Biological Studies on *Shigella dysenteriae* Toxins

by

NEELAM KUMARI
(L-79-85-132-M)

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To

My

Parents

CERTIFICATE I

This is to certify that this thesis entitled "Biological Studies on *Shigella dysenteriae* Toxins" submitted for the degree of Master of Science in the subject of Microbiology of the Punjab Agricultural University, is a bonafide research work carried out by Miss Neelam Kumari under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

Ajit Singh

(Dr Ajit Singh)
Major Advisor

CERTIFICATE II

This is to certify that the thesis entitled "Biological Studies on Shigella dysenteriae Toxins" submitted by Miss Neelam Kumari (L-79-BS-132-M) to the Punjab Agricultural University for the degree of Master of Science in the subject of Microbiology has been approved by the Student's Advisory Committee after an oral examination on the same, in collaboration with an External Examiner.

Ajit Singh
(Dr Ajit Singh)
Major Advisor

(Dr M.S.Kalra)
Head of the Department

11.2.87
(Dr A.S.Atwal)
Dean of Post-graduate Studies

R N Sinha 14/12/87
( )
External Examiner
(Dr. R.N. SINHA)
Division of Microbiology
N.D.R.I. Karnal

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Chapter I

INTRODUCTION

The problem of diarrhoeal disease is an enormous one on a global scale and is characteristically more severe, in terms of morbidity, mortality and economic impact, in the more underprivileged areas of the world. People in the lower economic strata are more affected by it.

In contrast to the "toxigenic diarrhoea", caused by Vibrio cholerae and Escherichia coli, the occurrence of watery diarrhoea with blood, inflammatory cells, mucus and ulcerative lesions of the colonic mucosa represent a cardinal feature of shigellosis. Shigella dysenteriae type-1 (Shiga's bacillus), is known to produce the most severe form of dysentery (Levine et al., 1973).

Shigellosis is said to be produced by the organism following penetration and multiplication of the bacteria in the gastrointestinal mucosa, which results in fever and alteration in intestinal motor functions. Shigellae and Salmonellae have been seen within the intestinal epithelial cells of experimentally infected animals by using electron microscopic, fluorescent antibody and routine histological techniques (La Brec et al., 1964; Takeuchi et al., 1965; Giannella et al., 1973). With electron microscopic techniques, a general picture of the

morphological events of penetration has been developed. After penetration and multiplication, a complex series of events occur which result in inflammation, ulceration, cramps, tenesmus, fever and fluid loss. These pathophysiological changes are due to the production of enterotoxin (Keusch *et al.*, 1970), neurotoxin (Engley, 1952) and circulating endotoxin (Koster *et al.*, 1978). The enterotoxin possesses the property of cytotoxicity, neurotoxicity and enterotoxicity (Keusch and Jacewicz, 1975). Although the precise role that *Shigella* endotoxin plays in the overall pathogenesis of dysentery has not been fully elucidated, but mucosal invasion and subsequent multiplication permit the expression of some endotoxin activity. In the penetration step of infection, the chemical composition of lipopolysaccharide (LPS) is of great significance (Gemski and Formal, 1975). The production of enterotoxin has been shown to be controlled by a plasmid transferable through conjugation, in *E.coli* (Gemski *et al.*, 1978) and in *Streptococcus faecalis* (Gurmeet, 1980).

Endotoxins are produced by gram-negative bacteria and are bound to the cell wall where they exist as high molecular weight complexes composed of polysaccharide, lipid and small quantities of protein. They remain attached to the cell surface and are released into the surrounding medium when the bacteria are lysed or disintegrate.

Lipopolysaccharides are known to be potent toxins. Their injections into higher organisms lead to fever, leukopenia, leukocytosis, local and generalized Shwartzman reactions (Sukhjit, 1980), non-specific resistance to infection, tumour necrosis, adjuvanticity and mitogenecity and in large doses shock and death. Endotoxin was found to have a marked effect on the intestinal water transport, serum constituents and serum enzymes.

Chemically, lipopolysaccharides consist of a hetero-polysaccharide (O-specific chain, core) and a covalently bound lipoidal component (lipid A). The O-specific chain determines the serological specificity, whereas the lipid A component represents the toxic principle of the molecule.

Although a lot of research has been carried out with Shigella dysenteriae lately, yet the exact biological role and nature of the endotoxin of this organism has not yet been explained.

The present investigation involves the following objectives :

1. Production of toxin by strain of Shigella dysenteriae and comparison of different media for the production of enterotoxin.

2. Effect of various chemical agents on the release of toxin from Shigella dysenteriae organism.

Chapter II

REVIEW OF LITERATURE

Shigellosis is a past plague and a modern menace. This epidemic is associated with considerable morbidity and mortality. It is an acute enteric infection caused by the four bacterial species, Shigella dysenteriae, Shigella flexneri, Shigella boydii and Shigella sonnei. Clinically, the disease is characterized by abdominal cramps, diarrhoea, fever and stools containing blood and mucus.

