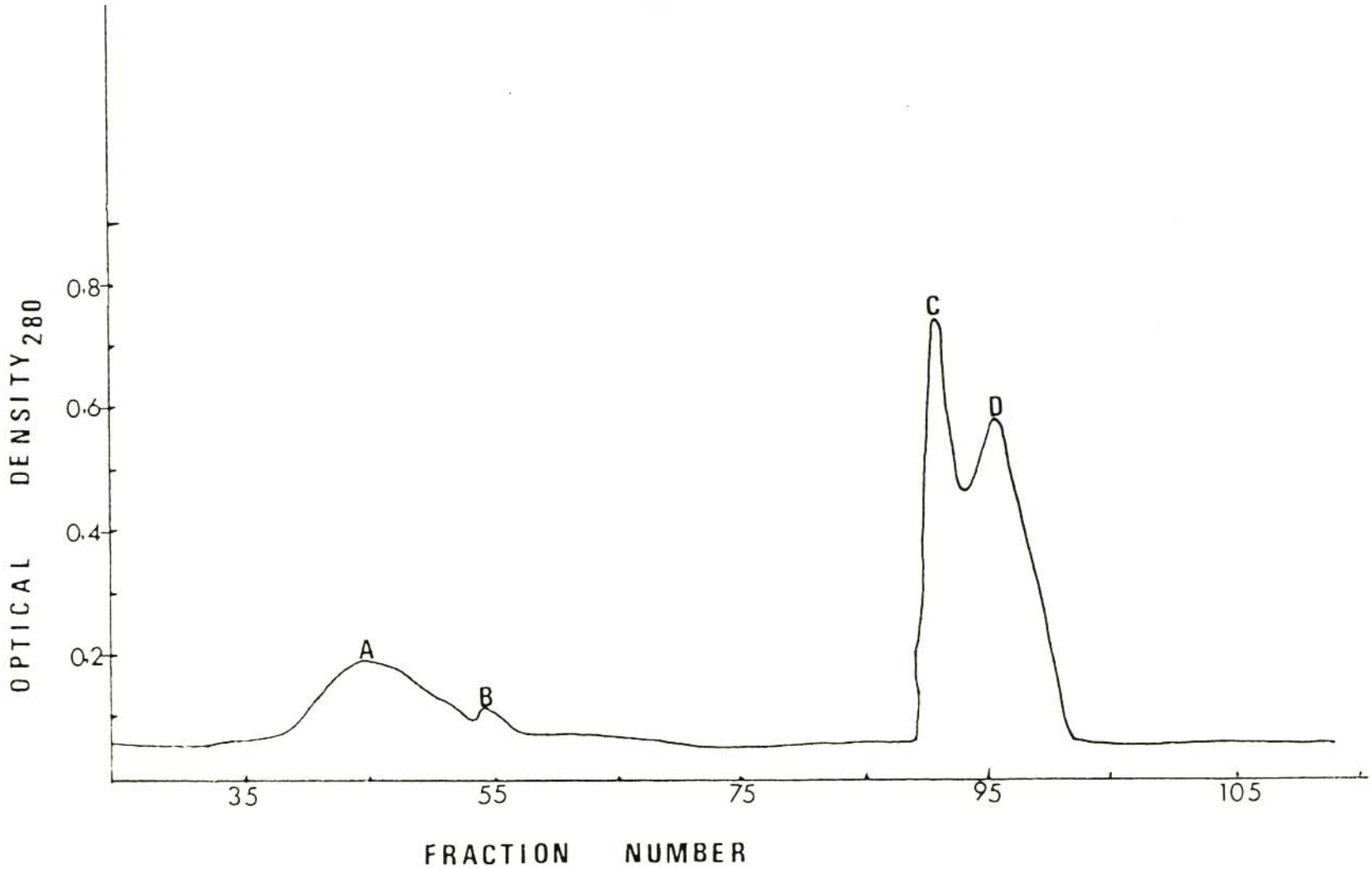


Fig. 4.5: Two step procedure of the conjugation of alkaline phosphatase to anti-IgG.

Column chromatography on Sephacryl S-200. Elution patterns of the alkaline phosphatase antihuman IgG conjugate prepared by the two step procedure. 60 x 16cm column equilibrated with 0,1M Tris-HC1 buffer, using a flow rate of 30ml/hour.

The enzymatic activity of the fractions is shown in Table 4.3.

Table 4.3 Enzymatic activity of the pooled fractions

Fraction Number	Peak	Absorbance at 405 nm
39-53	A	0,419-0,735
54-57	B	0,008-0,067
89-93	C	0,014-0,027
94-101	D	0,019-0,008

The results show that the fractions with the highest enzymatic activity are present in peak A, indicating thtat there is an antibody-enzyme conjugate. Peak C and D consists of unconjugated enzyme.

DISCUSSION:

Different conjugation procedures were used for the preparation of the antibody-enzyme conjugate to be used in ELISA. Horseradish peroxidase was conjugated using $NaIO_4$ as a coupling agent. Chromatography results show that the use of $NaIO_4$ gives a satisfactory conjugate as shown by the sharp peak, which is followed by small quantities of free antibodies and free enzyme. No polymerised antibodies or enzyme were detected.

Pooled fractions, when tested in the ELISA assay, showed enzyme activity in peak A. Traces of antibody enzyme conjugate were also detected in peak B.

The one step procedure for conjugation of alkaline phosphatase to anti-IgG using glutaraldehyde as a coupling reagent shows the presence of a high concentration of polymerised immunoglobulins despite the use of lysine. The chromatograph pattern shows two overlapping peaks for the polymerised-immunoglobulins and the enzyme-antibody conjugate, suggesting that they have nearly the same molecular weight. This preparation however shows that there is very low concentration of unconjugated material which exists either as a free enzyme or a unpolymerised free antibodies.

The chromatogram pattern of the two-step procedure shows that the conjugated material comes first in peak A, followed by unconjugated anti-IgG probably dimers of the immunoglobulin.

SECTION II

DETERMINATION OF OPTIMAL CONDITIONS FOR ELISA IN THE SERO-DIAGNOSIS OF TYPHOID FEVER

1. Introduction

A series of experiments were performed in order to determine the optimal conditions for ELISA. The effect of the following variables were determined: Temperature, incubation time, antigen concentration and antibody-enzyme conjugate concentration.

2. MATERIALS AND METHODS

Materials:

Antigens:

The following preparations of S. typhi were used as antigens: lipo-polysaccharides, lysate crude-extract and veronal buffer extract of Salmonella typhi prepared as described in Chapter 2. Commercial LPS from S. typhi was purchased from Sigma Chemical Co., St. Louis, Mo. USA.

Crude H and O antigens were obtained from the South African Institute of Medical Research (SAIMR) in Johannesburg.

Sera

Positive sera were obtained from the Annicke Institute for Tropical Diseases,

Tzaneen, SAIMR, Pietersburg and Tshilidzini Hospital, Venda.

Negative sera were obtained from donors at the University of the North and Sekororo in the North Eastern Transvaal.

Negative control serum was prepared in the laboratory. Positive control serum was obtained from SAIMR, Pietersburg. The positive control serum was positive by culture and had O-titres of 1:1600 and H- titres of 1:800.

Antisera

Anti-IgG conjugated to enzymes were prepared according to methods outlined in Section I of chapter 4. Commercial anti-IgG and enzyme-antiserum conjugates were obtained from Sigma Chemical Co. USA, and from Miles Research Products, Cape Town.

Washing solutions

(a) Phosphate buffered saline (PBS) pH 7.4 containing the following:

0,05% w/v Tween 20

0,02% w/v NaN_3

0,05% w/v Bovine serum albumin (BSA)

This washing solution was called PBSTB

(b) Phosphate buffered saline pH 7.4 containing the following:

0,05% w/v Tween 20

0,02% w/v NaN_3

0,05% w/v gelatin

This washing solution was called PBSTG.

(c) Phosphate buffered saline pH 7.4 containing the following:

0,05% w/v Tween 20

0,02% w/v NaN_3

This washing fluid was called PBST.

Buffers

(a) Diethanolamine buffer containing

1,015M diethanolamine

0,02% NaN_3

0,01% $MgC1. \ 6H_2 0$

This buffer was prepared fresh everyday.

(b) 0,05M Carbonate bicarbonate buffer pH 9.6

(c) PBS pH 7.4

Enzyme Substrates

(a) Substrate for Alkaline Phosphatase

1mg/ml 4-Nitrophenyl phosphate (BDH) in diethanolamine buffer.

(b) Substrate for HRPO

18,4mM borax

30,5mM succinic acid

Table of apparatus

APPARATUS	SUPPLIER
96 well flat bottomed mictrotitre plates	Flow Laboratories, T & C Scientific Supplies, Johannesburg
50ml - 250 ml 8-channel pipette	Flow Laboratories
10ml - 300ml Autodrop	Flow Laboratories
Hamilton microprocessed hand pipette diluter	Optical instruments, Johannesburg
Multiwash	Flow Laboratories
Multiscan	Flow Laboratories
Water vacuum pump	Protea Laboratories, Johannesburg
Zeiss Spectrophotometer	Optical instruments, Johannesburg

3. Methods

3.1 Dilutions

All dilutions were done with the microprocessed Hamilton syringe.

3.2 Preparation of negative control serum

Negative serum from a healthy donor who had never contracted typhoid fever was absorbed by live typhoid bacilli to remove any cross-reacting antibodies. The serum was centrifuged at 5 000g for 20 minutes divided into 5ml aliquots and stored at -20°C.

3.3 Washing procedures of plates

1. Automatic washing: Plates were washed using a Multiwash and tapped dry after each wash.

2. Aspiration washing: Plates were filled with 250µl of washing solution using an Autodrop or an 8-channel pipette. The plates were left to stand for 3 minutes and later aspirated using a vacuum system. The procedure was repeated twice.

3. Overflow washing: The plates were flooded with the washing fluid, left to stand for 3 minutes and then emptied by flicking out the contents. They were dried with absorbent paper.

3.4 Coating of plate with antigen

An antigen solution consisting of 8µg LPS/ml in 0,05M carbonate buffer was prepared and 200µl of this solution was added to each well of the microtitre plate. After incubation for 24 hours at 4°C or for 3 hours at 37°C, the plates were washed three times with PBSTB. After blotting, 250µl PBSTB was added to each well and the plates stored at 4°C.

3.5 ELISA Technique

ELISA was performed essentially as described by Engvall and Perlmann, (1972). The coated microtitre plates were washed twice with PBSTB. 200µl serum diluted in PBSTB was added to each well of the LPS coated plates which were then incubated for 24 hours at 37°C. The plates were washed by the methods described above. After each wash, the plates were dried by blotting. The anti-IgG enzyme conjugate diluted in PBST was added and the plates incubated for 2 hours at 37°C. After the plates were washed, 200µl of a solution of enzyme substrate was added. The enzyme reaction was performed at room temperature and stopped by the addition of either 3M NaOH or 1N HC1, depending on the enzyme used. The absorbance was read at 405nm and 490nm for alkaline phosphatase and horseradish peroxidase respectively.

RESULTS

Determination of optimal concentration of antigen to be used in ELISA.

Three different antigens were used:

(i) Laboratory prepared LPS antigen.

(ii) Commercial LPS antigen.

(iii) Laboratory prepared veronal buffer antigen.

Different concentrations of the antigens varying from 1µg/ml - 20µg/ml were used to coat the plates according to the method described in 3.4. ELISA assay was performed according to the procedure outlined in 3.5. Serum samples were diluted 1:200 with PBST. Commercial anti-IgG alkaline phosphatase conjugate was diluted 1:800 and substrate for alkaline phosphatase

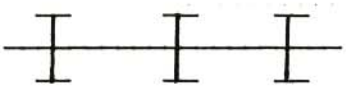
used at a concentration of 1mg/ml. The reaction was stopped by the addition of 50 µl 3M NaOH after 40 minutes at room temperature.

Results of this experimental work are shown in Fig. 4.4, Fig. 4.5 and Fig. 4.6. The commercial LPS preparation and the laboratory prepared LPS antigens give comparable results (Fig. 4.4 and Fig. 4.5). The results show that a concentration of 6 µg/ml to 20 µg/ml may be used with these antigens. Veronal buffer antigens results show that a concentration of 2 µg/ml to 8 µg/ml may be used for the experimental work.

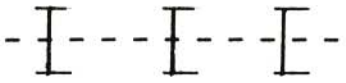
Throughout the experimental work, commercial LPS preparation and laboratory prepared LPS antigens were used at a concentration of 8 µg/ml. The use of veronal buffer antigen was discontinued since it was found to contain high concentration of proteins (chapter 2).

Fig. 4.4 Determination of optimal concentration of the laboratory prepared LPS for use in ELISA

Microtitre plates were coated with solutions of laboratory prepared LPS antigen using concentration varying from 1 ug/ml - 20 ug/ml and incubated overnight at 4°C. The plates were then incubated with 200ul of serum diluted 1:100 followed by incubation with the enzyme-anti-IgG conjugate for 2 hours at 37°C. Enzyme-substrate incubation was for 40 minutes at room temperature.



Mean absorbances obtained with 31 positive serum samples



Mean absorbance obtained with 10 negative serum samples

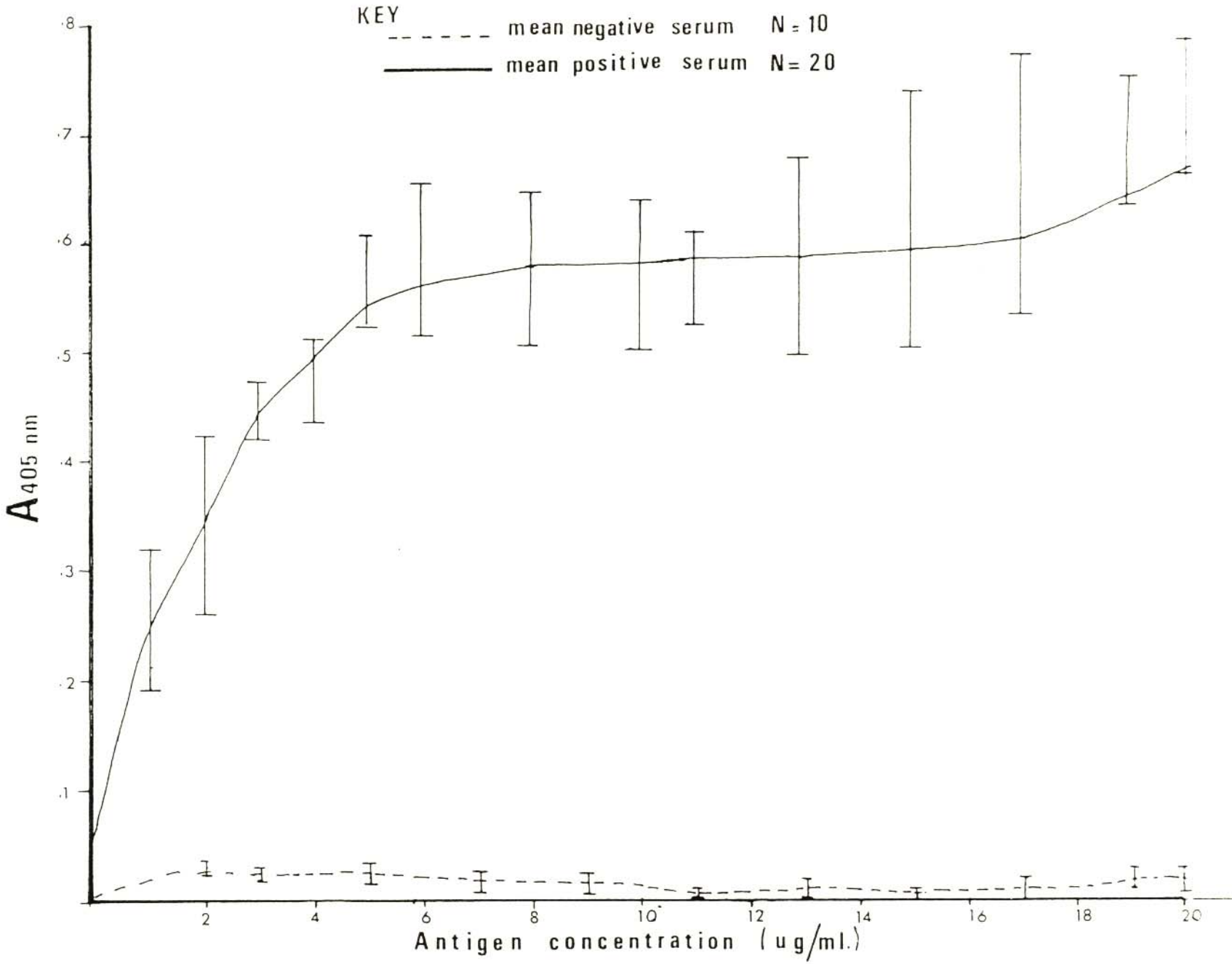
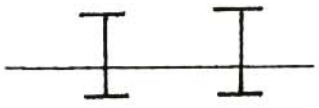


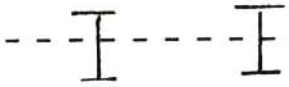
Fig. 4.4: Determination of optimal concentration of the laboratory prepared LPS for use in ELISA.

Fig. 4.5: Determination of optimal concentration of commercial LPS for use in ELISA

Plates were coated with solutions of commercial LPS antigen of different concentrations incubated with serum diluted 1:100 and followed by incubation with enzyme-anti-IgG conjugate for 2 hours at 37°C. Enzyme-substrate incubation was for 40 minutes.