Gastroenteritis or dysentery or diarrhoea is among the oldest clinical problems in the world's human population. Even in ancient palestinian and biblical writings, reference to this are found. A disease 'Sangraeni' which was characterized by propulsive diarrhoea was described by Sushruta (5th century, B.C.) in India.

Diarrhoea was also attributed to teething, exposure to cold and consumption of spoiled milk. A report of diarrhoea in prisoners of IInd World War in India is also there. The characteristic passage of light coloured stools of high fat content, flatulent-dyspepsia, sore tongue and emaciation was also reported.

Toxigenic *Shigella dysenteriae*

Shiga's bacillus, *Shigella dysenteriae* or (Shigae) (1898), occurs less frequently in outbreaks of bacillary dysentery than does Flexner's bacillus (*Shigella paradysenteriae*) but the disease that it gives rise to is much more severe, as well as the abdominal pain, diarrhoea and tenesmus common to all forms of bacillary dysenteriae, there is often a "profound toxemia" ending in collapse (Wilson and Miles, 1964).

An outbreak of shigellosis in Chandigarh of which 63.5 per cent isolates were of *Sh.dysenteriae* type-1, among a total of 955 stool samples has been reported by Arya *et al.* (1977). Mata *et al.* (1970) and Gangarosa *et al.* (1970) reported a strain of *Sh.dysenteriae* type-1 responsible for a severe pandemic with high morbidity and mortality rates in five countries of Central America and this was having multiple drug resistance.

Sh.dysenteriae type-1 was one of the most important causes of diarrhoeal illness in and around Bangladesh (Rahaman *et al.*, 1974). Rahaman *et al.* (1975) reported another case of same infection in the Coral Islands which displayed a multiple drug resistance.

Khan *et al.* (1979) reported various serotypes of *Shigella* from an outbreak of *Shigella gastroenteritis* at Lucknow and *Sh.flexneri* was the most common isolate (84.9%)

followed by Sh.dysenteriae type-1 (8.0%); Sh.boydii (5.2%) and Sh.sonnei (1.9%). Naik et al. (1978) also reported an outbreak of dysentery in a village around Sawantwadi. From 262 acute dysentery cases, 52 strains of Sh.dysenteriae type-1, 4 strains of Sh.flexneri type 3C and a strain of Sh.sonnei were isolated. Most of these strains were resistant to common antibiotics but sensitive to ampicillin and furoxone.

Enterotoxin

Toxins are classified broadly as :

i) Extracellular toxins or exotoxins which diffuse readily from the living bacteria into the surrounding medium.

ii) Intracellular toxins or endotoxins which are retained within the bacterial until they die and disintegrate.

Exotoxins are usually proteins which can be readily inactivated by heating at 60-80°C. The exotoxins are secreted by bacteria and can easily be converted to toxoids and be neutralized by antitoxins. In contrast, endotoxins are lipo-polysaccharides which are relatively heat resistant and do not form toxoids readily. They are also very difficult to neutralize with antiendotoxin antibodies. Exotoxins generally exert a specific and restricted biological function affecting a particular cell type or tissue, whereas endotoxin produces

a vast array of biological effects manifested by their action on different cell types and tissues.

Sh.dysenteriae had been considered to differ from other members of dysentery group by elaborating an exotoxin which had neurotoxic, enterotoxic and cytotoxic properties (Keusch and Jacewicz, 1975).

Boivin and Mesrobeanu (1937) first succeeded in separating the two toxins by chemical methods. They showed that the neurotoxin was protein in nature and precipitable by trichloroacetic acid, while the enterotoxin was soluble in trichloroacetic acid.

Heat-stable and Heat-labile Enterotoxins

The definition of LT and ST is still arbitrary, generally enterotoxin that can withstand 100°C for 15 minutes is considered heat-stable (ST), whereas LT preparation is destroyed at 60°C for 30 minutes.

Van Heyningen and Gladstone (1953) and Vicari et al. (1960) reported that the toxin was a thermolabile neurotoxin until Keusch et al. (1970) reported that the toxin was enterotoxin and was destroyed upon heating at 60°C. Ketyi et al. (1978) reported a heat-stable enterotoxin from Sh.flexneri by the mouse suckling test and permeability test. Ketyi et al. (1978) reported that Sh.dysenteriae type-1 failed to give these tests and, therefore, served as negative controls. The

heat-labile tests like mouse pad odema, delayed permeability factor reaction and Chinese Hamster ovary cell elongation were all negative in case of Sh. flexneri confirming the production of a heat-stable enterotoxin.

Cultural Conditions for the Production of Toxin

Medium :

The choice of the medium is an important consideration in the production of enterotoxin. The methods for the production of enterotoxin have changed markedly since Dack et al. (1930) first produced enterotoxin in veal infusion broth. Meat infusion broths such as veal (Woolpert and Dack, 1933), beef, heart (Burnet, 1930) and brain heart (Casman and Bennet, 1963; Mcleon et al., 1968) were used for the production of enterotoxin.

Dubos and Geiger (1946) showed that the presence of iron above a small optimal value (about 0.15 ug Fe/ml) inhibited toxin production, For toxin production on a large scale, Van Heyningen and Gladstone (1953) used the casein hydrolysate (CCY) medium in which the iron content had been reduced by adsorption on calcium phosphate to a level of 0.05 ug/ml.

Evans et al. (1972) proposed liquid broth containing two per cent casamino acids for the production of enterotoxin by E. coli. Sahney (1979) employed the medium given by Evans et al. (1972) for the production of enterotoxin from Shigella dysenteriae.

pH :

pH of the medium is also important although enterotoxin has been produced over a wide pH range. The suitable pH for enterotoxin production was an alkaline pH (Keusch et al., 1972). At pH 8.0 the toxin produced maximum fluid accumulation in rabbit ileal loops showing the maximum yield at this pH. Sahney (1979) employed pH 8.5 for the production of enterotoxin from Shigella dysenteriae.

Temperature :

Enterotoxins being protein in nature, get denatured at high temperature. Keusch et al. (1972); McIver et al.(1975); Ketyi et al, (1978) observed that the best temperature for toxin production was 37°C. At 60°C, partial inhibition in toxin production was achieved, while at a temperature of 90°C there was 85 to 100 per cent inhibition in toxin production. Enterotoxin was produced at 37°C from E.coli (Solomon, 1978), from Shigella dysenteriae (Sahney, 1979) and from Streptococcus faecalis (Gurmeet, 1980).

Aeration :

McCartney and Olitsky (1923) showed that aeration of cultures of Sh.dysenteriae was necessary for the production of the "exotoxin". Keusch et al. (1970) used shaked peptone broth

for enhancing toxin production. McIver et al. (1975) reported that stirring at a rate of 250 rpm yielded substantial amount of toxin. Van Heyningen and Gladstone (1953) and Ketyi et al. (1978 .) reported that aeration of cultures resulted into good yields of toxin.

Properties of Toxin

Shigella dysenteriae exotoxin is a protein with properties as shown in Table :

Properties of Shigella dysenteriae exotoxin*

LD_{50}/mg (mouse)	40,000
LD_{50}/mg (rabbit)	4,00,000
Lf/mg	5,500
L+/mg (5 unit level)	4,200
N content	15.7%
Electrophoretic mobility - u_{30} (barbital buffer pH 8.8 u = 0.1)	2.78×10^{-5} cm^2/second/V
Weight average sedimentation co-efficient, $Sm_{20,W}$	4.80S ± 1%
Diffusion co-efficient $D_{20,W}$	5.7×10^{-7} cm^2/second ± 5%
Frictional co-efficient, f/f_o	1.26
Average molecular weight	82,000

*From Van Heyningen and Gladstone (1953) and Baldwin (1953)

Van Heyningen and Gladstone (1953) showed that the toxin is insoluble in distilled water and soluble in aqueous salt solution and that the solubility of the toxin in salt solution has a high temperature coefficient. They also reported that when a 1 per cent solution of toxin in 1 per cent NaCl was cooled to -5°C, a precipitate formed and redissolved on warming.

Keusch et al. (1972) showed that the toxin was active at an alkaline pH, heat-labile and sensitive to proteolytic enzymes. Maximum activity of the toxin was at pH 8.0, however, the activity was considerably diminished at pH 5.0 or 6.0 (Sahney, 1979). Similar observations were reported by Solomon (1978) for E.coli enterotoxin. Temperature had pronounced effect on the activity of Sh.dysenteriae enterotoxin and the maximum stability was at 37°C (Sahney, 1979), however, a complete loss of activity occurred at 100°C.

Finkelstein et al. (1966); Duncan and Strong (1969) Kubota and Liu (1971) and Lariviere et al. (1973) reported that the enterotoxin of V.cholerae, Cl.perferingens, Pseudomonas aeruginosa and E.coli get inactivated by the proteolytic enzymes. Proteolytic enzymes papain, trypsin reduced the activity of the Sh.dysenteriae toxin, whereas amylolytic enzymes rhozyme, α-amylase, β-amylase did not alter its activity (Sahney, 1979) The author also reported that drugs like aspirin, indomethacin, furoxone and septran inhibited the enterotoxin induced intestinal secretions.

Keusch et al. (1972) reported that Sh.dysenteriae do possess enterotoxic activity, and this is associated with neurotoxic and cytotoxic activities of this organism.

Enterotoxic Properties

For the organism to show enterotoxic properties, it must produce fluid accumulation in the intestine. Sh. dysenteriae, an invasive organism, penetrated the intestinal mucosa (Labrec et al., 1964; Gemski et al., 1972 ; Formal et al., 1976) and thus resulted in the abnormal fluid accumulation which was not reabsorbed by the colon leading to diarrhoea. Gemski et al. (1972) reported that invasiveness is an essential step before toxin production.