Mean absorbances obtained with 20 positive serum samples



Mean absorbances obtained with 10 negative serum samples

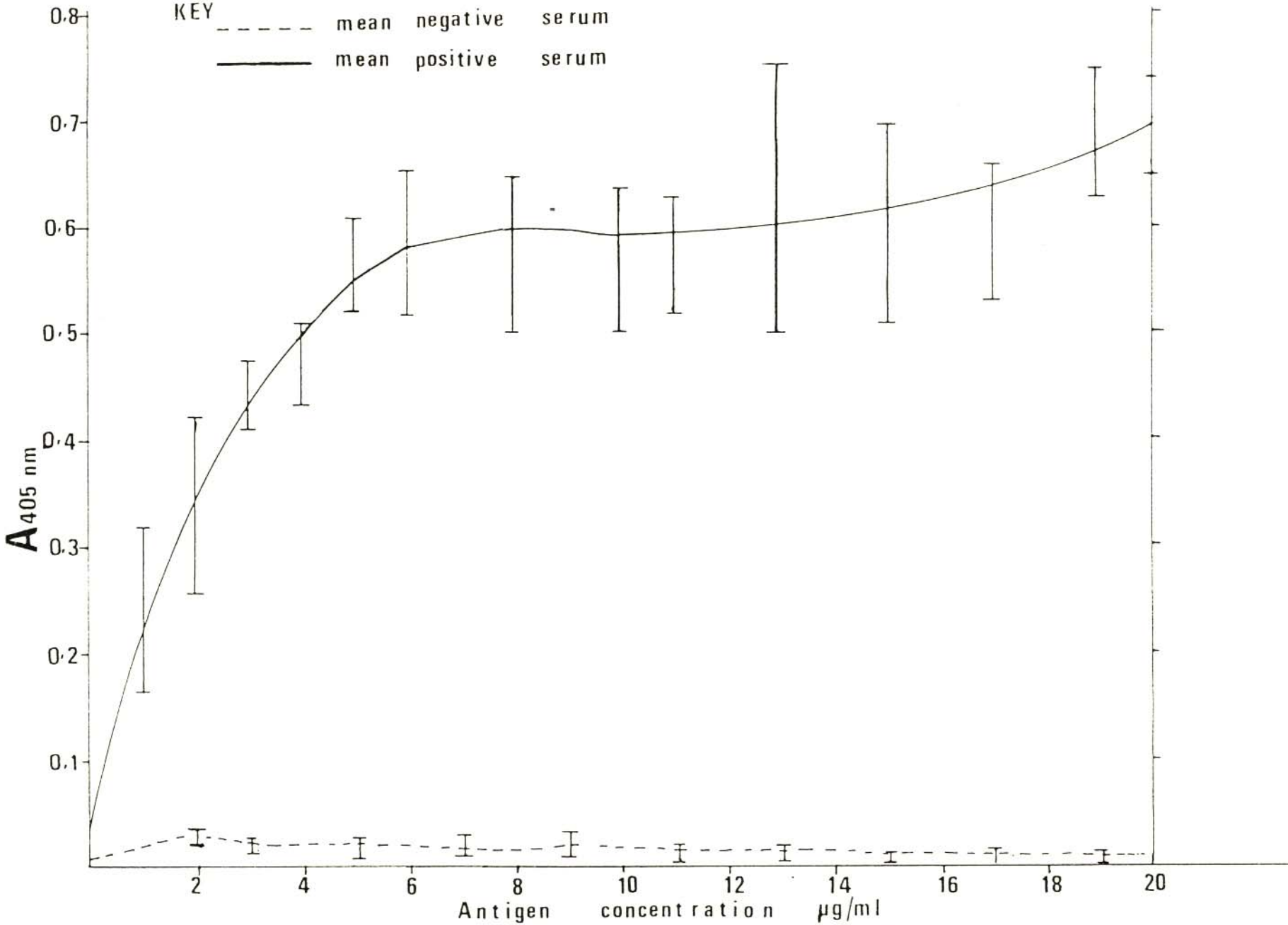


Fig. 4.5 Determination of optimal concentration of commercial LPS for use in ELISA.

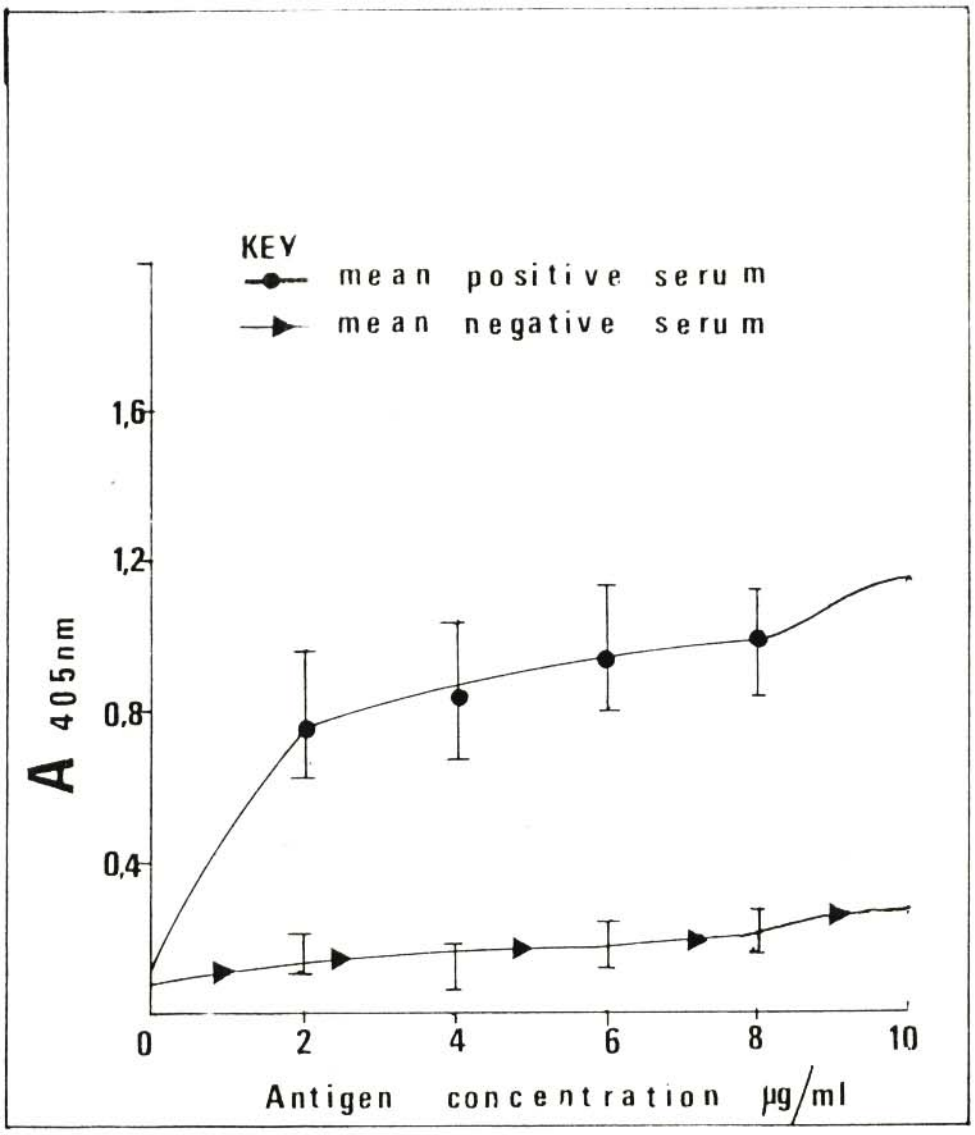
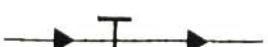


Fig. 4.6: Determination of optimal concentration of crude veronal buffer antigen for use in ELISA

Plates were coated with solutions of veronal buffer antigen of different concentrations and incubated with serum diluted 1:100. Enzyme-anti-IgG conjugate was added and the plates incubated for 2 hours at 37°C. Enzyme-substrate incubation was for 40 minutes at room temperature.

Mean absorbances obtained with 12 positive serum samples



Mean absorbances obtained with 10 negative serum

Effect of time and temperature on antigen coating of plates

Plates were coated with 8µg/ml of LPS and incubated under the following conditions:

One batch of coated plates was incubated for 24 hours at 4°C, a second batch, incubated for 3 hours at 37°C and the last batch was left at room temperature for 3 hours. ELISA was performed with negative and positive samples as described previously. Results are shown in Table 4.4. The amount of antigen that binds to the microtitre plates is high after 24 hours incubation at 4°C, slightly lower after 3 hours at 37°C and significantly lower after 3 hours incubation at room temperature.

For coating of antigen to the microtitre plates, the incubation time is 24 hours at 4°C in all the experimental procedures in the following sections unless otherwise stated.

Table 4.4: Effect of time and temperature on antigen-binding to plates

	Batch 1	Batch 2	Batch 3
Sample No	24 hours at 4°C	3 hours at 37°C	3 hours at room temperature
1	0,871	0,791	0,312
2	1,017	0,981	0,247
3	0,987	0,999	0,332
4	0,842	0,871	0,317
5	0,926	0,934	0,412
6	0,727	0,817	0,324
7	0,917	0,912	0,417
8	1,201	0,991	0,296
9	1,118	1,012	0,371
10	0,201	0,118	0,127
11	0,171	0,113	0,111
12	0,018	0,001	0,091
13	0,098	0,005	0,045
14	0,101	0,081	0,061
15	0,124	0,095	0,067
	$X_1 = 0,956$	$Y_1 = 0,923$	$Z_1 = 0,336$
	$S_1 = \pm 0,144$	$S_1 = \pm 0,082$	$S_1 = \pm 0,055$
	$X_2 = 0,119$	$Y_2 = 0,067$	$Z_2 = 0,084$
	$S_2 = \pm 0,064$	$S_2 = \pm 0,053$	$S_2 = \pm 0,032$

The test was performed at different temperatures and for different periods of time as indicated. 1:100 serum dilution, and 1:800 conjugate dilutions are used. The colour was developed

for 45 minutes at room temperature.

Sample No 1 to No 9 are positive

Sample No 10 to No 15 are negative

X_1, Y_1, Z_1 = mean for positive samples

S_1 = standard deviation for positive samples

X_2, Y_2, Z_2 = mean for negative samples

S_2 = standard deviation for negative samples

Serum dilution

To determine the optimal serum dilution, 10 positive and 10 negative sera were diluted with PBS Using a Hamilton diluter. Serum dilutions were from 1:1 to 1:10000 and the plates incubated for 2 hours at 37°C. Anti-IgG-alkaline phosphatase conjugate diluted 1:800 was added and the plates incubated for 2 hours at 37°C. The results of this work are shown in Fig. 4.7. Absorbance readings obtained when the serum was diluted between 1:10000 and 1:100 show a linear relationship. The highest absorbance is reached at a dilution of 1:100, with a dilution of less than 1:100, there is a decrease in the absorbance. From the results shown in Fig. 4.7 a dilution of 1:200 was taken because it is on the linear part of the graph and moreover it allows for a serum with high concentration of antibodies.

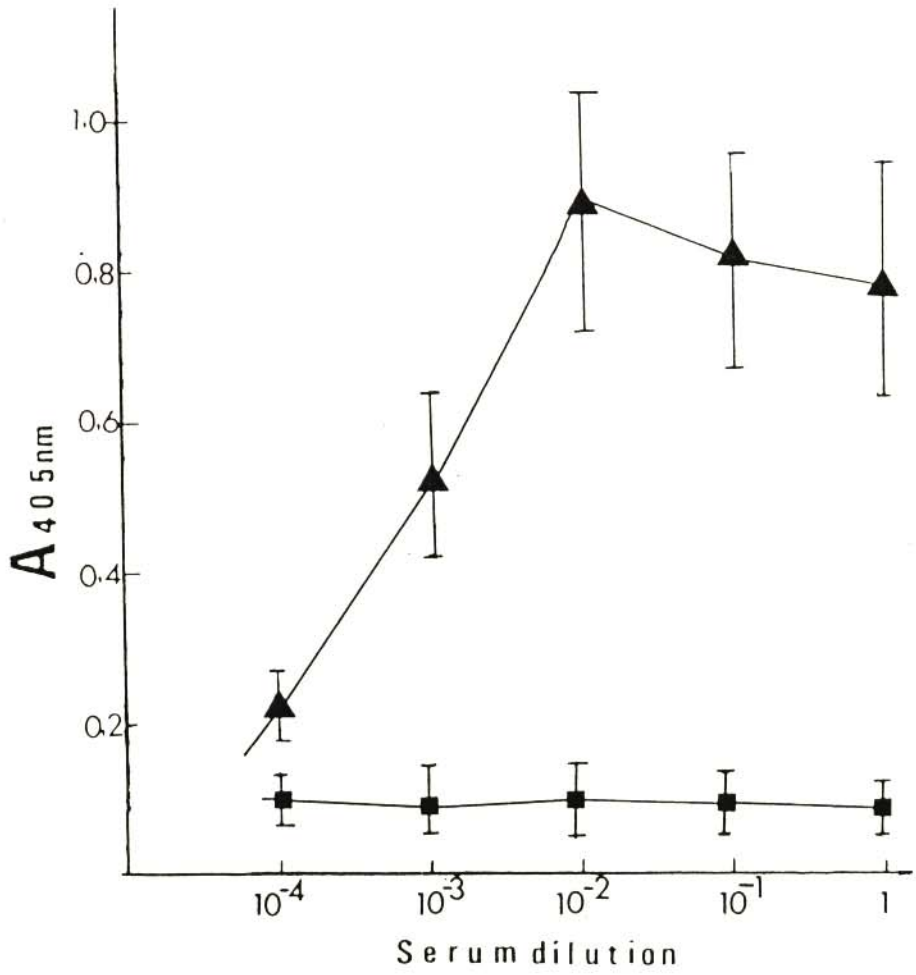
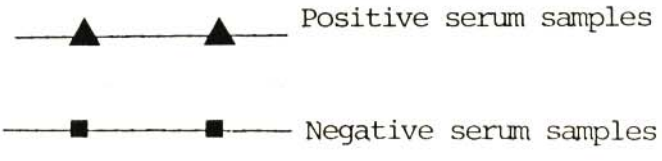


Fig. 4.2 : Determination of optimal serum dilution for use in ELISA

For the determination of optimal serum dilution, 10 positive and 10 negative serum samples were diluted by a Hamilton dilutor with PBS at different dilutions. After incubation for 2 hours at 37°C, enzyme-IgG conjugate was added and incubated for 2 hours at 37°C. The enzyme-substrate incubation was for 45 minutes at room temperature.

Key:



Determination of the incubation time needed for optimal antibody-antigen reaction.

Positive and negative serum samples diluted 1:200 in PBS were allowed to react with the solid phase from 0-150 minutes at 37°C. The results are shown in Fig. 4.8. From the results, it can be seen that from 0-30 minutes, there is no constancy in the absorbance while an incubation time of 60 minutes and longer gives constant readings. For the experimental work, an incubation time of 2 hours was used in order to allow the antibody-antigen reaction to stabilise.

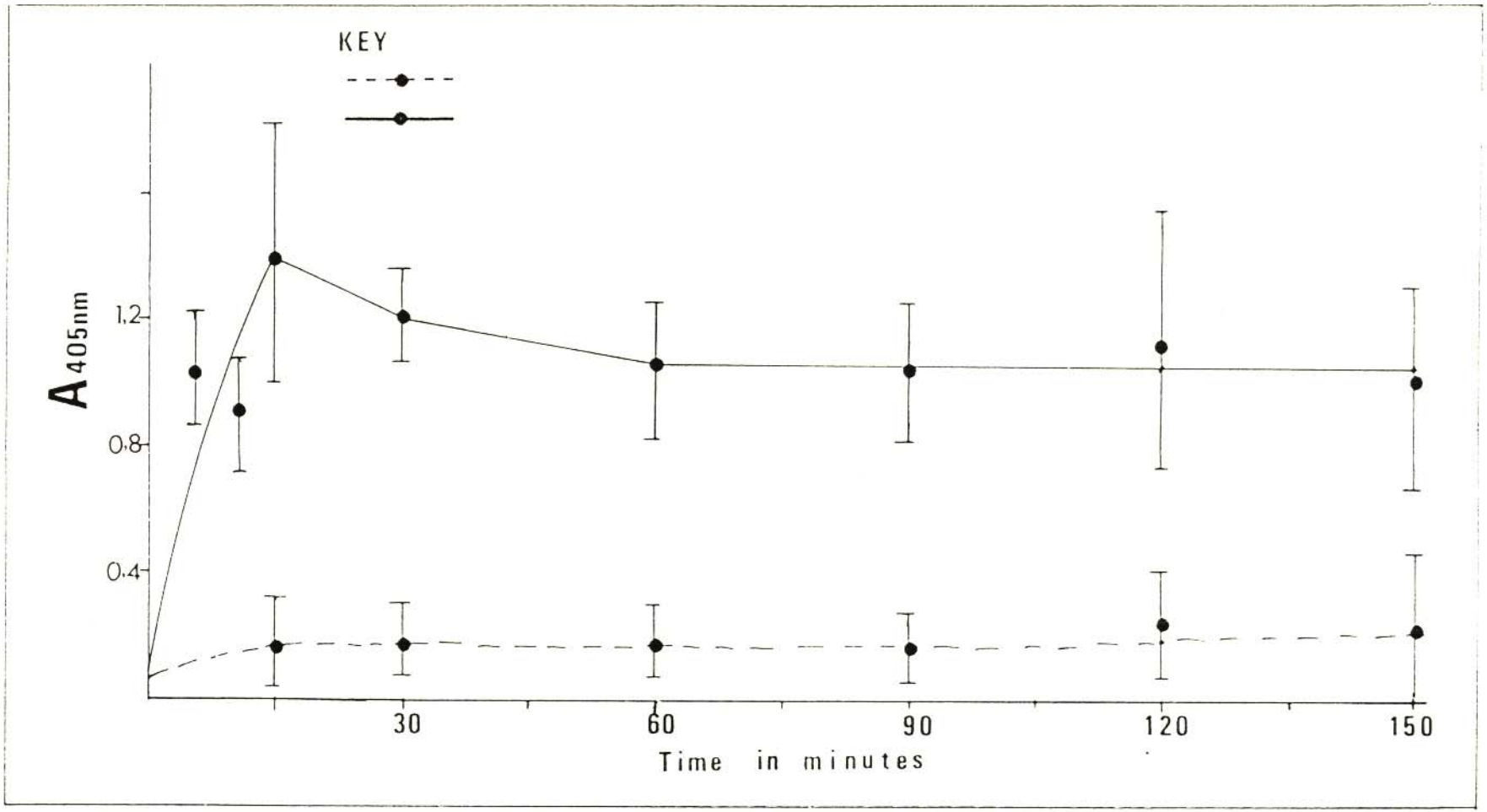


Fig. 4.8 : Determination of incubation time needed for optimal antibody-antigen reaction.