Levine et al. (1973) showed that Shigella had two potential pathogenic modes of action : invasiveness and enterotoxin which together resulted in the intestinal changes of both water and electrolyte. Gemski et al. (1972) reported that Sh.flexneri hybrids with O-related sub-units of E.coli retained virulence suggesting that the chemical composition and structure of the O-side chain of somatic antigen represented one determining factor for bacterial penetration of mucosal epithelial cells. Keusch and Jacewicz (1977) suggested that mammalian cells possess a toxin receptor and oligomeric 1-4 linked N-acetyl D-glucosamine was involved in it.

Shigella penetrate the intestinal mucosa unlike Cholera and E.coli and it does not stimulate the production of adenyl cyclase, a property common to both Cholera and E.coli enterotoxins (Flores et al., 1974).

Steinberg et al. (1972) compared the effects of Shigella dysenteriae toxin and cholera toxin and observed the response of jejunum to it. Shigella toxin secreted fluid similar in composition to cholera toxin and the rates of secretion were also similar in the period of linear response but the Shigella toxin secretion diminished after 5 hours and was completely ceased to excrete by 2.5 hours but the loops having cholera toxin continued to secrete. Rout et al. (1975) reported that the most consistent response occurred in the colon and the response of small intestine was more variable.

Donowitz (1975) reported that glucose stimulation of Na transport is partially inhibited by Sh.dysenteriae enterotoxin. Further, Na transport is diminished across Shigella enterotoxin-treated ileal mucosa. Binder and Whiting (1977) found that the enterotoxin of Sh.dysenteriae inhibited the amino acid and sugar transport in the small intestine. Powell et al. (1971) reported that intraluminal glucose in Salmonella enterocolitis stimulates the net lumen to blood transport of sodium and water and thus increase their absorption both in vivo and vitro.

In different enterotoxin producing bacteria, it has been reported that the enterotoxin production is genetically

controlled either by plasmid or chromosome. Smith and Halls (1968) and Smith and Gyles (1970) reported that enterotoxin production in E.coli is controlled by a transferable plasmid designated as ENT^{+}. Isolation and characterization of enterotoxin deficient mutants of E.coli was done by Silva et al. (1978). They reported that genes controlling the production of two types of enterotoxin, heat-labile (LT) and heat-stabile (ST) are present in plasmids.

Cytotoxicity

Vicari et al. (1960) showed that a preparation of the toxin, purified by the method of Van Heyningen and Gladstone (1953), exerted specific cytopathogenic effects on cells in tissue cultures. KB cells, normal human liver cells and monkey kidney cells showed similar degenerative processes. Mesrobeanu et al. (1962) confirmed that the exotoxin of Sh.dysenteriae had a cytotoxic action on tissue cultures of human embryonic and KB cells. Sh.dysenteriae type-1 enterotoxin was cytotoxic to Hela cells monolayers and resulted in rapid detachment of cells from glass surface (Keusch et al., 1972). Keusch (1973) assayed the enterotoxin of Sh.dysenteriae to Hela cells and observed that it contained significant biological activity in this system. It was most active on Hela cells, intermediate to rhesus monkey kidney monolayers and unaffected on WI-38 cells suggesting monolayers to serve as a model system to study

the properties and mode of action of Shigella toxin. Takeda et al. (1977) studied the effect of culture filtrates of Sh. dysenteriae on Chinese hamster ovary (CHO) cells and found that the toxin induced morphological changes in these cells.

Neurotoxicity

The exotoxin of Sh.dysenteriae has been called a neurotoxin ever since its discovery because it produced unmistakable neurological symptoms in the rabbit, the animal that was most susceptible to it. A rabbit that received i/v injections of one or two fatal doses of toxin showed no overt signs of injury for the first two days. Muscular weakness then first appears in the forelimbs and causes them to splay out. During the next 24-48 hours, the paralysis increases and extends to the hind limbs and the abdominal muscles. By the fourth or fifth day, the animals are entirely paralysed and prostrated with complete loss of muscle tone; often there is a marked retraction of the head. These neurological symptoms are accompanied by changes in the central nervous system.

Bridgwater et al. (1955) used a highly purified sample of Sh.dysenteriae toxin prepared by Van Heyningen and Gladstone (1953) for a study of the effect of the toxin in the rabbit. They found in rabbits that died after receiving 2 LD_{50} i/v, that there were macroscopic changes in the spinal cord,

especially in the cervical enlargement, but some times also in the lumbar enlargement. The cord was swollen and on section, extensive areas of softening with numerous petechial hemorrhages in the grey matter were found but the white matter was found to be unaffected.

Cavanagh et al, (1956) pointed out that the neurological effects of Sh.dysenteriae exotoxin in the rabbit tended to obscure intestinal effects which were frequent, though less constant. They also studied the effect of the highly purified toxin of Van Heyningen and Gladstone (1953) on a number of animal species. In mice, there was flaccid paralysis and some times convulsions accompanied by distinct focal changes in the histology of the brain and the spinal cord scattered at random through the neuraxis. In the hamster, which was more susceptible than the mouse, there was no paralysis and convulsions and no diarrhoea. There were no lesions in the central nervous system. The guinea pig also showed no histological abnormality in the central nervous system or the viscera.