Plates were coated with 8µg/ml of commercial LPS antigen and incubated with 1:200 dilution of serum followed by incubation over periods of time varying between 15 and 150 minutes at 37°C. Alkaline phosphatase conjugated to anti-IgG diluted at 1:800 in PBS was added and incubated for 2 hours at 37°C. Enzyme-substrate incubation was for 45 minutes.

—●——● 20 positive serum samples

- -●- - -● - 10 negative serum samples

Determination of optimal concentration of enzyme-antibody-conjugate

To determine the optimal concentration enzyme-antibody-conjugate, a series of dilutions were performed using a Hamilton diluter. Both the laboratory prepared conjugate and the commercially available enzyme-antibody-conjugates were used.

Results of the different concentrations of conjugates are shown in Fig. 4.9, 4.10, 4.11 and 4.12. Fig. 4.9 shows the results when using the commercially available enzyme-anti-IgG. The four positive serum samples were run together and it can be seen that dilution of not more than 1:1000 should be used. In order to obtain the optimum antibody-enzyme conjugate, the 1:800 dilution was used since at this concentration there is enough antibody-enzyme conjugate and the system is saturated. However, the saturation of this assay will depend on the amount of antibody present. If there are more antibodies in the sample, the saturation point can shift to lie at a dilution less than 1:1000. The laboratory prepared conjugate does not show saturation of the system. (Fig. 4.11 and 4.12). There is a gradual fall with increasing dilutions. For this prepartion, an arbitrary dilution of 1:10 was taken.

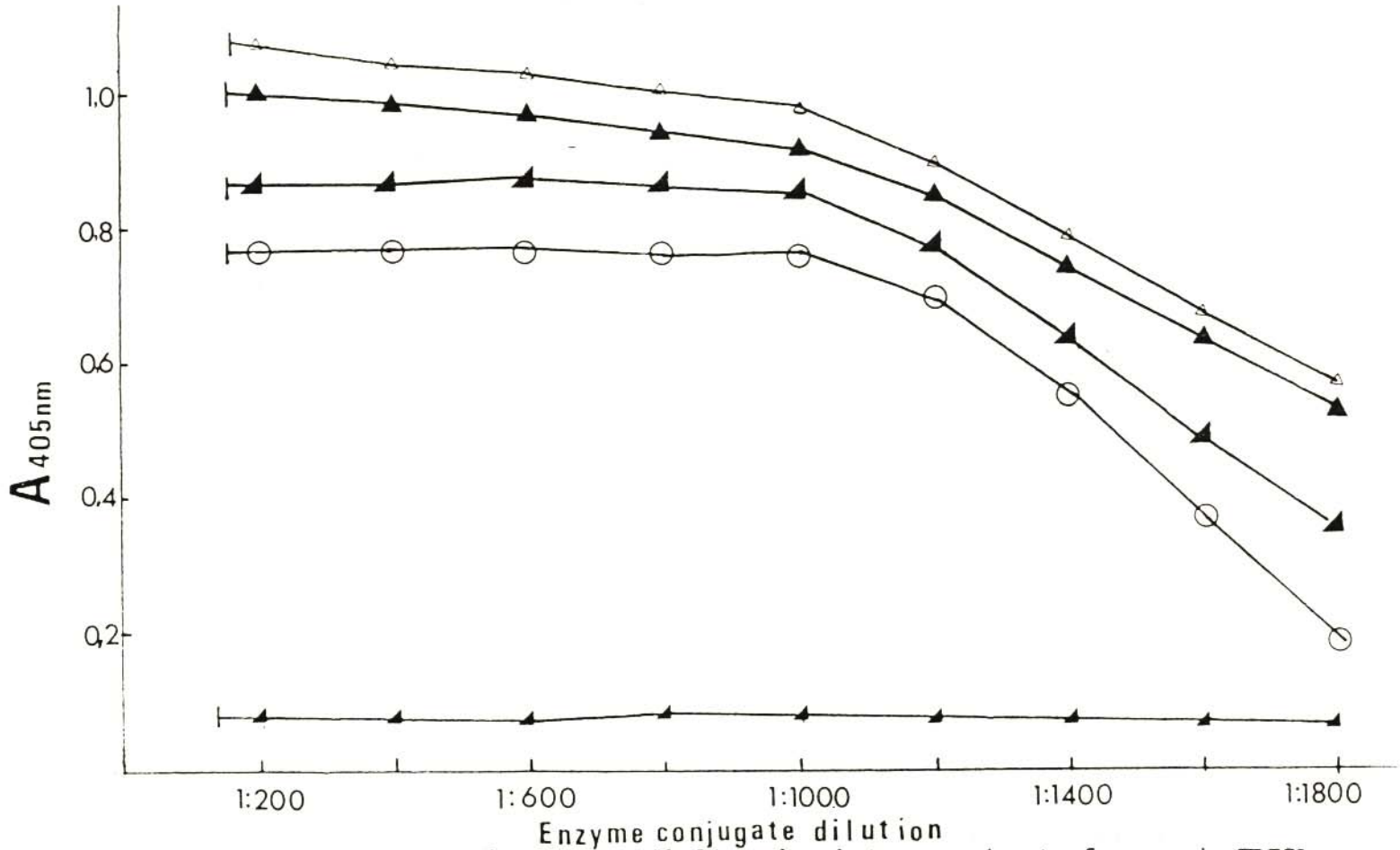


Fig. 4.9 : Determination of optimal concentration of anti-IgG-alkaline phosphatase conjugate for use in ELISA

Dilution of commercial anti-IgG-alkaline phosphatase conjugate. The experiment was performed as follows: Plates coated with 8µg/ml of LPS according to the procedure outlined in 3.3 were incubated with serum diluted 1:200. Diluted commercial anti-IgG-alkaline phosphatase was added and later incubated for 2 hours. Enzyme-substrate incubation was for 45 minutes. Dilutions were done by using an automatic diluter.

Key:

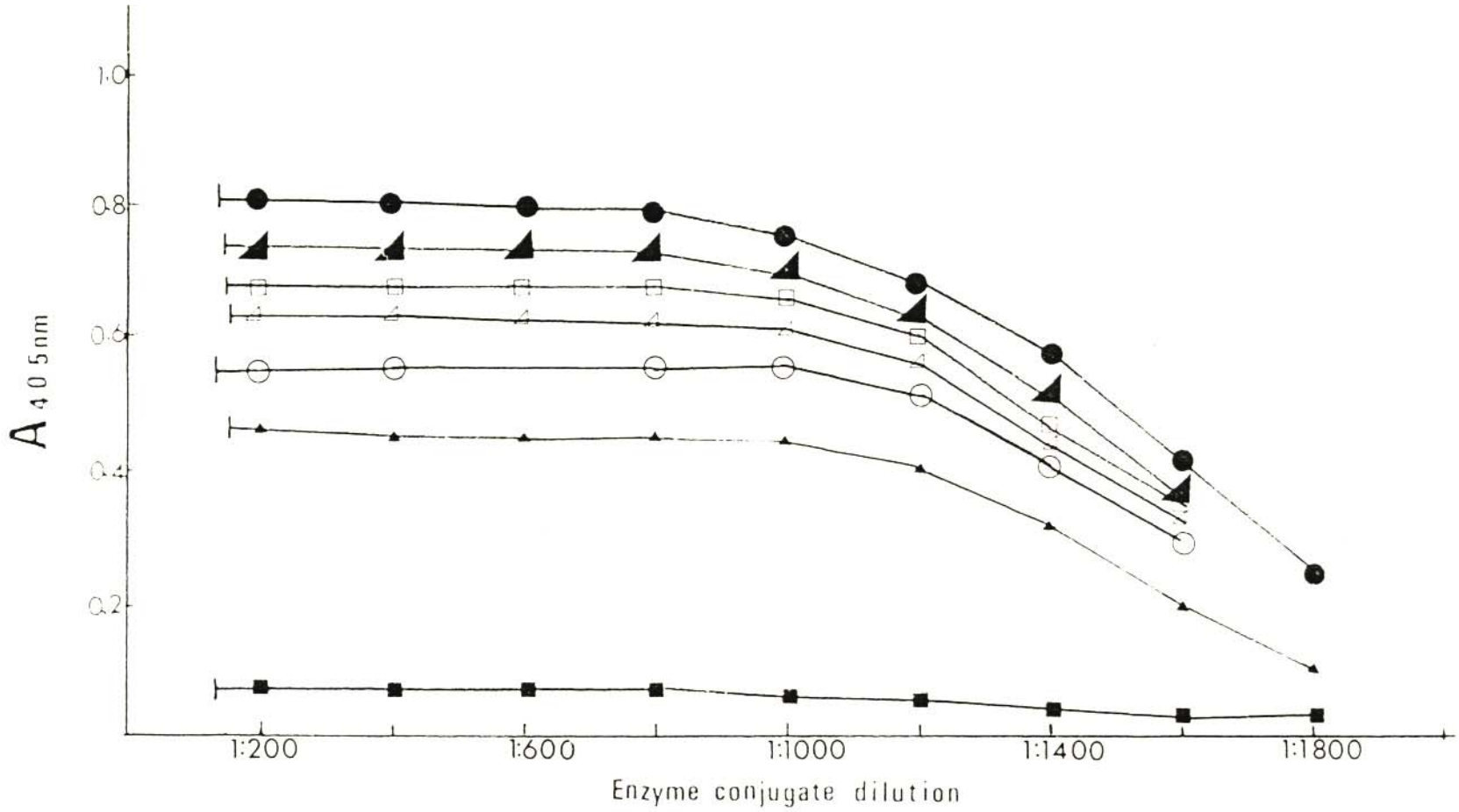


Fig. 4.10 : Determination of optimal concentration of commercial anti-IgG-alkaline phosphatase conjugate for us ELISA

Dilution of commercial anti-IgM-alkaline phosphatase conjugate. The different dilutions were performed according to the procedure in 4.5 and outlined in Fig. 4.9

Key: Negative serum

Positive serum samples

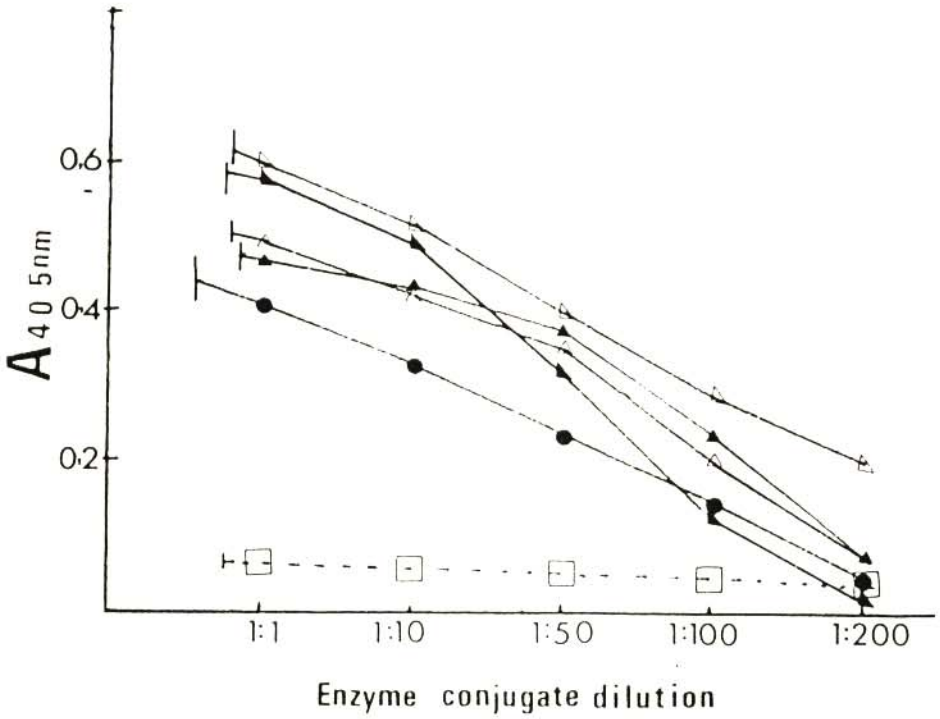


Fig. 4.11 : Determination of optimal concentration of laboratory prepared anti-IgG-alkaline phosphatase conjugate

The procedure of this experimental part was done according to the outlined methods in section 4.5. Dilutions were done by a microprocessed automatic hand pipette.

Key: Negative serum sample

Positive serum samples

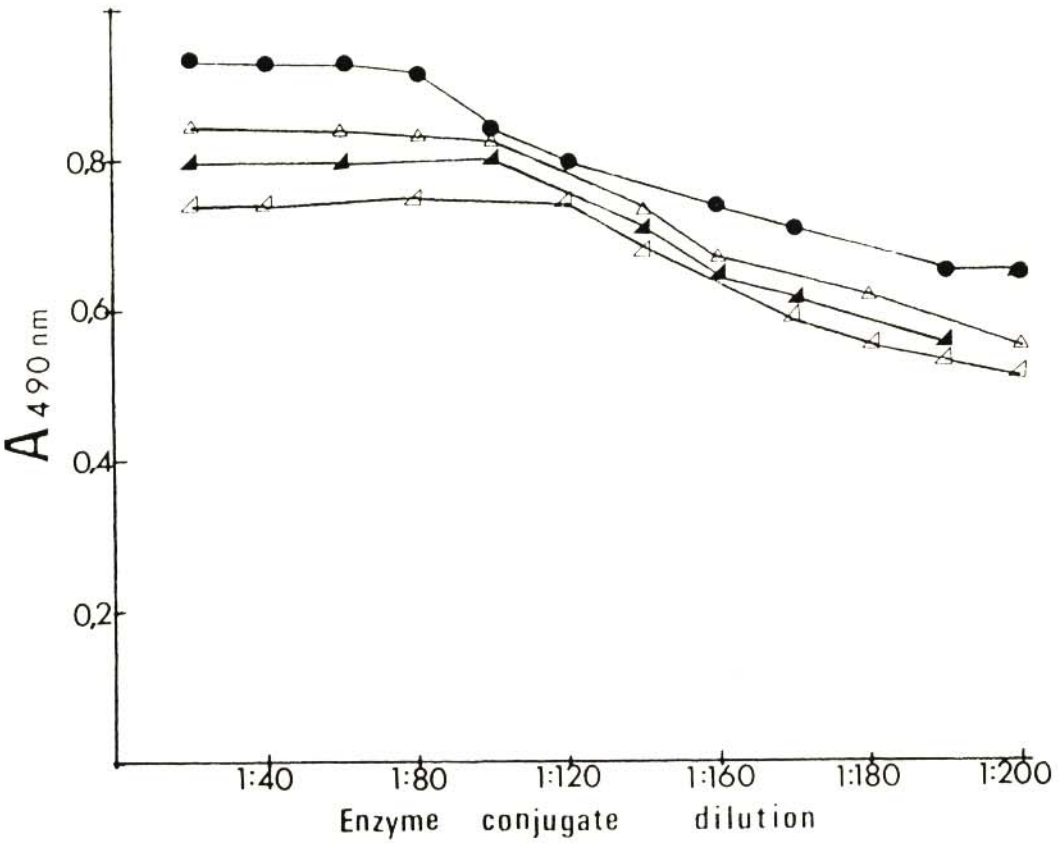
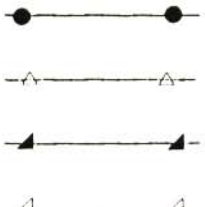


Fig. 4.12 : Determination of optimal concentration of laboratory prepared anti-IgG horseradish peroxidase conjugate

The experiment was performed as follows: Plates coated with 8µg/ml of commercial LPS according to the procedure in 3.3 were incubated with positive serum samples diluted 1:200. Different concentrations of the anti-IgG horseradish peroxidase was added and the plates incubated for 2 hours at room temperature. Enzyme substrate was added and the plates incubated for 45 minutes in the dark. The reaction was stopped by the addition of 50µl of 1N HCL.

Key:



Positive serum samples

Determination of the optimal incubation time for antibody-anti-antibody reaction with various concentrations of the conjugate

To determine the optimal incubation time for the enzyme-antibody conjugate, the following experimental work was performed. Plates coated according to the methods outlined in section 3.3 were incubated for 2 hours with positive sera diluted in 1:200.

Various dilutions of the enzyme-antibody-conjugate were made and incubated before the enzyme substrate was added. The results are shown in Fig. 4.13, Fig. 4.14 and Fig. 4.15.

For the laboratory prepared enzyme-conjugate a very low dilution gives the same results as those obtained from the commercial preparations during the same incubation time. For the laboratory preparation, dilutions of 1:1 to 1:100 were used.

From the results, it can be seen that the commercial preparation can be used at a high dilution within an incubation period of 45 to 120 minutes. However, the longer the incubation period, the more stable the results are. For the commercial preparation, anti IgG-alkaline phosphatase conjugate was used at dilutions 1:200 to 1:1500 and incubated for 2 hours. However the incubation time can be decreased by about 50% to 50 minutes and still give reproducible and accurate results. This reduction in time will help to reduce the time in which the whole assay can be performed.

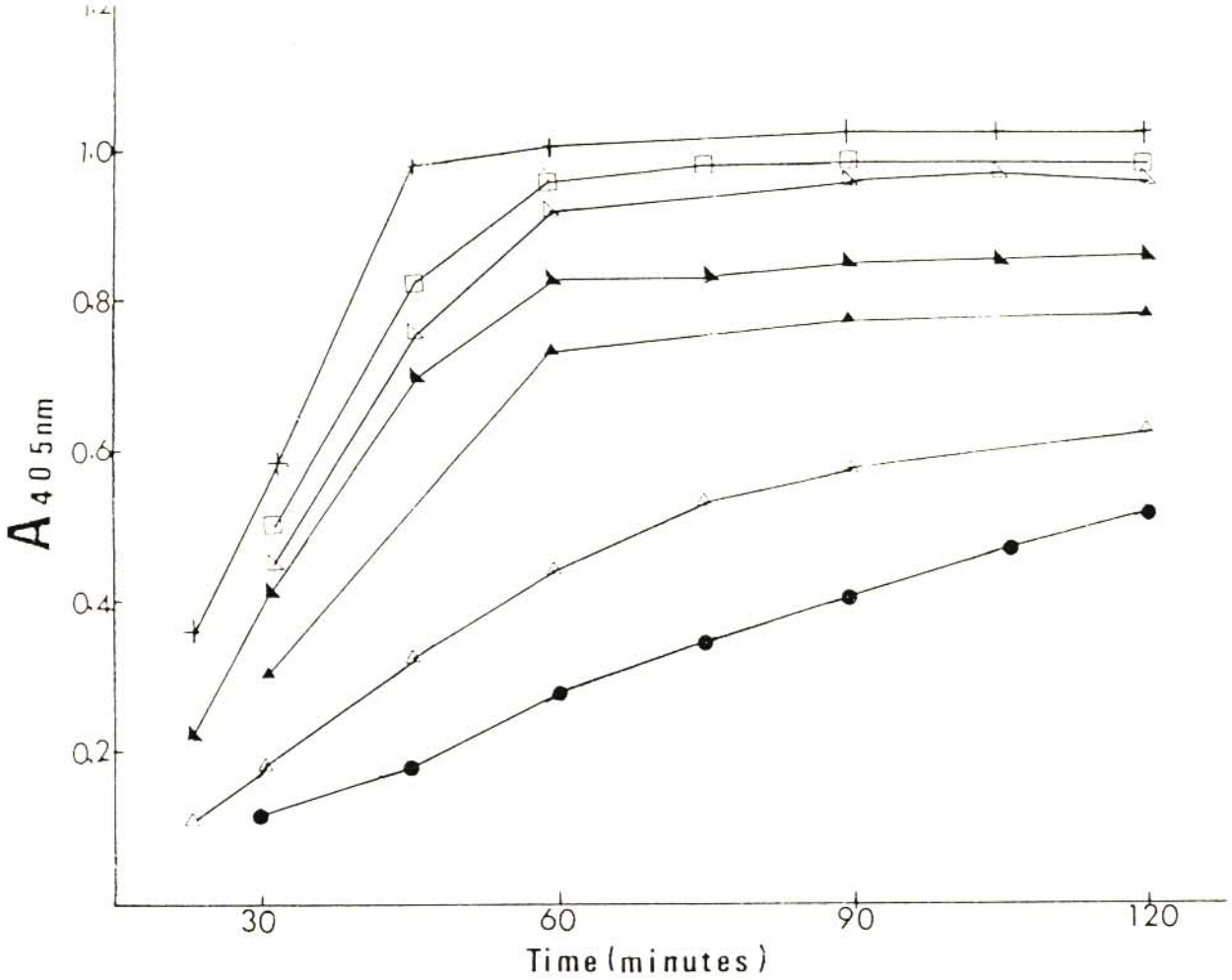


Fig. 4.13 : Effect of incubation time and dilution on laboratory prepared anti-IgG-alkaline phosphatase conjugate

Incubation time for the laboratory prepared enzyme-conjugate at different dilutions. The experiment was done according to the methods as outlined in section 3.3. of the methods. Different dilutions of enzyme-conjugate was used and incubated for different length of times. The figure shows the results obtained when the enzyme-conjugate prepared in the laboratory using a two step glutaraldehyde method, was tested.

1:1 1:10 1:100

1:2 1:20

1:4 1:50

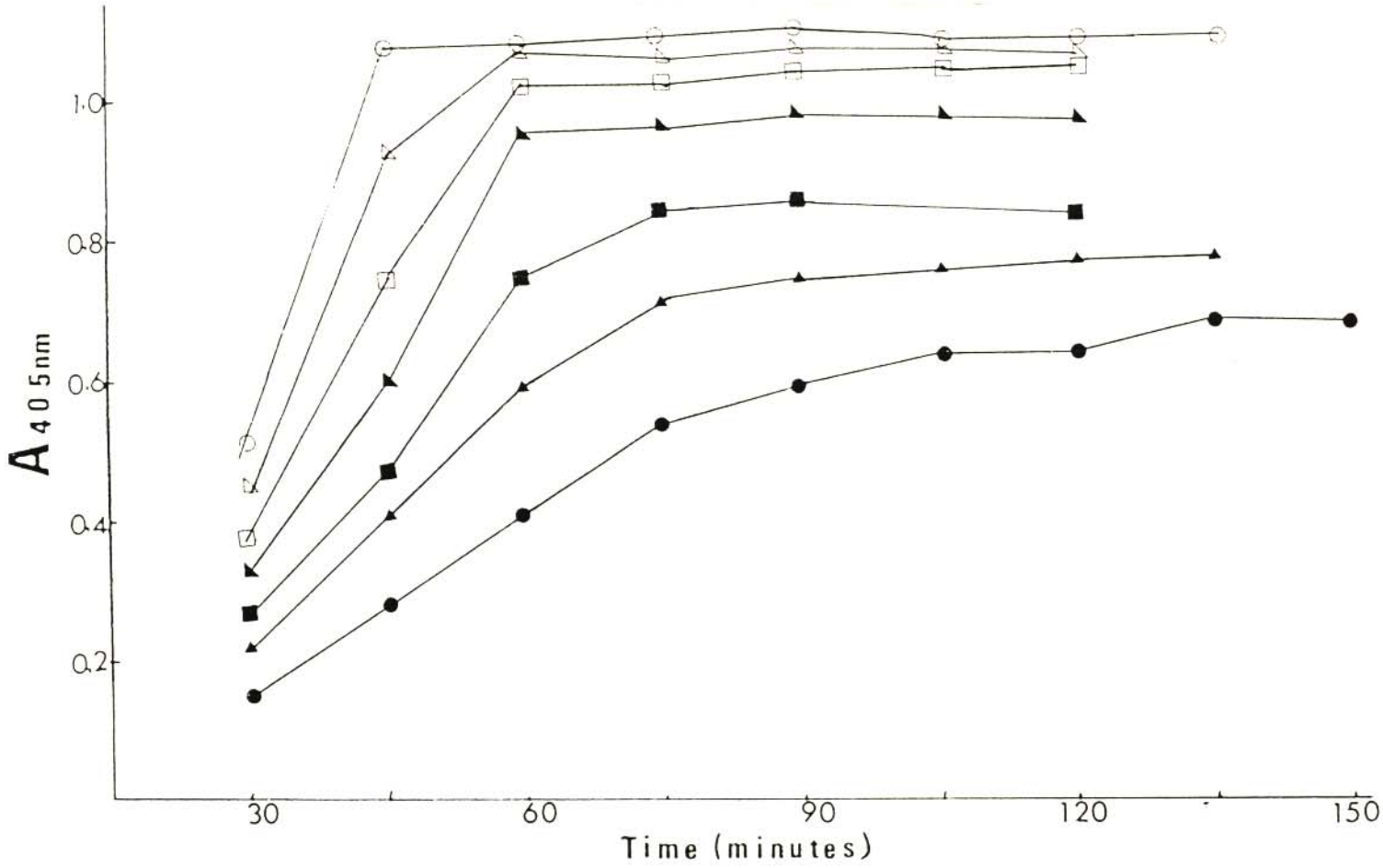


Fig. 4.14 : Effect of incubation and dilution on a commercial anti-IgG enzyme conjugate

Commercial anti-IgG conjugated to alkaline phosphatase was diluted at various dilutions, and incubated at different lengths of times according to the procedure outlined in 4.6. The same positive serum was used in all the above experiments.
Key to dilutions:

1:200 1:800 1:1500
1:400 1:1000
1:600 1:1200

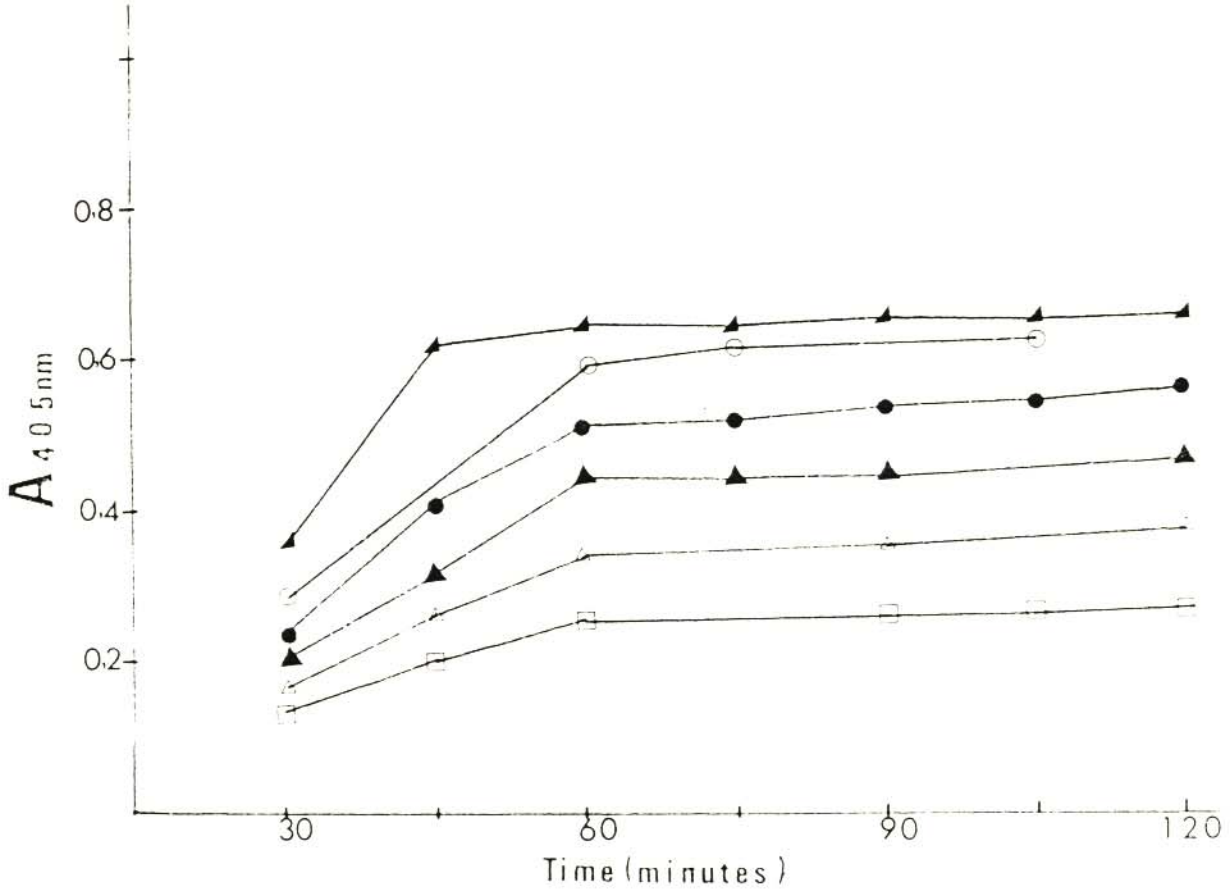


Fig. 4.15 : Effect of conjugate incubation time and dilution on commercial anti IgM alkaline phosphatase conjugate.

Commercial available anti-IgM alkaline phosphatase conjugate was used at various concentrations for different periods of time.

Key to dilutions:

1:600
1:800
1:1000
1:1200
1:1500
1:2000

Colour development

To determine the incubation time for the chromogenic substrate, positive and negative serum samples were diluted 1:200, incubated with anti-IgG-alkaline phosphatase diluted 1:800. Different antigenic concentrations were used. Enzyme-substrate incubation was done at timed intervals when the reaction was stopped and read. The results are shown in Fig. 4.16 and Fig. 4.17.

From the results, it can be seen that there is a linear relationship between incubation time and absorbance. For the experimental work, the enzyme-substrate incubation time used was 45 minutes with an antigen concentration of 8µg/ml and a substrate concentration of 1mg/ml. The incubation period of 45 minutes was taken because it gave a clear difference between positive and negative specimens.

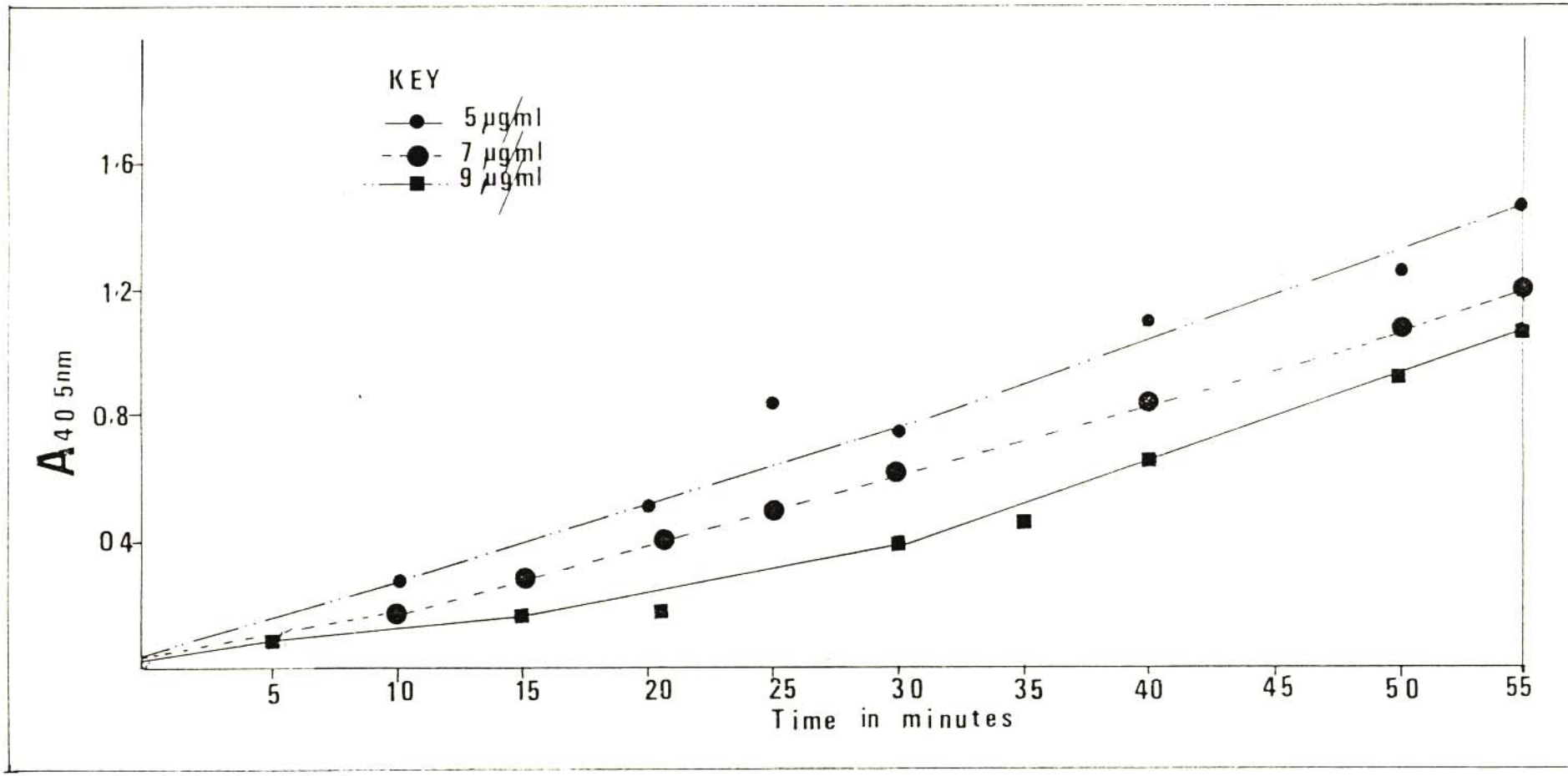


Fig. 4.16 : The effect of varying the enzyme-substrate incubation time using commercial LPS

The assay was performed as described in section 3.5. Microtitre plates were coated with different concentrations of commercial LPS antigens. Positive serum samples were incubated for 2 hours and then the anti-IgG alkaline phosphatase conjugate was incubated for 2 hours.

Two hundred ul of para-nitrophenyl phosphate was added and the plates incubated at varying lengths of time as indicated in the graph.