Endotoxin

Physical state of lipopolysaccharide

The physical state of LPS plays an important role in its endotoxic properties.

The cell wall LPS of gram-negative bacteria are acidic amphipathic macromolecules. They contain a heteropoly-

saccharide, the O-specific chain, which is linked to a central heteropolysaccharide, the core, which in turn is linked to an acylated oligosaccharide of lipidic character, termed lipid A. When suspended in aqueous solutions, LPS tend to form aggregates probably due to hydrophobic forces present in the lipid A region.

Shands et al. (1967) studied the morphology of LPS isolated from smooth and rough strains of Salmonella typhimurium and found that they possess a bilayer structure very much like natural membranes, each half of which is composed of polysaccharide moieties covalently linked with lipid A. In aqueous solution, the two layers are held together by hydrophobic interactions to produce a bilayer, the hydrophobic lipid moieties are buried in the centre while the hydrophilic polysaccharide regions are exposed to the surrounding water.

Rothfield and Pearlmann (1966) examined LPS of S. typhimurium under the electron microscope using negative staining technique and observed hollow spheroid structures and as endotoxin are amphipathic molecules, these authors proposed that the spheroid consisted of a leaflet of LPS arranged either in bimolecular or monomolecular fashion with the lipid regions inside and the polar polysaccharide moieties exposed to both the inner and the outer surface of the leaflet.

Chemical Structure of Endotoxin

Lipid A :

The chemical structure of *Salmonella* lipid A has been studied extensively in recent years by Gmeiner *et al.* (1969, 1971), Rietschel *et al.* (1972). Lipid A contains glucosamine, phosphate and long chain fatty acids (Rietschel *et al.*, 1972; Hase and Rietschel, 1976). Rietschel *et al.* (1972) showed that it was made up of β-1, 6-linked D-glucosamine disaccharide units, which were 1-4' interlinked by pyrophosphate bridges. Lauric, myristic, palmitic and 3-D-hydroxymyristic acid represent the long-chain fatty acid constituents in lipid A of *Salmonella*.

Westphal *et al.* (1952), Westphal and Luderitz (1954) claimed that the lipid component was responsible for endotoxic activities of LPS. Ribi *et al.* (1961) isolated preparations which were poor in lipid A but still possessed potent endotoxic activities suggesting endotoxic properties did not reside within a lipid A region. Kim and Watson (1967), however, has not attributed the endotoxicity to the polysaccharide part only.

O-specific chain :

The cells of a bacterial species produce a family of similar, but not identical, lipopolysaccharide molecule. Kauffman *et al.* (1961) noted that *Salmonella* species

contained five basal sugars : 2-keto-3-deoxyoctonate (KDO), heptose, glucose, galactose, and N-acetylglucosamine. The same five sugars were present in Shigella, but their arrangement was different (Luderitz et al., 1968).

Simmons (1969) studied in detail the structure of the O-specific side chain of various serotypes of Shigella flexneri. These chains consisted of repeat units which contained a primary unbranched chain of N-acetyl-glucosamine and rhamnose to which secondary glucosyl side chain was added. Dmitriev et al. (1977) released the specific polysaccharide from Sh. dysenteriae type-5 LPS by mild acidic hydrolysis and then purified it by gel chromatography on Sephadex G-50 and found that polysaccharide was built up of residues of D-mannose, 2-acetamido-2-deoxy-D-glucose,3-O-(D-1-carboxyethyl)-L-rhamnose (rhamnolactylic acid) and O-acetyl groups in the ratio of 2 : 1 : 1 : 1.

New sugar derivatives other than the recognised carbohydrates have been identified. These include neutral sugars (fructose, tetrose) and additional glycerol in Vibrio cholerae (Jann et al., 1973), amino sugars (4 amino arabinose) in Salmonella and E.coli (Volk et al., 1970; Rooney and Goldfine, 1972) and uronic acids (glucouronic acid) in Shigella boydii. Other substituents of sugars are the O-acetyl group, the phosphate group, lactic acid linked as an ether (as in muramic acid) to glucose in Shigella dysenteriae (Dmitriev et al.,1976).

Shigella dysenteriae LPS has been classified into chemotypes on the basis of sugar constituents (Dmitriev et al., 1973). Such classification has been done on Shigella flexneri by Lindberg et al. (1973) and on Shigella boydii by Dmitriev et al. (1973).

The LPS of S.minnesota R595 contains only lipid A and 2-keto-3-deoxyoctonate (KDO) and is devoid of O-polysaccharide but is biologically as active as the parent LPS from the wild type (smooth) (Luderitz et al., 1966; Kim and Watson, 1967; Kasai and Nowotny, 1967), suggesting that polysaccharide part is not essential for endotoxicity. Chemical modification of glycolipid by succinylation which results in the esterification of free hydroxyl group of KDO did not diminish the toxicity of glycolipid suggesting that KDO residues did not contribute to endotoxicity (Rietschel et al., 1972). The removal of KDO from glycolipid by acid hydrolysis and subsequent complexing with bovine serum albumen (BSA) led to the formation of lipid A - BSA complexes which were also toxic (Galanos et al., 1972).

These findings suggest that the polysaccharide moiety of LPS, although playing an important role in immune reactions during infections, do not play any major role in endotoxicity. Kabir et al. (1978) suggested that they might increase the affinity of LPS towards water being hydrophillic in character, thus increasing the endotoxic potency of lipid A.

Isolation and Purification

Present information suggests that the LPS is bound in the cell wall primarily by physical forces, i.e. hydrophobic, ionic, or both (Wheat, 1964). Several methods have been used for the isolation of LPS from gram-negative bacteria. Moderate to good yields may be obtained by extracting cells with hypertonic sodium chloride and sodium citrate (Raynaud and Digeon, 1960; Digeon et al., 1952). These methods also involve the extraction of the bacteria with organic reagents : (i) trichloroacetic acid (Boivin and Mesrobeanu, 1935), (ii) diethylene glycol (Morgon and Partridge, 1942), (iii) aqueous pyridine (Goebel et al., 1945), (iv) dimethyl sulfoxide (Adams, 1967), (v) aqueous ether (Fukusi et al., 1964). All of these methods, besides extracting LPS, also remove other structural components of the cell wall such as phospholipid, lipoprotein and protein.

The most widely adopted extraction procedure has been the phenol-water method of Palmer and Gerlough (1940) which was modified by Westphal and Jann (1965). According to this procedure, dried bacteria are treated with a mixture of 90 per cent phenol and water (1 : 1, v/v) at 68°C for a short period of time. Under these conditions, the endotoxin is found in the water phase as a complex with nucleic acid. From this aqueous phase, they can be isolated with the aid of cetavlon (cetyl trimethyl ammonium bromide, Westphal and Jann, 1965).

If the bacteria are pretreated with TCA, phenol-water will not lead to the extraction of nucleic acid (O'Neill and Todd, 1961). Galanos *et al.* (1969) have modified the phenol-water method for the extraction of incomplete LPS from rough (R) mutants. This procedure involves the extraction of LPS with phenol/chloroform/petroleum ether at room temperature.

Lethality

It is doubtful that pre-oral administration of any reasonable amount of endotoxin could produce death (Berczi, 1968; Berczi *et al.*, 1968). Most mammals will respond with lethal shock if given a sufficient amount of endotoxin by a suitable parenteral route. Susceptibility to this effect varies greatly among different species. Rabbits are markedly susceptible and mice relatively resistant. Ribi *et al.* (1959) found that it took more than five times as much of a given endotoxin, on a micro-gram per animal basis, to kill mice as it did to kill rabbits of a similar degree of maturity. Smith and Thomas (1954) showed that infant rabbits were 50 times as resistant as mature rabbits but Uhr (1962) found that new born guinea pigs were highly susceptible and that resistance increased with age in this species.

Chick embryos are extremely susceptible during a critical stage of development between 8 and 12 days of incubation; thereafter resistance increases rapidly and is marked before

hatching time (Smith and Thomas, 1956; Finkelstein and Ramm, 1962; Finkelstein, 1964). Berczi et al. (1966) and Jordan and Hinshaw (1964) confirmed that chickens were markedly resistant to purified endotoxin. Markley and Smallman (1970) suggested the role of uric acid in birds' resistance to endotoxin. Sturkie (1965), however, suggested the role of three blood-coagulation factors and Hageman factors in chickens resistance to endotoxin. Germ-free mice are more resistant than normal mice (Jensen et al., 1963) and mice harbouring large numbers of gram-negative bacteria in the gut are more susceptible than those that do not (Schaedler and Dubos, 1962).

Pyrogenecity

Fever can provide the most sensitive biological response to the toxic LPS constituent of gram-negative cell walls that is generally called endotoxin.

Galancs et al. (1977) found that a few nanograms of endotoxin per kg of body weight raises the temperature of man and experimental animals, i.e. rabbit, cat, dog, etc. Kim and Watson (1967) reported that the polysaccharide portion of LPS did not contribute specifically to pyrogenecity. Rietschel et al. (1973) showed that pyrogenic principle was clearly embedded within their lipid A component. Galanos et al. (1972) by employing soluble free lipid A in complex form with bovine serum albumen and human serum albumen, revealed pyrogenic

activity of lipid A which was in the order of that of intact endotoxin demonstrating that the part of the endotoxin molecule responsible for pyrogenic activity is lipid A.

The time lag between intravenous injection of endotoxin and onset of fever suggested that its action was not a direct one on temperature regulating centres in the brain (Beeson, 1947). This delay was about 15 to 30 minutes in the rabbit and about 45 to 90 minutes in man. Endotoxin injected directly into the hypothalmus of rabbits did not produce fever (Grant et al., 1955).