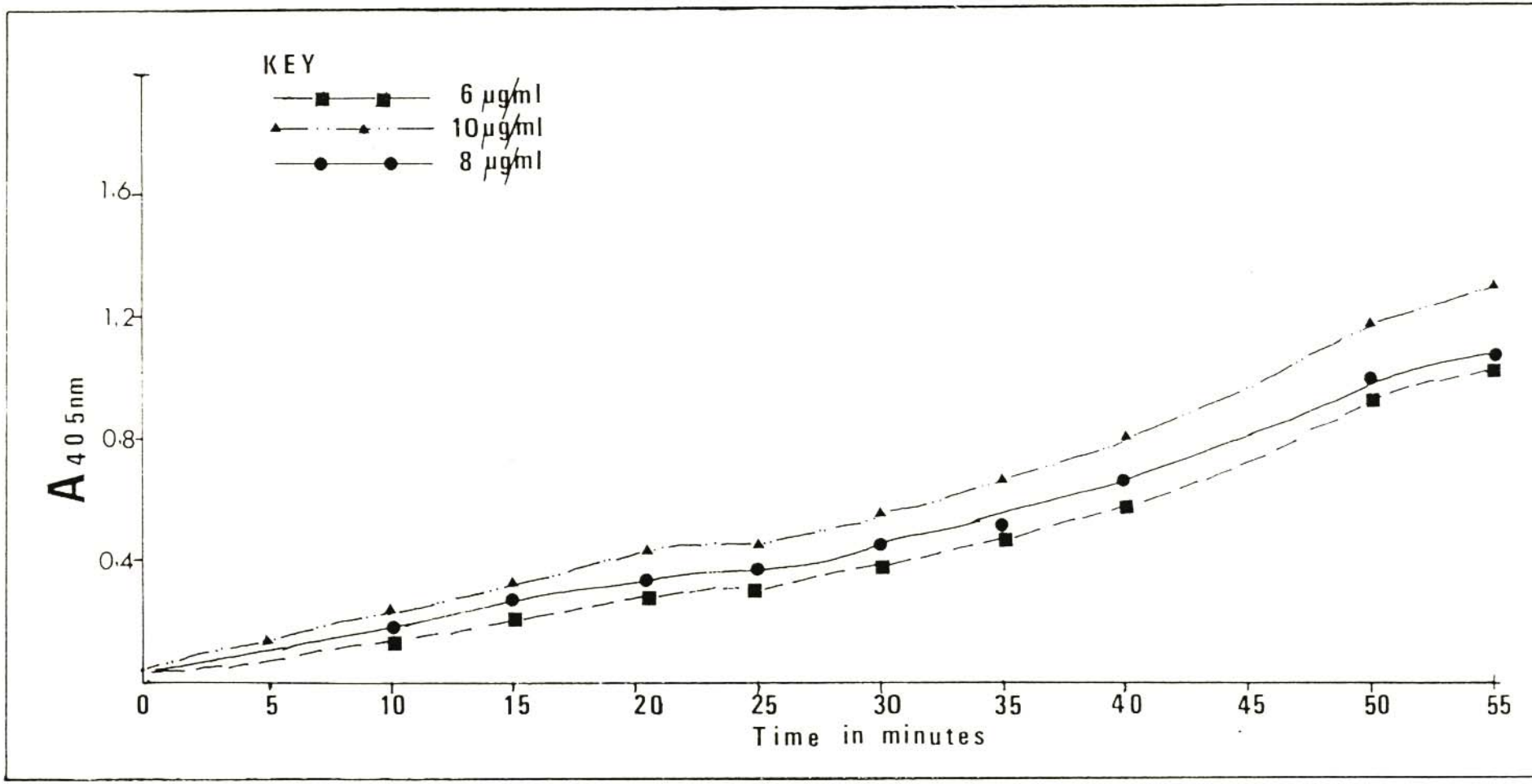


Fig. 4. 17: The effect of varying enzyme-substrate incubation time using laboratory prepared LPS of different concentrations

The assay was performed according to the procedure outlined in secion 3.5.

Microtitre plates coated with 10ug/ml, 8ug/ml and 6ug/ml of prepared LPS were incubated for 2 hours with a positive serum sample diluted 1:200.

Anti-IgG conjugated to alkaline phosphatase was added and the plates incubated for 2 hours.

Two hundred ml of para-nitrophenyl phosphate was added and the plates incubated as indicated for varying lengths of time.

DISCUSSION

In designing an assay for use in the laboratory, standardised and optimal conditions for the "assay geometry" should be determined. The term assay geometry refers to the steps in the assay, their sequence and the equipment needed to perform these steps (Morris et al., 1982).

Reduction of blank readings

During the initial stages of this work, considerable difficulty was experienced with high blank readings. High blank readings were also found when tubes were used. This was overcome by adding 1% bovine serum albumin (or gelatin) to the washing solutions, and to PBS used for diluting the serum.

Coating with Antigen

The method widely used to render antigen insoluble in ELISA is passive adsorption to the inner surface of disposable microtitre plates or cuvette blocks (Viljanen et al., 1982; Conradie et al., 1980; Lunde et al., 1979). Plates were coated for varying periods of time, all other variables being constant. The results indicate that the maximum antigen coating for the assay was obtained after 24 hours at 4°C. Another interesting parameter to be assessed is the pH of the coating buffer. Carlsson et al., (1972) found that the variation of pH from 6-10 did not have any significant effect on antigen coating. However, the optimal pH for bacterial antigens was found to be a pH of 9-10.

Concentration of the Antigen

The amount of the antigen used for coating the plates is critical for Elisa. If the antigen concentration is too low, then there

is insufficient antigen for the binding of the antibody and the sensitivity of ELISA is decreased. On the other hand, using a high antigen concentration is uneconomical. It is therefore essential that the optimal concentration for each system be established. In the present work, the optimal concentration is 8µg/ml which compares well with the antigen concentration reported in other experimental work with ELISA (Gripenberg et al., 1978; Engvall & Perlmann, 1972; Holmgren and Soennerholm, 1978). A concentration between 6µg/ml and 10µg/ml could be used in this experiment but 8µg/ml was chosen as it is the mean. Veronal buffer extract antigen can be used in lower concentrations of about 2µg/ml.

The optimal serum dilutions for ELISA

The optimal serum dilution should be chosen in such a way that a prozone phenomenon is avoided. The prozone phenomenon was reported by Carlsson and Lindberg, (1978) and Gripenberg et al., (1978). In the experimental work a serum dilution of 1:200 was used because it was the lowest dilution on the linear part of the graph and it also gave the greatest difference between the positive and negative serum samples. From the graph, higher serum dilutions e.g. 1:100 falls on the linear part of the graph but lower serum dilution e.g. 1:10 produces the prozone phenomenon. The absorbance reading shows a saturated system which decreased with a high concentration of antibodies. The reason for this is not understood. It seems that the more free antibodies there are in the system, the greater the likelikehood there is that the antigens-antibody complex will become detached from the well.

The optimal time for antibody-antigen reactions

Incubation of the serum can either be done at room temperature or at 37°C.

In the experimental work all the incubations were done at 37°C in order to reduce the reaction time. Figure 4.8 shows that the reaction was completed within 30 minutes, however the absorbance only started to stabilize after 60 minutes. To allow for the proper antibody-antigen interaction an incubation of 2 hours should be used. However, the incubation time may be decreased to one hour at 37°C. A decrease of antibody binding to LPS coated tubes, which was observed by Carlsson and Lindberg (1978) when the incubation period was more than two hours was not evident in the present investigation. Carlsson and Lindberg (1978) reported that the decrease in binding could be due to the detachment of LPS antigen-antibody complexes from the tube. The same authors found no detachment using protein or polysaccharide antigens.

Concentration of enzyme-antibody conjugate

Positive and negative serum samples assayed at 2 hours show that a dilution of the conjugate of 1:800 to 1:1000 can be used in the assay. The higher conjugate concentration for the two commercial conjugates gave the same results. When using the laboratory prepared enzyme-antibody conjugate, there is linearity from 1:1 to 1:200. This indicates that the enzyme-conjugate prepared was not pure and an arbitrary 1:10 dilution was taken in order to determine the optimum enzyme-conjugate incubation time. For the commercial enzyme-antibody conjugate a dilution of 1:800 was taken because it is on the saturated part of the graph to make allowance for any loss of enzymatic activity due to storage, and to make allowance for a serum containing a high concentration of antibodies.

Reaction time for enzyme-antibody conjugate with the serum

At high concentrations of enzyme-antibody conjugate, binding is completed within 45 minutes. However the decision to use such high concentrations was taken after considering the results of **page** 88. The time allowed for the binding of both commercial conjugate and laboratory prepared conjugate was two hours. The time can still be decreased to 60 minutes without reducing the accuracy of the results.

CONCLUSION

The first section of this chapter covers the preparation of the anti-antibody-enzyme conjugate which is used as a marker for ELISA. The preparation is not worth the effort since commercial conjugates are readily available at reasonable prices. It was decided to use the commercial preparation since it gives a higher enzymatic activity as compared to the laboratory prepared conjugate. The laboratory prepared conjugate was used at a low dilution compared to the high dilutions necessary in the case of the commercial preparations. Moreover, to prepare antibody-enzyme conjugate is laborious and time consuming.

The second section of this chapter has established the optimal conditions for ELISA. It is necessary to determine these conditions in order to minimise the cost of the assay, to cut the time required to perform the test and to ascertain the reproducibility and accuracy of the results.

It must be pointed out that each and every laboratory have to determine its optimal working conditions for ELISA because for some unknown reason, conditions described by one laboratory may not be valid in another laboratory (Conradie, 1981, personal communication).

The specificity of the ELISA depends on the purity of the antigen used. In the present study, different antigens are compared and the results indicate that the more specific antigen gives more reliable, reproducible and accurate readings than when crude extracts are used. The quality of enzyme-

antibody conjugate is also of importance. This was tried but the prepared conjugate were of inferior quality. So far no requirements for conjugates in terms of enzyme/protein ratio, absence of labelled immunoglobulins and free enzyme have been specified.

It must pointed out that negative and positive control specimens should always be used in the assay. This helps to reduce the errors due to day to day variations.

CHAPTER 5

ELISA IN THE SERODIAGNOSIS OF S.TYPHI INFECTIONS

When an antigen enters the body, it normally induces the formation of specific antibodies. These antibodies are mainly IgM and IgG and they can be detected in the serum of people who are suffering from the disease caused by the antigen. If the serum containing these antibodies is allowed to react with the antigen *in vitro* and the antibodies quantitated using an enzyme as a marker, the test will show if an active infection is present or not.

This chapter sets out to test the application of ELISA in the serodiagnosis of typhoid fever. The following experimental work was undertaken:

(i) Testing of the specificity of the assay.
(ii) How antigen purity affects the results of ELISA.
(iii) The use of microtitre plates as compared with the use of tubes.
(iv) Standard curve for quantitation of IgG antibodies.
(v) A survey on the normal population in an endemic typhoid area.
(vi) Reproducibility of ELISA.
(vii) Comparison of ELISA with the Widal test.
(viii) Comparison of ELISA with bacteriologically proven cases of typhoid fever.
(ix) Survey using ELISA on patients admitted on clinical grounds.

MATERIALS AND METHODS

All the materials used in this section are the same as described in

Chapter 4 Section II.

Methods:

The experimental work was done essentially as described in Chapter 4 Section II.

Microtitre plates were coated with 8µg/ml antigen solution and incubated for 24 hours at 4°C. Plates were washed three times and stored at 4°C before use.

200µl of serum diluted 1:200 with PBST was added to each well and plates incubated for 2 hours at 37°C. After washing the microtitre plates three times, alkaline phosphatase conjugated to either anti IgG or to anti-IgM was added and the plates incubated for 2 hours at 37°C. Alkaline phosphatase conjugated to anti-IgG was used in all the sections. The plates were washed three times after which a solution of para-nitro-phenyl phosphata was added. After an incubation period of 45 minutes, at room temperature, the absorbance of the specimens were read using a multiscan.

Results

1. Determination of specificity of ELISA

Specificity of the assay was studied by absorption experiments. Positive and negative serum samples were diluted 1:200 and preincubated for 30 minutes at 37°C with either 10µg/ml of Salmonella typhi or E. coli acetone

killed bacteria. PBS was used as a control. After incubation the samples were centrifuged at 3000 rpm to remove bound antibodies. The results of this work are shown in Table 5.1.

From the result it can be seen that the mean absorbance of samples absorbed by S. typhi is X_1=0,117 where as the mean absorbance of samples absorbed by E. coli is X_2=0,719 and the mean of unabsorbed samples is 0,726. This shows that the absorption of the serum samples by specific pathogens reduces the amount of specific antibodies considerably.

Table 5.1 Specificity of ELISA

Sample	S. typhi	E. Coli	Unabsorbed
1	0,041	0,743	0,753
2	0,147	0,858	0,861
3	0,221	0,715	0,894
4	0,31	1,113	0,917
5	0,246	0,952	0,895
6	0,014	0,678	0,695
7	0,05	1,023	1,116
8	0,077	0,98	0,987
9	0,035	0,015	0,064
10	0,006	0,116	0,076
	X_1=0,117	X_2=0,719	0,726

Serum samples were preincubated for 30 minutes at 37°C either with 10 µg/ml of Salmonella typhi antigen or E. coli with PBS as control before assaying with ELISA.

Serum sample No. 9 and 10 are negative when assayed by Widal test.

Serum sample No. 1 to 8 are positive when assayed by Widal test.

Comparison of the LPS purified by centrifugation and LPS purified by enzyme treatment.

(a) Laboratory preparations of LPS antigens as described in Chapter 2 were used. Ribonuclease treated LPS were used to coat one set of plates, and centrifuged LPS were used for the coating of a second set of plates. ELISA was performed as described in the methods. Results of this work is shown in Table 5.2.

The results show that a high correlation exists between ribonuclease treated LPS and centrifuged LPS in their application in ELISA. The different methods of removing RNAs, seem to give the same results.

Table 5.2 Comparison of centrifuged LPS and enzyme treated LPS

Sample No	Ribonuclease treated LPS	Centrifuged LPS
1	0,215	0,228
2	0,187	0,18
3	0,175	0,28
4	0,101	0,099
5	0,271	0,463
6	0,281	0,201
7	0,779	0,814
8	0,883	0,885
9	0,675	0,712
10	0,772	0,779
11	1,015	1,063
Mean	=0,485	=0,525
	Rank correlation	=0,96

Comparison of ribonuclease treated LPS and centrifuged LPS in the ELISA. Ribonuclease treatment was done after the extraction by phenol-water. Centrifuged LPS fraction was purified by centrifugation at 105 000g for removal of nucleic acid.

(b) The following antigens were used to coat separate batches of plates:

(i) Veronal buffer extract

(ii) Commercial LPS

(iii) Lysate antigenic preparation

(iv) Centrifuged laboratory prepared LPS

Serum specimens diluted 1:200 were tested simultaneously in all the plates using alkaline phosphatase conjugated to anti-IgG, diluted 1:800. Results of this work are shown in Table 5.3.

The correlations between absorbances obtained when using different antigens are indicated in Table 5.3. e.g. 12 refers to correlation between absorbance in columns 1 and 2.

Table 5.3 : Comparison of different antigen preparations for their possible use in serodiagnosis of typhoid fever

Sample No	Veronal extract 1	Commercial LPS 2	Lysate 3	Prepared LPS 4	Rank Correlation
1	0,987	0,797	0,996	0,811	12=0,93
2	0,115	0,275	0,299	0,245	
3	0,032	0,002	0,074	0,121	
4	0,101	0,312	0,094	0,093	23=0,85
5	0,122	0,451	0,185	0,172	
6	0,141	0,482	0,208	0,361	
7	0,120	0,052	0,098	0,108	34=0,88
8	0,044	0,221	0,132	0,242	
9	000	0,021	0,061	0,068	24=0,79
10	0,206	0,421	0,274	0,492	
11	0,088	0,025	0,000	0,111	14=0,78
Mean	X_1=0,255	X_2=0,321	X_3=0,288	X_4=0,305	

The readings indicate the ELISA titre obtained after performing the test under the same experimental conditions except that a separate plate was used for each type of antigen.

Serum sample No. 3,4,5,7,9 and 11 were negative as determined by the Widal test.

Serum sample No. 1,2,6,8, and 10 were positive as determined by the Widal test.

(c) Comparison of laboratory prepared LPS with Widal 0 antigens

Salmonella typhi 0 antigens normally used for the titration of antibodies in the Widal test were compared with the laboratory prepared LPS antigens.

Results of the work are shown in Table 5.4.

The results show that the two antigens correlate well. A rank correlation of 0,8 was obtained. However, the titres for the crude 0 antigens are higher than those for the LPS antigens. This may be an indication of a high absorbance of the contaminants in the 0 antigens.

Table 5.4 Comparison of laboratory prepared LPS with Widal-O antigens

Sample	LPS	Crude (O)
1	0,194	0,428
2	0,335	0,880
3	0,510	0,581
4	0,189	0,441
5	0,566	0,537
6	0,276	0,756
7	1,155	1,795
8	0,420	0,642
9	0,149	0,879
10	0,216	0,139
11	1,144	1,006
12	0,908	0,533
13	0,211	0,572
14	0,320	0,831
15	1,103	1,619
16	1,654	1,712
17	0,302	0,468
18	1,096	1,586
19	0,768	1,559
20	1,408	1,733
21	1,021	1,638
22	Control Negative 0,075	
23	Control Positive 1,071	

Results showing the ELISA titres after coating the plates with either purified LPS or with crude antigens used in Widal tests. Crude antigens were supplied by S.A.I.M.R. (Pietersburg). The procedure of the test is described in the text. Rank correlation for the experiment was found to be 0,8.

Serum sample No. 4,6,10, and 13 were negative by Widal test, whereas the rest were positive by Widal test.

Comparison of the use of tubes with the use of microtitre plates in ELISA

The application of ELISA for serodiagnosis of typhoid fever can be extended to small laboratories if tubes are used instead of microtitre plates. When tubes are used the absorbance can be read in a spectrophotometer which is readily available in contrast to the more expensive multiscan which has to be used with microtitre plates. The use of microtitre plates can also be expensive where only one or two serum samples are tested daily.

To investigate the applicability of the use of tubes in ELISA, 5ml polyethylene tubes with tops (Protea Laboratories) were used. The volume of the steps was increased to 1ml as compared to 200µl used with microtitre plates. The experimental part followed the same procedure as previously.

The results of the enzyme-substrate reaction were read in a Zeiss spectrophotometer at 400nm for the alkaline phosphatase conjugate. The results of this experimental work is shown in Table 5.5. The correlation between the test tubes and the plates was calculated according to the formula = $1 - \frac{6\sum D^2}{N(N^2-1)}$

Table 5.5 Comparison of the use of tube with the use of microtitre plates

	Tubes		Plates	
Sample No	IgM	IgG	IgM	IgG
1	1,344	1,414	0,375	0,767
2	0,512	1,825	0,431	0,843
3	0,758	0,833	0,761	0,831
4	0,881	1,689	0,271	0,985
5	0,444	0,539	0,187	0,271
6	0,398	0,307	0,071	0,198
7	0,871	1,234	0,231	0,594
8	0,109	0,215	0,663	1,157
9	0,826	1,571	0,591	1,325
10	0,143	0,168	0,898	1,173
11	0,452	0,955	0,702	0,995
12	0,829	1,595	0,204	0,543
13	1,074	1,196	0,673	0,972
14	0,333	0,544	0,964	0,185
15	0,181	0,511	0,087	0,171
16	0,271	0,813	0,061	0,207
17	1,227	1,831	0,797	0,091
	X=0,63	X_2=1,014	X_3=0,42	X_4=0,67

Results of ELISA using microtitre plates compared with ELISA using plastic test tubes. The procedure was carried out as indicated in the text and the tubes were read at 400nm in single beam Zeiss spectrophotometer (Model PM 2K; Optical Instruments, Johannesburg). Microtitre plates were read in a multiscan spectrophotometer at 405nm specifically designed for microtitre plates.

Serum No. 14, 15 and 16 were negative by Widal test, and the rest were positive by Widal test.

Preparation of a standard curve for IgG

In order to determine the concentration of IgG antibodies in the serum, a standard curve is needed. For this purpose plates were coated with varying concentrations of human IgG according to the methods described in Section II. Plates were incubated with enzyme-anti-IgG conjugate diluted 1:800 for 2 hours at 37°C after which the substrate was added and the absorbance read after 45 minutes incubation at room temperature. The results of the standardisation from 4 different plates with n = 30 are shown in Fig. 5.1. The absorbance versus concentration, when plotted using an ordinary linear scale, gave a sigmoid non-linear curve. Absorbances were converted into reciprocals and later plotted. This change the graph equation from $y=mx + c$ to $1/y = mx + c$ where y is equal to $\frac{1}{mx + c}$. By calculating $\frac{1}{y}$, for a specific sample, the corresponding concentration of antibody present can then be read off from the graph. Although the graph shows a linear relationship it can be seen that when the antibody concentration is very low, the absorbance approaches zero i.e. the reciprocal of absorbance will become very large. From a concentration of 10^{-6}, the linearity of the graph is lost and the smaller the change in concentration, the greater will be the reciprocal of the absorbance. When the concentration is high, the reciprocal of absorbance becomes very small, i.e. the absorbance will increase above the 1,999 reading which cannot be read with an ordinary spectrophotometer. At a concentration of 10^{-1}ug/ml, the linearity of the y and x variables is lost and there is a piling of points, a greater change in concentration will correspond to a very small change in reciprocal of absorbance and this will approach zero. From this experiment, it must be realised that the amount of the antibody that is coated on the plate will determine the results. The second variable to be considered is the amount of antibody-enzyme conjugate available. The antibody-enzyme conjugate should always be in excess of the antibody in the

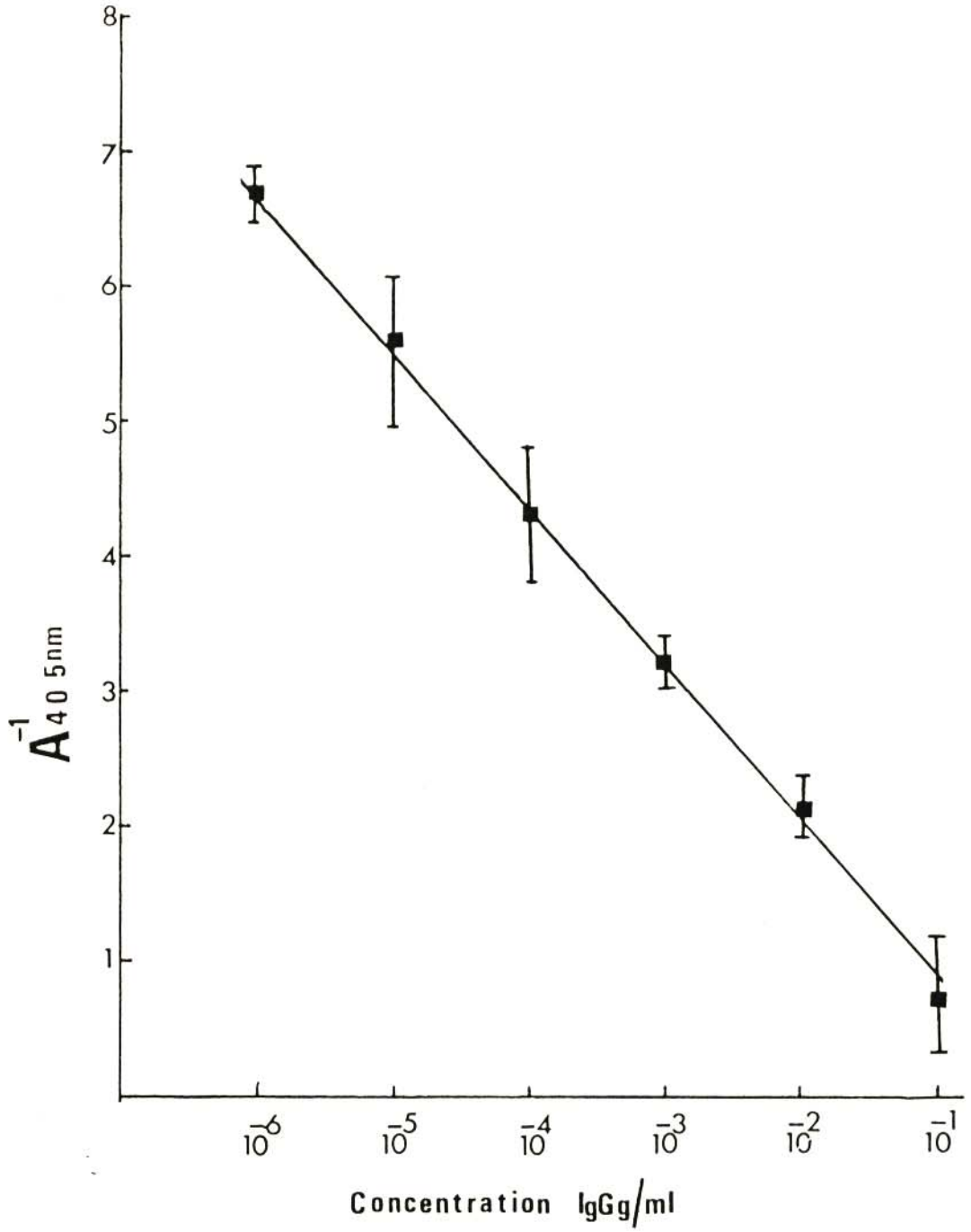


Fig. 5.1: Standard curve

Standard curve obtained from 30 assays. The plates were coated with IgG according to the procedure outlined in the methods and later incubated with the enzyme-antibody conjugate diluted 1:800. Incubation time for the enzyme-substrate reaction at room temperature was 45 minutes.

Determination of reproducibility of ELISA

To assess the reproducibility, positive and negative serum samples were tested by ELISA using 8 coated plates. Plates were coated with 8µg/ml of LPS in carbonate buffer as outlined in Section II of Chapter 4. Serum samples were diluted 1:200 in PBS and later incubated with enzyme-anti-IgG-conjugate for 2 hours at 37°C. Enzyme-substrate incubation time was 45 minutes at room temperature. The results of this work is shown in Table 5.6. Coefficient of variation (cv) between the different readings was calculated as follows, C.V. = $\frac{S}{X}$ where S is the standard deviation and X is the mean obtained. The results show a great coefficient of variation in the negative serum samples of 0,49 as compared with a low coefficient of variation in the positive serum samples. In general, the coefficient of variation between the absorbance of the same specimen was not great if the absorbance were rounded to the nearest second decimal point.

Reproducibility was secondly examined when two different individuals perform the test using the same conditions within a five week interval. Serum samples were first tested and then stored at -20°C until the second test was performed by a different individual.

Results of this work are shown in Table 5.7. From the results, coefficient of variation can be seen to range from 0,212 to 0,13. The coefficient of variation of the negative control is high. The test samples however, if divided into negative and positive can be seen to have a high coefficient of variation in the negative and a very low coefficient of variation in the positive

group. The average mean of the variation is 0,054 with a standard deviation of 0,052 which is quite high.

One factor to be observed from the experimental results is the storage time which seems to have no detrimental effect on the antibody titres. It can be concluded that the serum samples, if stored at -20°C can be tested at least after $\pm$ 5 weeks later and still give the same result.

Table 5.6 Reproducibility of ELISA

Sample No	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Plate 6	Plate 7	Plate 8	CV
1	0,762	0,771	0,751	0,759	0,81	0,744	0,739	0,73	0,032
2	0,089	0,054	0,104	0,062	0,085	0,071	0,055	0,087	0,23
3	1,067	1,119	1,166	1,081	1,11	1,98	1,093	1,152	0,03
4	1,287	1,217	1,263	1,63	1,118				0,057
5	0,012	0,058	0,055	0,040	0,036	0,020	0,026	0,023	0,49
6	0,128	0,097	0,114	0,111	0,112	0,121	0,116	0,107	0,08
7	0,071	0,076	0,066	0,084	0,064	0,064	0,069	0,087	0,112
8	0,166	0,128	0,179	0,139	0,16	0,163	0,136	0,153	0,4
9	0,436	0,449	0,454	0,434	0,491	0,458	0,452	0,524	0,065
10	1,055	1,046	1,088	1,162	1,116				0,052
11	1,354	1,067	1,085	1,239	1,239	1,213	1,152	1,269	0,083
12	1,474	1,508	1,230	1,431	1,485	1,531	1,411	4,434	0,065
13	1,042	1,029	1,015	1,084	0,995	1,05	0,996	1,109	0,045
14	0,132	0,101	0,184	0,114	0,117	0,099	0,096	0,104	0,245
15	0,753	0,64	0,704	0,807	0,812	0,792	0,797	0,752	0,079
16	0,496	0,502	0,45	504	0,525	0,424	0,494	0,574	0,095
17	0,871	0,901	0,969	0,892	0,929	0,873	0,915	0,974	0,044
18	0,077	0,091	0,062	0,078	0,107	0,083	0,107	0,089	0,177

Reproducibility of ELISA when done on the same day at the same time under the same conditions. Antibody titre of sera were tested by ELISA using different plates from the same batch and an enzyme-conjugate dilution of 1:800.

The coefficient of variation was calculated according to the formula $CV = \frac{S}{X}$ where S is the standard deviation and X is the statistical mean:

Specimen No 17 is a positive control

and No 18 is a negative control.

Serum sample No. 2,5,6,7,8 and 18 were negative by Widal test and the rest were positive by Widal test.

Table 5.7 Reproducibility of 23 serum specimens tested after a period of 5 weeks.

Sample No	1st Week	5th Week	CV
1	0,934	0,886	0,037
2	0,437	0,397	0,067
3	0,221	0,232	0,038
4	1,234	1,014	0,138
5	1,043	1,035	0,003
6	0,951	0,996	0,033
7	1,072	1,131	0,039
8	1,221	1,194	0,016
9	1,736	1,694	0,017
10	0,211	0,271	0,173
11	0,993	0,991	0,014
12	0,069	0,051	0,212
13	0,715	0,747	0,031
14	0,713	0,707	0,005
15	1,106	1,071	0,023
16	1,313	1,393	0,042
17	1,166	1,081	0,023
18	0,501	0,449	0,079
19	0,949	0,957	0,059
20	0,858	0,924	0,052
21	1,015	1,126	0,073
22	1,084	1,125	0,026
23	0,758	0,779	0,019
Positive control	1,071	1,113	0,027
Negative control	0,047	0,097	0,429

Mean CV = 0,054

Positive and negative sera were tested according to the procedure outlined in the methods by two individuals with an interval of 5 weeks. Positive specimens show a low coefficient of variation whereas the negative serum samples show an increased coefficient of variation.

Comparison between the ELISA assay and Widal test

Serum samples from patients with suspected typhoid fever were assayed by both the Widal test and by the ELISA test, in an endeavour, to determine the correlation between titres obtained by the Widal agglutination test and ELISA titres and to establish cut-off points in the ELISA assay which are associated with positive Widal tests.

The Widal agglutination test was done at the S.A. Institute of Medical Research in Pietersburg and in Duiwelskloof, and by the S.A. Institute for Tropical Diseases in Tzaneen. The specimens were stored at -20°C until the ELISA assay was performed. The Widal test was done in the following way: Serum was diluted as follows: 1;50, 1:100, 1:200 and 1:400. Salmonella typhi antigen was added and the mixture incubated for 18 hours at 50°C. The highest dilution giving a positive reaction was taken as the Widal titre. The highest positive reaction with crude 0 antigens is called 0-titre and the H antigens give an H-titre.

ELISA was done according to the procedure outlined in the methods in Section 3.5 of Chapter 4. The absorbance (reading) was taken as the ELISA titre. The results of this work are shown in Table 4.9. Coefficient of correlation was calculated according to the following formula:

$$r = 1 - \frac{6 \Sigma D^2}{N(n^2-D)}$$

D is the deviation of the ranking, and N is the number of test performed.

From the results, it can be seen that there is a high spearman's ranking correlation between 0 Widal titres and IgM ELISA titres. A correlation of 0,799 between H titres and IgM titres was found. There is a low correlation between IgG serum antibodies and the two Widal titres.

Table 5.8 ELISA titre vs the Widal titre

ELISA		Widal	
1 IgG	2 IgM	3 0	4 H
0,516	0,131	800	800
0,618	0,361	1600	1600
0,766	0,394	400	400
0,775	0,972	100	100
0,768	0,109	50	N
0,513	0,421	200	1600
0,951	0,221	800	400
0,836	0,241	1600	1600
0,151	0,071	N	N
0,723	0,138	200	100
0,344	0,494	400	1600
0,454	0,471	1600	1600
0,582	1,441	400	800
0,246	0,096	N	N
0,629	0,465	800	1600
0,707	0,114	100	400
0,509	0,125	100	50
0,751	0,065	N	N
0,606	0,219	100	N
0,671	0,221	800	400
0,775	0,064	50	N
0,811	0,112	200	100
0,406	0,121	100	N
0,416	0,121	100	N
0,768	0,109	50	N
0,634	0,031	N	N
0,951	0,221	800	400
0,723	0,138	200	100 Negative control
0,184	0,051	N	N Positive control
0,837	0,474	800	1600

Results of ELISA titre as compared with Widal titre.

Rank correlations were obtained using the following formula:

$$1 - \frac{6 \sum D^2}{N(n^2 - 1)}$$ where D is the deviation of

the ranking and N is the number of tests. The procedure used in ELISA was as described in the text. Widal tests were done at Tzaneen Institute of Tropical Diseases and at S.A.I.R. (Pietersburg).

$r_{13} = 0,143$ $r_{14} = 0,17$

$r_{23} = 0,799$ $r_{24} = 0,84$

Correlation between ELISA titres and patients with proven typhoid fever

Serum specimens were obtained from 28 patients who had clinically diagnosed typhoid fever which had been confirmed by culture. Serum from healthy students were used as controls.

The serum samples were tested by ELISA according to the procedure outlined in Section 3.5 of Chapter 4 using alkaline phosphatase conjugated to anti-IgM and anti-IgG as a marker enzyme. The results of this work are shown in Table 5.9.

Very high titres were obtained using either IgM or IgG in serum from patients with typhoid fever.

Table 5.9 Comparison of ELISA titres with clinically diagnosed and bacteriologically confirmed samples

Sample No	IgM titre	IgG titre	Blood culture	Stool culture
1	0,517	0,767	+	-ve
2	1,049	0,534	+	+
3	0,537	1,235	+	-ve
4	0,956	0,562	+	-ve
5	1,059	0,801	+	+
6	0,886	0,773	+	-ve
7	0,624	1,207	+	-ve
8	0,086	0,113	-	-ve
9	0,624	0,815	+	-ve
10	0,864	0,565	+	-ve
11	0,872	1,071	+	-ve
12	0,157	0,784	-ve	+
13	0,344	0,66	+	-ve
14	0,114	0,719	-ve	-ve
15	0,502	0,446	+	+
16	0,424	0,689	+	+
17	0,712	1,142	+	-ve
18	0,807	1,021	+	-
19	0,495	1,027	+	-
20	0,062	0,194	-ve	-ve
21	0,574	0,503	+	-ve
22	1,316	0,617	+	+
23	0,876	1,119	+	-ve
24	1,028	0,866	+	-ve
25	0,785	0,914	+	+
26	0,465	0,987	+	-
27	0,812	0,871	+	-
28	0,736	0,903	+	-ve
29	0,672	0,869	-ve	+
30	0,558	0,974	-ve	+

Serum samples No 8 and No 20 are the two negative specimens from healthy donors. The bacteriological isolations were done at S.A.I.M.R., Pietersburg and Duiwelskloof and at the S.A. I. Tropical Diseases, Tzaneen. Samples were tested according to the ELISA procedure as already described. Alkaline phosphatase was used as a marker for the system.