Pyrogenic response increases with age in rabbits (Watson and Kim, 1963) and in man (McCabe, 1963). The first event in the pathogenesis of endotoxin induced fever is the release of an endogenous mediator (endogenous pyrogen) from stimulated granulocytes (Kass and Wolff, 1973), macrophages (Kass and Wolff, 1973; Bodel and Miller, 1976) and Kupffer cells (Dinarello et al., 1968). Endogenous pyrogen, a low molecular weight protein, is believed to pass the blood brain barrier and to alter the "set-point" of temperature regulating neurons in the anterior hypothalamus.

Philipp Dormston and Siegert (1975) found that intraventricular injection of prostaglandin E_2 or a derivative of cyclic AMP (cAMP) caused high fever in cats and rabbits. A significant increase in the concentration of these molecules

was detected in the cerebrospinal fluid of cats and rabbits during fever caused by intravenous lipid A (endotoxin) or intraventricular endogenous pyrogen indicating that both prostaglandin E_2 and cAMP play an essential role in the set-point elevation (Feldberg, 1975; Philipp Dormston and Siegert, 1975). Szekely (1980) also suggested the role of prostaglandins and monoamines in endotoxin fever in the new born kittens. Cefalo et al. (1980) have suggested the role of prostaglandins in endotoxemia. Feldberg (1975) gave the possibility that lipid A activates prostaglandin synthetase directly or alternatively that it stimulates leukocytes, invading the ventricular walls to severe endogenous pyrogen.

When lipid A is injected into the cerebral ventricles, cats, rabbits, and rats react with high fever, indicating a direct effect of lipid A on the thermoregulatory system (Feldberg, 1975). Bodel (1974) studied the mechanism of endogenous pyrogen production. A dose response assay of monocyte pyrogen in rabbits indicated a linear relationship of temperature elevation to dose of pyrogen at lower doses. Puromycin, an inhibitor of protein synthesis, prevented both initiation and continuation of pyrogen production and release. Heikkila and Brown (1979) found that hypothermia induced following injection of bacterial pyrogen to rabbits was associated with a disaggregation of brain polysomes to monosomes. Direct elevation of the body temperature to levels

similar to that found after pyrogen administration resulted in a brain polysome shift and since polysome disaggregation was not found in the kidney, the brain may be more sensitive to elevation in body temperature. Szekely et al. (1973) suggested that brown adipose tissue acted as a source of heat during pyrogen induced fever.

Hypothermia

Kampschmidt and Upchurch (1969) found that endotoxin even in small doses produced a biphasic hypothermia in restrained rats. Szekely and Szelenyi (1977) found that when 20-50 ug/kg of E.coli endotoxin was given i.p. to 0-3 day old rabbits, 0-3 day old guinea pigs, 3-6 day old kittens, it resulted in fever, while in 8-10 day old rats, a reversible fall of body temperature was observed. Greer and Rietschel (1978) reported that mice responded to LPS with a dose dependent, monophasic, hypothermia reaching a maximum at 2 hour post-injection. Wardlaw et al. (1971) found a similar activity with endotoxins from Salmonella typhi and Bordetella pertussis, but Shigella flexneri was less active.

Berry (1966) established that the primary effect was the interference by endotoxin with the thermoregulatory mechanism of the mice. Feldberg and Saxena (1975) found that intraventricular injections of lipid A caused long-lasting fever in rats suggesting that central system responsible for the generation

of lipid A fever is as well developed in rats as it is in rabbits and cats. In rats, however, intravenously administered lipid A (or the endogenous pyrogen induced by it) does not reach the responsive brain tissue because it seems to be unable to pass the blood-brain barrier.

Effect of endotoxin on blood picture

Konickova et al. (1980) observed changes in the rabbits blood picture in the first 24 hours after endotoxin administration. Granulocytes almost vanished from the blood stream immediately after injection and in 24 hour granulocytopenia was succeeded by marked granulocytosis, changes in lymphocytes were smaller, platelet count also fell but the red blood picture remained virtually the same. Gale et al. (1977) reported that endotoxin administration resulted in a substantial lymphocytopenia with reduction of both thymus-dependent (T) and bone marrow derived (B) lymphocytes and that absolute number of circulating B lymphocytes decreased to a greater extent than T lymphocytes.

Ramsey et al. (1980) showed that lipid A, like intact endotoxin, can stimulate platelet and fibrinogen production and induced leukopenia but the doses required are high. Alving et al. (1979) demonstrated that leukopenia occurred after the infusion of endotoxin at all dose levels (0.1 to 50.0 ug/kg body mass), the lowest dose that caused

thrombocytopenia was 0.5 ug/kg. Jain and Lasmanis (1978) studied leukocytic changes in cows given intravenous injections of E.coli endotoxin, and found that it produced dose dependent leukocytic and febrile responses. Injection of 5-20 ug of endotoxin induced a neutrophilia between 4-8 hours, whereas 50-500 ug produced a severe neutropenia for 4-6 hours followed by a gradual increase in neutrophils alongwith slight to moderate left shift. Higher doses caused progressively severe lymphopenia.