Key: + - positive isolations
-ve - no isolations

ELISA titres of a population in an area endemic for typhoid fever

In order to evaluate the ELISA test on people who are living in an endemic area but who had no typhoid fever infection, samples were obtained from subjects in Sekororo. The donors were women and school children who were taking part in a nutrition survey run by the department. Serum samples from healthy donors were tested by ELISA according to the procedure outlined in Section 3.5 of Chapter 4 of the methods. The enzyme-antibody conjugate used for this work was alkaline phosphatase conjugated to either anti-IgG or anti-Ig ..

The results of this work are shown in Table 5.10. The two means of the values are low with a very low standard deviation. There is an increased titre of IgG as compared to IgM.

Table 5.10 ELISA titres of a population in an area endemic for typhoid fever

Sample No	IgM	IgG
1	0,033	0,178
2	0,032	0,058
3	0,011	0,099
4	0,025	0,107
5	0,004	0,111
6	0,027	0,198
7	0,007	0,001
8	0,004	0,121
9	0,012	0,321
10	0,007	0,160
11	0,016	0,109
12	0,000	0,107
13	0,014	0,181
14	0,005	0,211
15	0,021	0,101
16	0,078	0,107
	X_1 = 0,019	X_2 = 0,133
	S = $\pm$0,019	S = $\pm$0,074

Results of ELISA done to test serum from people who visited a clinic in Sekororo for antenatal clinics and school children.

RESULTS OF A SURVEY ON PATIENTS ADMITTED TO THE LETABA HOSPITAL WITH SUSPECTED TYPHOID FEVER

Sera were obtained from patients who were admitted to the Letaba Hospital. Blood, stools and urine were collected from the patients. Cultures for S. typhi were performed at the Institute of Tropical Diseases in Tzaneen as well as the Widal test. The results are shown in Table 5.10.

The sera were collected and stored at -20°C and later tested for the presence of IgM and IgG antibodies using the ELISA technique. The results are shown in Table 5.10.

Variables which are included in the table are the ELISA titres, the Widal titres, the basis of admission, results of stool culture, blood culture and the interval between admission and the blood collection. Admissions were done on clinical grounds and the diagnosis of typhoid fever confirmed by a rising Widal titre and/or by a positive culture.

It is interesting to note that specimen No. 24 has a negative Widal titre, a high ELISA titre and a positive blood culture.

When using ELISA for determination of the concentration of antibodies, one gets a good indication if active infection is present or not. Serum specimens taken three months after discharge has a low IgM titre, but a significantly increased IgG titre which shows that the concentration of IgM antibodies is increased in the initial stages of the disease, subsides with time, while IgG stays elevated.

RESULTS OF A SURVEY ON PATIENTS ADMITTED TO THE LETABA HOSPITAL WITH SUSPECTED TYPHOID FEVER

Sere were obtained from patients who were admitted to the Letaba Hospital. Blood, stools and urine were collected from the patients. Cultures for S. typhi were performed at the Institute of Tropical Diseases in Tzaneen as well as the Widal test. The results are shown in Table 5.10.

The sere were collected and stored at -20°C and later tested for the presence of IgM and IgG antibodies using the ELISA technique. The results are shown in Table 5.10.

Variables which are included in the table are the ELISA titres, the Widal titres, the basis of admission, results of stool culture, blood culture and the interval between admission and the blood collection. Admissions were done on clinical grounds and the diagnosis of typhoid fever confirmed by a rising Widal titre and/or by a positive culture.

It is interesting to note that speciman No. 24 has a negative Widal titre, a high ELISA titre and a positive blood culture.

When using ELISA for determination of the concentration of antibodies, one gets a good indication if active infection is present or not. Serum specimens taken three months after discharge has a low IgM titre, but a significantly increased IgG titre which shows that the concentration of IgM antibodies is increased in the initial stages of the disease, subsides with time, while IgG stays elevated.

Table 5.10 Survey done on patients admitted at Letaba Hospital, Tzaneen.

Sample No	ELISA TITRE		WIDAL TITRE					Time at which specimen was taken after admission
	IgM titre	IgG titre	O- titre	H- titre	Basis of admission	Blood Culture	Stool Culture	
1	0,098	0,741	400	200	Clin	-	-	20 days
2	0,041	0,834	-	-	Clin	-ve	-	30 days
3	0,728	0,923	-	-	Clin	+ve	-ve	7 days
4	0,123	0,566	-	-	Out patnt	-	-	-
5	0,416	0,725	100	-ve	Clin	-ve	-ve	1 day
6	0,197	0,766	400	200	Clin	-ve	-	5 days
7	0,311	0,987	N	N	Clin	-ve	-	1 day
8	0,709	1,035	50	100	Clin	+ve	-	21 days
9	0,849	1,056	100	50	R W	+ve	-	1 day
10	0,534	1,264	400	400	W	+ve	+ve	7 days
11	0,667	0,995	200	100	Bact.	+ve	+ve	5 days
12	0,701	1,086	400	1600	Clin	+ve	+ve	1 day
13	0,783	0,982	400	200	Clin & W	-ve	-ve	5 days
14	0,835	1,103	400	400	Clin	+ve	+ve	1 day
15	0,401	1,094	N	100	-	+ve	+ve	1 day
16	0,762	1,139	100	1600	W	-ve	-ve	1 day
17	0,397	0,865	100	50	W	+ve	-	10 days
18	0,624	1,694	1600	400	Clin	+ve	+ve	21 days
19	0,058	0,586	N	N	W & Clin	-ve	-ve	90 days ±
20	0,001	0,587	N	N	-	-ve	-ve	-
21	0,677	0,944	800	400	W & Clin	+ve	-	1 day
22	0,931	0,988	N	N	Clin	+ve	-	day
23	0,166	0,705	100	200		-ve	-ve	10 months
24	0,492	0,886	N	N	Clin	+ve	-	6 days ±
25	0,429	0,987	100	400	Clin	+ve	-ve	1 day
26	0,314	0,421	1600	400	H.W.	+ve		10 days ±
27	0,114	0,404	-	-	-	-	-	-
28	0,297	0,049	-	-	-	-	-	12 months
29	0,492	0,886	N	N	Clin & W	-ve	-	15 days
30	0,525	0,715	N	200	Clin & W	+ve	-	1 day±
31	0,117	0,337	400	200	Urine culture	-	+ve	1 day±

Sample No	ELISA TITRE		WIDAL TITRE		Basis of admission	Blood Culture	Stool Culture	Time at which specimen was taken after admission
	IgM titre	IgG titre	O- titre	H- titre				
32	0,887	1,203	100	400	W	+ve	-	15 days ±
33	0,563	0,814	100	200	W	+ve	+ve	1 day ±
34	0,876	1,046	200	400	W	+	-	6 days ±
35	0,013	0,223	-	-	Clin	+	-	5 days ±
36	0,427	0,631	400	1600	W	+	-	1 day ±
37	0,069	0,099	400	800	-	-	-	4 months
38	0,821	1,217	-	-	Clin	+ve	-	8 days ±
39	0,656	0,985	N	N	Clin	+ve	-	5 days ±
40	0,021	0,174	N	N	Clin	-	-	4 months
41	0,317	0,725	100	50	Clin	+ve		1 day
42	0,805	0,971	400	1600	W	+ve	+ve	3 days ±
43	0,055	0,124	200	200	-	-	-	-
44	0,627	0,989	400	400	W	+ve	-	1 day ±

Serum samples were tested by ELISA without prior knowledge of other variables - like Widal titre etc. Samples were collected from S.A. Institute of Tropical Diseases in Tzaneen and transported in a frozen state and kept at -20°C until tested. Samples were tested according to the procedure outlined in Section 3.4 of the method.

—— indicates that the culture was not done

+ve indicates a positive culture and -ve indicates a negative culture, N in the Widal column indicates a negative Widal titre.

DISCUSSION

Early diagnosis of typhoid fever is important for the proper treatment of patients. The existing serological methods, however useful, suffer from certain disadvantages as was outlined in the introduction. The recently introduced ELISA technique is rapidly becoming the most widely utilised serological method for the diagnosis of infectious diseases. It was therefore decided to establish if ELISA could be used for the early diagnosis of typhoid fever.

One is faced with several problems in designing an assay that measures the amount of antibodies by using antibody-antigen interaction; Firstly one has to have an efficient method of separating the free or unbound adsorbed antigens. Secondly, non-specific adsorptions of any other protein to the solid-phase has to be avoided. Thirdly, the antigens used should be pure and standardised and lastly, the optimal conditions of the "assay geometry" should be determined. The term "assay geometry" refers to the steps in the assay, their sequence and the equipment needed to perform these steps (Morris et al., 1982).

Specificity of ELISA

The ELISA allows specific determination of S. typhi antibodies. The results indicate that the assay is specific for antibodies against S. typhi. It could be interesting to assay a serum positive for specific strain or Salmonella e.g. S. strousberg, S. typhimurium or S. johannesburg. During the experimental work a single serum specimen which was positive for S. paratyphi by culture, was tested and found to be negative. However, more of this experimental

work need to be done to ascertain if any cross reactivity occurs with species closely related to S. typhi.

Expression of Results

Quantitative determination of antibody concentration is usually expressed in titres. An ELISA titre was defined as the highest dilution which gave an absorbance of 2.0 in 100 min at 405 nm (Carlsson et al., 1972). However, the procedure used above was found to be laborious, more especially if the test is used in routine diagnostic work where many specimens are tested. In this investigation, the ELISA titre is defined as an absorbance at 405 nm after an incubation time of 45 minutes of a serum sample diluted 1:200, and an enzyme-antibody conjugate diluted 1:800. Many manipulation steps were avoided to reduce the chance of high error in the test. All the tests were performed in quadriplicate with a negative and positive control serum in duplicate. A sample is considered to be positive if it produced an absorbance of ,25.deviation from the control negative serum level. This way of expressing results has also been used by Engvall and Ljungström (1975) and Veldkamp and Visser, (1975). Control sera have to be included since batch-to-batch variations is known to occur due to the different adhesive properties of polystyrene microtitre plates (Ruitenberg and Brosi, 1975; and Conradie, personal communication).

Standard curve for determination of serum IgG

Composite standard curves for the direct quantitation of antibodies can be constructed using ELISA (Paperniak et al., 1982, Conradie and Mbhele, 1980). The standard curve is usually constructed from purified immunoglobulin class

e.g. IgG. The serum samples however, are heterogeneous mixtures of antibodies e.g. IgM, IgG, IgA, etc. In the assay there is a competition between IgM and IgG antibodies for the binding sites available on the antigen. This feature may explain the prozone phenomenon. Hamilton et al., (1981) found that the competition between IgM and IgG antibodies may be a problem in assaying class specific antibodies, moreover, the affinity of the antibodies are different. IgM has been found to have five high affinity sites and five low affinity sites (de Preval, 1982).

The results indicate that the IgM titre is low compared to the IgG titre. On the basis of the above discussion this does not mean that there is a low concentration of IgM antibodies in the serum but may only indicate the relative concentration of IgM as compared to IgG antibodies in the serum.

ELISA in diagnosis of Typhoid fever

Antibodies to S. typhi belonging to the IgM class are known to exist only transiently in the early phase of the immune response (Uhr and Finkelstein, 1967). Thus the application of ELISA in assaying IgM antibodies offers two interesting possibilities.

(i) High levels of specific IgM antibodies would enable a diagnosis to be made from a single early phase serum sample.

(ii) It could also be used to distinguish between primary infections and reinfections and help to study the clinical differences between the two groups.

This study shows that the measurement of S. typhi specific IgM and IgG provides a useful tool in the diagnosis of typhoid fever. The most important finding in the experimental work is the high correlation between positive sera detected by using ELISA and patients who were clinically diagnosed. Also the ELISA test gave negative results in samples of people who were not suffering from typhoid fever.

The correlation between the IgM titre using ELISA and the O-titre using the Widal test was found to be 0,8, while a low correlation between IgG O and H widal titres was found. The reason for the low titre correlation cannot be explained. It must be pointed out that single Widal titres may be misleading because diagnostic Widal titres are the ones which rise (Somerville et al., 1981; Wicks et al., 1974). Widal titres may be elevated in people who are always in contact with the disease although they do not suffer any overt symptoms and in people who had been vaccinated against S. typhi. (Lewis, personal communication). In this instance it was not established as to whether a rise in IgG ELISA titre is also necessary for the test to be diagnostic.

A blind study performed on cases which were clinically diagnosed as having typhoid fever and where S. typhi was isolated after admission, shows that 86% had a positive IgM ELISA tire, whereas 96% had a positive IgG ELISA titre. 73% of all the cases admitted were found to have a positive IgM ELISA titre, whereas 82% had a positive IgG ELISA titre. Some of the cases which had a negative IgG and IgM ELISA titre were very old cases who were admitted for observation in order to trace carriers and thus did not have an active infection.

Because of the short time taken to obtain results, ELISA can help in confirming the clinical diagnosis of typhoid fever and only if there are any doubts, need a culture be set up. Some of the cases had a high Widal titre and yet were negative on cultures. Although they were genuine typhoid fever cases, proven treatment, no cultures could be found. It has been reported by Wicks et al., (1974), Bai et al., (1980) and by Hook (1974) that about 5 - 10 percentage failure is obtained with the culture method.

Patient No. 31 had a positive urine culture, reasonably diagnostic Widal titres but a low Elisa titre. It is unlikely that the specimen was taken earlier before even IgM antibodies could be formed because of urine isolations. Smith (1973) has reported that urine isolations is usually positive in asymptomatic carriers. Repeat specimens in such instances will obviously eliminate these doubts.

The use of peroxidase labelled conjugate was found to yield the same results as when using alkaline phosphatase as a marker enzyme. The colour produced by the peroxidase substrate can be discriminated visually into positive or negative, and the results were accurate, reproducible and stable. However, the substrate for peroxidase, o-phenylenediamine is reported to be mutagenic (Voogd et al., 1980). Moreover, peroxidase is easy to conjugate as outlined in Chapter 3. The choice of using alkaline phosphatase was taken to avoid the mutagenic activity of most of peroxidase substrates, although peroxidase is being widely used in enzyme-immuno assays with no immediate overt problems.

At present, isolation of S. typhi in body fluids still remains the most accurate method of confirming.

However, it is known that endotoxins are released by S. typhi during infection and by determining their presence in the serum one should get an indication of the presence or absence of an active disease. It may be possible to detect endotoxins by the use of the ELISA technique which would enable one to diagnose S. typhi infection with the same accuracy as with the culture method.

The present work has shown that ELISA can be used in the diagnosis of typhoid fever and that it is quicker than the Widal test which can only yield results after 18 hours and furthermore the results in the Widal test are subjectively interpreted. ELISA is reproducible, accurate and can be automated to a considerable extent by means of diluters, dispensers, plate washing machines and plate readers. This makes ELISA more suitable for the processing of a large number of samples without the need of a highly trained personnel.

Before ELISA can be recommended for general use in the diagnosis of typhoid fever, the following has to be established:

1) The change of antibody concentration during illness using ELISA.
2) Determination of the dominant antibody in cases of acute and chronic typhoid fever and the concentration of antibody in these cases.
3) The concentrations of antibody in subjects who have been vaccinated within a period of 4 - 14 months.

Conclusion

The inherent magnification of an enzyme-substrate reaction allows for the measurement of small quantities of enzyme bound to antibodies by a relatively simple procedure. ELISA has proved to be well suited for detection and quantitation of the antibody response to S. typhi infections. Compared with the Widal test, the results show that ELISA is more sensitive, produces more accurate results, has a higher reproducibility, and the results are obtained within a relatively short time. Another added advantage of ELISA is the quantitation of class specific immunoglobulins, using very small amounts of serum. The instrumentation required is simple, the procedure can be automated and results are read objectively.

The comparison between ELISA and the isolation of S. typhi by culture procedures show a high correlation indicating that ELISA may be used in place of bacteriological isolations which is time consuming. Results using ELISA can be obtained within a very short period as compared to the long period needed for the isolation of S. typhi. Isolations also need preparations of sterile media which add to the complexity of the whole procedure. This is the reason why bacteriological isolations can only be done in bigger centres where there are trained personnel, whereas ELISA can be used by an untrained person.

The results of the comparison between ELISA and the clinically diagnosed typhoid fever patients show a high correlation. Class specific antibodies may indicate whether the patient has a primary infection or a reinfection.

From the work covered here, it is clear that ELISA is easily applicable for use in the diagnosis of typhoid fever. It is also clear, however, that optimal conditions of the assay be derived before applying this method in a quantitative manner for detection of antibodies against infectious diseases.

Future efforts should be directed towards the automation of the ELISA and the application of this assay in other infectious diseases. The availability of this system is slowly improving the abilities of the physicians in the diagnosis and management of patients with infectious diseases.

The fact that all kinds of antigens adhere to plastic walls, makes ELISA a 'darling' in the diagnostic world.

LITERATURE CITED

ASHCROFT, M.T., MORRISON RITCHIE, J. and NICHOLSON, C.C. (1964). Am. J. Hyg., 79, 196.

ASHCROFT, M.T., SINGH, B., NICHOLSON, C.C., RITCHIE, J.M., SORBRYAN, E. and WILLIAMS, F. (1967). Lancent, 2, 1056.

AVRAMEAS, S. and URIEL, J. (1966). C.R. Acad. Sci., Paris, 262, 2543.

AVRAMEAS, S. (1969). Immunochem., 6, 43.

AVRAMEAS, S. and TERNYNCK, T. (1971). Immunochem., 8, 1175.

AVRAMEAS, S., TERNYNCK, T. and GUESDON, J.L. (1978). Scand. J. Immunol., 8, 7.

BAI, K.T., RAYON, A.J. and UNNISA, A. (1980). Indian Journal of Pub. Hy., 24, 38.

BAKER, E.E., WHITESIDE, R.E., BASH, R. and DEROW, M.A. (1959). J. Immunol., 83, 680.

BARBER, C. (1961). Arch. Roum Pathol. Exp. Microbiol., 20, 541.

BIOVIN, A., MESROBEANU, U. and MESROBEANU, K. (1933). C.R. Soc., 113, 490.

BLANDER, R.V., MACKANESS, A.B. and COLLINS, F.M. (1966). J. Exp. Med., 124, 573.

BOORSMA,D. and STREEFKERK, J.G. (1976). J. Histochem. Cytochem., 24, 481.

BUDD, W. (1873). Typhoid Fever, Longmans, London, quoted by Huckstep R.L. In Typhoid infections and other Salmonella infections.

CAMPBELL, F.C., ERIKSSON, B.L. and ANGORN, I.B. (1980). S.A.M.J., 57, 793.

CARLSSON, H.E., LINDBERG, A.A. and HAMMARSTRÖM, S. (1972). Infection and Immunity, 6, 703.

CARLSSON, H.E. and LINDBERG, A.A., (1978). Scand. J. Immunol., 8, suppl. 7,97.

CARR, J.G. (1892). In Principles and Practice of Medicine 16th Ed. (Edited by Osler, P. xiii), Appleton Century Company, London.

CHANDRA, R.K. (1981). Nutrition Review, 39, 225.

CHAU, P.Y., TSANG, R.S.W., LAM, S.K., LA BROOY, J.T. and ROWLEY, D. (1981). Clin. Exp. Immunol., 46, 515.

CHEN, C.H., JOHNSON, A.G., KASAI, N., KEY, B.A., LEVIN, J. and NOWOTNY, A. (1973). J. Infect. Dis. 1285, 43.

CHERNOKHVOSTOVA, E., LUXEMBERG, K.I. STARSHINOVA, V. and et al., (1969). Clin. Exp. Immuno., 4, 407.

CHORDIKER, W.N. and TOMASI, T.B., Jnr. (1963). J. Clin. Invest., 42, 1080.

COLLINS, F.M. and CARTER, P.B. (1974). Amer. J. Clin. Nutr., 27, 1424.

CONRADIE, J.D. and MBHELE, B.E.L. (1980). S.A.M.J. 57, 282.

CRABKE, P.A., CARBONARA, A.O. and HEREMANS, J.F. (1965). Lab. Invest., 14, 235.

DAVIS, D.A.L. (1958). Journal of Gen. Microbiol. 16, 118.

DEBRE, R., LARNY, M., BONNET, H. and BROCA, R. (1935). Bull. Mem. Soc. Med. Hop., 51, 1723.

DE VILLIERS, A.B., DUPREE, R.H., DIEKINSON, C. and BEELER, M.F. (1965). Am. Journal of Clin. Path., 44, 410.

DE PREVAL, C., (1982). In Immunology 2nd Edition (Edited by J. Bach and R.S. Schwartx) Wiley and Sons.

EBERTH, C.J. (1980). Virchows Arch. 81, 58.

EDWARD, P.R., EWING, W.H. (1952). In Identification of Enterobacteriaceae 1st Ed. Minneapolis : Burgers Publ. Co.

ENGVALL, E. and PERLMANN, P. (1971). Immunochem., 8, 871.

ENGVALL, E. and PERLMANN, P. (1972). J. Immunol., 109, 129.

ENGVALL, E., LJUNGSTRÖM, I. (1975). Acta Path. Microbiol. Scand. Sect. e. 83, 231.

ENGVALL, E., RUOSLAHTI, E, (1979). In Immunoassays in the Clinical Laboratory, Alan-Liss, p. 89, New York.

EWING, W.H. (1974).In differentiation of Enterobacteriaceae by biochemical reactions. DHEW. Publ. No, CDC 75, 8098.

FELBER, J.P. (1978). Adv. Clin. chem., 20 129.

FELIX, A., Pitt, M., (1934). Lancet, 2, 186.

FUKAZAWA, Y., SHINODA, T., YOMODA, T. and TSUCHIYE, T. (1970). Infect. Immun., 1, 219.

GAFIN, S.C., NASIMA BADSHA, BROCK-UTNE, J.G., VORSTER, B.J. and CONRADIE, J.D. (1982). Annal. Clin. Bioch., 19, 191.

GAFFKY, G. (1896). Mitt Gesund AMT. Berl. 2 quoted by Wilson and Miles in Principles of Bacteriology and Immunity.

GANNON, P.J., SURGALLA, M.J., FITZPATRICK, J.E. and NETER, E. (1980). J. Clin. Microbiol., 12, 60.

GILMAN, R.H., HORNICK, R.B., WOODWARD, W.E., DUPONT, H.L. and et al. (1977). J. Infect. Dis., 136, 717.

GIRARD, J.P. and DE KALBERMATTEN, A. (1970). Eur. J. Clin. Invest., 1, 188.

GRIPENBERG, M., LINDER, E., KURKI, P., ENGVALL, E. (1978). Scand. J. Immunol., 7, 151.

GUENTZEL, M.N. and BERRY, L.J. (1975). Infect. Immun., 11, 890.

GUPTA, A.K., RAO, K.M. (1979). Immun. methods, 30, 349.

HAMILTON, R.G., HUSSAIN, R., OTTESEN, E.A. and ADKINSON, N.F. Jnr. (1981). J. Immunol. Methods, 44, 101.

HEATH, E.C., MAYER, R.M., EDSTROM, R.D. and BEAUDREAU, C.A. (1966). Ann. N.Y. Acad. Sci., 133, 315.

HELLERQVIST, C.G., LINDBERG, B., SVENSON, S. and et al. (1968), Carbohydrate Research, 8, 43.

HELLERQVIST, C.G., LINDBERG, B., SVENSON, S. and et al. (1969). Acta Chem. Scand., 23, 1588.

HEREMANS, T.F., VAERMAN, J.P. (1971). Proc. 1st. Int. Congr. Immunol., 875.

HERNER, E.E. and CLAYTON-HOPKINS, J.A. (1978). Clin. Chem., 24, 1275.

HOLMGREN, J., SVENNERHOLM, A.M. (1978). Scand. J. Immunol. 8, Supp. 7, 111.

HOLMQUIST, L. (1980). J. Immunol. Methods, 34, 243.

HOOK, E.W. (1974). Salmonella Infections in Harrison's Principles of Internal Medicine 7th Ed. P. 803, McGraw Hill, Tokyo.

HORNICK, R.B. and WOODWARD, T.E. (1966). Trans. Amer. Clin. Climat. Ass.,

HORNICK, R.B., GREISMAN, S.E., WOODWARD, T.E., et al. (1970). N. Engl. J. Med. 283, 686.

HUCKSTEP, R.L. (1962). Typhoid fever and other Salmonella infections. E. and S. Livingstone Ltd., London.

JAWETZ, E., MELNICK, J.L., ADELBERG, E.A. (1980). In Review of Medical Microbiology, Lange Medical Publ., California.

JENNER, E. (1850). On the identity and non-identity of typhoid and typhus fever. John Churchill, London.

KAUFFMAN, F. (1966). In the Bacteriology of Enterobacteriaceae p. 400. Munksguard.

KENDALL, F.E. (1937). J. Clin. Invest., 16, 921.

KOFFLER, H. and SMITH, R.W. (1971). In Cellular Antigens (Edited by Nowotny, A.) Heidelberg, New York.

KOMINAMI, G., FUJISAKA, I., YAMAUCHI, A. and KONO, M. (1980). Clin. Chim. Acta, 103, 381.

LANDY, M., JOHNSON, A., WEBSTER, M. and SAGIN, J. (1955). J. Immunol. 74, 755.

LANGE, S., ELWING, H., LARSSON, P. and NYGREN, H. (1980). J. Clin. Microbiol., 12, 637.

LEVINE, M.M., DUPONT, H.L., HORNICK, R.B. and et al. (1976). J. Infect. Dis., 133, 429.

LEVINE, M.M., GRADOS, O., GILMAN, R.H. and et al. (1978). Am. Soc. Trop. Med. Hyg., 27, 795.

LILIENFIELD, D.E. and LILIENFIELD, A.M. (1977). Am. Jnrl. Epidemiology, 106, 445.

LING, C.C., TAUR, S.S., HSEUH, P.C. and YANG, S.Y. (1940). Chinese Medical Journal, 57, 11.

LOSPALLUTO, J., MILLER, W., Jr., DORWARD, B. and FINK, C.W. (1962). J. Clin. Invest., 41, 1415.

LUDERITZ, O., STAUB, A.M. and WESTPHAL, I. (1966). Bacteriological Rev., 30, 192.

LUDERITZ, O., JANN, K. and WHEAT, R. (1968). Comp. Biochem., 26A, 105.

LUNDE, M.M., OTTSEN, E.A. and CH_EVER, A.W. (1979). Am. J. Trop. Med. Hyg. 28, 87.

MACKANESS, G.B. (1971). Cellular Immunity, Ann. Inst. Pasteur, Paris, 120, 428.

MEANS, G.E. and FEENEY, R.E. (1971). Chemical modifications of proteins. San Fransisco, Holden Day Inc.

MILLER, C.P., BORNHOFF, M. and RIFKIN, D. (1956). Trans. Amer. Climat. Ass., 68, 51

MORELLO, J.A. and BAKER, E.E. (1965). J. Invest. D., 115, 131.

MORGAN, W.T.J. and PATRIDGE, S.M. (1941). Biochem. J.,35, 114.

MORRIS, R.E., THOMAS, P.T. and HONG, R., (1982). Human Immunol., 5, 1.

NAKANE, P.J. and PIERCE, G.B. (1966). J. Histochem. Cytochem.,114, 929.

NAKANE, P.K. and KAWAOI, A. (1974). J. Histochem. Cytochem., 22, 2084.

NAKANE, O. (1979). In Immunoassays in the Clinical Laboratory (Edited by Alan A.R. Liss) New York.

NELSON, B.W. and ROANTREE, R.J. (1967). J. Gen. microbiol., 48, 179.

NETTER, F.H. (1975). Ciba Collection Vol. I part III Ciba Rochester, New York.

NOWOTNY, A. (1966). Nature London, 210, 278.

NOWOTNY, A. (1979). In Basic Exercises in Immunology, 2nd Ed. p. 69, Springer-Verlag, Berlin.

OSBORN, M.J. (1963). Proc. Natl. Acad. Sci., US 50, 499.

ORSKOV, F. and ORSKOV, I. (1962). Acta Path. Microbiol. Scand.,55, 99.

OSLER, W. (1912). The principles and practice of medicine. Appleton-Century, p. 147, New York.

PAL. S.B. (1978). Enzyme Labelled immunoassay of hormone and drugs. Walter de Gruyter, Berlin.

PALMER, J.W. and GERLOUGH, T.D. (1940). Science, 92, 155.

PAPIERNIAK, C.K., KLEGERMAN, M.E., BOYER, K.M. and et al.(1982). J. Lab. Clin. Med., 100, 385.

PFEIFFER, A. (1885). Deutsch Med. Wschrll. quoted Hornick et al. in N. Engl. J. Med., 283, 686.

PLUMMER, D.T. (1978). An Introduction to Practical Biochemistry 2nd Ed. McGraw-Hill, U.K.

POLISH TYPHOID COMMITTEE, (1966). Bull. WHO., 34, 211.

RICHARDS, F. and KNOWLES, J. (1968). J. Molecular. Biol.,37, 231.

ROBERTSON, R.P. WAHDAN, M.F. and RAASCH, R.O. (1968). New Engl. J. Med., 278, 171.

ROTHFIELD, K. and HORECKER, B.L. (1964). Proc. Natl. Acad., Sci., U.S. 52, 939.

ROTHFIELD, L. and TAKESHITA, N. (1965). Biochem. Biophys., Res. Commun., 20. 521.

ROWLAND, H.A.K. (1961). J. Trop. Med. Hyg., 64, 143.

ROWLEY, D. and WARDLAW, A.C. (1958). J. Gen. Microbiol., 18,529.

RUBENSTEIN, K.E. (1978). Scand. J. Immunol. 8 Suppl. 7, 57.

RUITENBURG, E.J. and BROSI, B.J. and STEERENBERG, P.A. (1976). J. Clin. Microbiol., 3, 541.

RUITENBURG, E.J. and BROSI, B.J. (1978). Scand. J. Immunol. 8, Suppl. 7, 63.

SCHROEDER, S.A. (1968). J. Amer. Med. Ass., 206, 839.

SENEWIRATNE , B. and SENEWIRATNE, J. (1977). Gastroenterology, 73, 233.

SKELLEY, D.S., BROWN, L.P. and BESCH, P.K. (1973). Clin. Chem. 19, 146.

SMITH, J. (1973). Medicine, 6, 458.

SOMERVILLE, P.C., LEWIS, T.M., KOORNHOF, H.J. et al., (1981). S.A.M.J., 57, 851.

SONNENWIRTH, A.C., JARETT, J. (1980). Gradwohl's clinical laboratory methods & diagnosis Vol. 2. 8th Ed. p. 1731, Mosby, U.S.

SPRINZ, H., GAGAROSA, E.J., WILLIAMS, M. and et al., (1966). Amer. J. Dig. Dis., 11, 615.

STRAUSS, C.A. (1974). Proceeding of the Mine Medical Officers Association of S.A. 54, 1.

STUART, A.E. and COLLEE, J.G. (1967). J. Path. & Bact., 94, 429.

TENEBAUM, M.J., ARCHER, G.L. (1989). S.A.M.J., 73, 140.

THOMAS, L. (1956). J. Exp. Med., 104, 865.

TROUSSEAU, A., (1826). Arch., gen. med. quoted by Huckstep in Typhoid Fever

and other Salmonella infections.

TSANG, R.S.W. and CHAU, P.Y. (1981). B.M.J., 282, 1505.

TSANG, R.S.W., CHAU, P.Y., LAM, S.K., LA BROOY, J.T. and ROWLEY, D. (1981). Clin. exp. Immun., 46, 508.

UHR, J.W. and FINKELSTEIN, M.S. (1967). Prog. Allergy, 10, 37.

VAN WEENEN, B.K., and SCHUURS, A.H.W. (1971). FEBS letters, 15, 232.

VELDKAMP, J.and VISSER, A.M. (1975). British J. Vernereal. Dis. 51, 227.

VILJANEN, M.K., RUUSKANEN, O., GRANDBERG, C., SALMI, T.T. (1982). Scand. J. Infect. Dis., 14, 117.

VOLLER, A., BIDWELL, D.E. and BARTLETT, A. (1976). 1st Int. Symp. on Immuno-enzymatic Techniques (Editors: Feldman, et al., P. 167),Amsterdam.

VOOGD, C.E., VAN DER STEL., J.J. and JACOBS, J.J.J.A.A. (1980). J. Immunol. Methods, 36, 55.

WAGNER, H.M., Jnr. LIO, M. and HORNICK, R.B. (1963). J. Clin. Invest., 42, 427.

WAHDAN, M.J., SIPPEL, J.E., MIKHAIL, I.A. and et al. (1975). Bull. WHO., 52, 69.

WALKER, W.A. (1976). Pediatrics, 57, 901.

WALKER, W.A. and ISSELBACHER, J.J. (1977). N. Engl. J. Med., 297, 767.

WARREN, J.W. and HORNICK, R.B. (1979). Amer. Rev. Med., 30, 457.

WEIBULL, C. (1950). Acta Chem. Scand., 4, 342.

WIDANZ, W.P., JACKSON, A.L. and LANDY, M. (1964). Proc. Soc. Exp.Biol. Med., 116, 832.

WEIL, A.J. and SAPHRA, I. (1953). In Salmonellae and Shigella : Laboratory diagnosis correlated with clinical manifestations and epidemiology. Springfield, Illinois.

WESTPHAL, O., LUDERITZ, O. and BISTER, F. (1952). Z. Natuurforsch. 7B, 148.

WESTPHAL, O. and LUDERITZ, O., (1954). Angew. Chem. 66,407.

WICKEN, A.J. and KNOX, K.W. (1980). Biochem. Biophys. Acta, 604. 1.

WICKS, A.C.B., HOLMES, G.S. and DAVIDSON, K. (1971). Quart. J. Med., 40, 341.

WICKS, A.C.B., CRUICKSHANK, J.G. and MUSEWE, N. (1974). S.A.M.J., 48, 1368.

WILLIAMS, R.C. and GIBBONS, R.J. (1972). Science, 177, 697.

WILLIS, T. (1864). Practice of Physich. 234. London quoted by Wilson and Miles.

WILSON, G.S. and MILES, A.A. (1964). Topley's and Wilson's Principles of Bacteriology and Immunity, 5th Ed. p. 2005. (Edited by E. Arnold) London.

WOODWARD, T.E. (1970). S.A.M.J., 44, 99.

YOLKEN, R.H., WYATT, R.G., BARBOUR, B.A. and et al. (1978). J. Clin. Microbiol. 8, 283.

YOLKEN, R.H. and STOPA, P.J. (1980). J. Clin. Microbiol., 11, 546.

YOLKEN, R.H. (1982). Review of Infect. Dis., 41, 35.

YOGOSLAV TYPHOID COMMISSION. (1962). Bull. WHO, 26, 357.

YUGOSLAV TYPHOID COMMISSION. (1964). Bull. WHO, 30, 623.