Kampschmidt and Upchurch (1980) reported that mature neutrophils left the rat humerus at about the same rate after an injection of endotoxin or leucocytic endogenous mediator (LEM). The neutrophils appeared promptly, in the circulating neutrophil pool after an injection of LEM but not after an endotoxin injection.

Effect of Endotoxin on Plasma Changes

One of the early effects of endotoxemia is a decrease in serum lipids (Schrade, 1942). However, Butler et al. (1977) reported increase in plasma free fatty acid levels after 24 hours of E.coli endotoxin injection in fowls. Total blood lipids decrease sharply but cholesterol levels are stable in the early period after endotoxin injection (Schrade, 1942). After several hours, serum lipids apparently rise; these

include serum cholesterol (Foldvari and Kertai, 1967), triglycerides and free fatty acids (Hirsch et al (1964), total fatty acids, total cholesterol (LeQuire et al., 1959).

The serum concentration of serotonin fell immediately after endotoxin injection (Rosenberg et al., 1959). Kampschmidt and Upchurch(1962) reported marked hypoferremia within 16 hours of E.coli or Serratia marcescens LPS injection. The i/v injection of E.coli LPS produced a reduction in the plasma Zn concentration in chickens which was maximum after 2.5 - 9 hours and was still detectable after 24 hours (Butler and Curtis, 1974). Later, it was reported that i/v injection of E.coli endotoxin into 8-9 week old disease free fowls (0.025 - 3.0 mg/kg) produced successive fall in the plasma K^+ and Ca^{++} levels during the 9 hours following the injection. This change in the K^+ concentration was accompanied by a slight rise in that of Na^+ and in some cases a slight reduction in the Mg^{++} concentration was detected (Butler and Curtis,1975).

Weil (1963) found elevated concentration of lactate, urea, potassium, amylase and catecholamines after endotexemia.

Effect of Endotoxin on Enzymes

Butler et al. (1977) reported a transient increase in the activity of acid phosphatase, aryl sulfatase and β-glucouronidase in the plasma of chickens aged 9-11 weeks, during 2 hours of E.coli endotoxin injection followed by a

fall in their activity. McGivney and Bradley (1978) showed that endotoxin caused a two-fold increase within 4 hours of specific activities of β-glucouronidase or acid phosphatase but not of catalase.

Griel et al. (1975) showed increased levels of SGOT and serum ornithine carbamyl transferase after E.coli endotoxin administration intravenously. Serum levels of aldolase, glutamic-oxaloacetic transaminase, isocitric dehydrogenase and phosphohexose isomerase were all elevated above normal levels in 180-250 g rats 2 to 4 hours after injection of 7.5 mg of S.typhimurium LPS (Woodward et al., 1969), but returned to normal levels after 24 to 36 hours. Similar changes in serum levels of arginase and glutamic-oxaloacetic transaminase were reported by Kumate et al. (1958) in endotoxin poisoned rats.

McGivney and Bradley (1979) observed a marked decrease in the activities of mitochondrial malate dehydrogenase, succinate dehydrogenase and adenylate kinase in African Green monkey kidney (vero) cells and primary cultures of mouse liver cells within 2 hours after exposure of LPS. Vesell et al. (1960) observed increases in serum lactic dehydrogenase activity following endotoxin poisoning, while Konttinen et al. (1964) measured increases in serum levels of glutamic-oxaloacetic transaminase, lactic dehydrogenase, and α-hydroxybutyric dehydrogenase in rabbits given E.coli LPS intravenously.

Aballi et al. (1978) studied the effect of single preparatory dose of gram-negative endotoxin in the liver and found increase in lactic dehydrogenase (LDH) isoenzyme 5, and SGOT, SGPT and 5' nucleotidase, Sakaguchi et al. (1979) reported that activity of hepatic phosphorylase in the endotoxin-poisoned mice at 2 hour was slightly higher than that in the control mice but this difference was not significant after 18 hours. Glucose-6-phosphatase activity in the poisoned mice increased by 2 hours p.i., but decreased by 18 hours. The activity of hepatic and skeletal LDH declined while that of cardiac LDH was markedly activated after endotoxin injection and serum LDH activity in the poisoned mice increased about 1.75 fold by 16 hour p.i. They also showed similar elevation in the activities of transaminases and malate dehydrogenases in the mouse serum 16 hours p.i.

Yoshida and Hayaishi (1978) found 30- to 50-fold increase in activity of indoleamine 2,3-dioxygenase {indoleamine : oxygen 2,3-oxidoreductase (decyclizing)} in the supernatant fraction of the mice lung homogenate after an i.p. administration of bacterial (from E.coli or Salmonella abortus-equi) LPS. Bhattacharjee and Phylactos (1978) reported decrease in prostaglandin synthetase activity in the kidney medulla of rabbits after i.v. administration of Shigella endotoxin.

Effect of Drugs Against Endotoxin

Reports of the endotoxin-neutralizing activities of polymyxin-B sulfate at the molecular (Koike et al., 1969; Lopes and Inniss, 1969) and cellular (Bannatyne et al.,1977; Corrigan et al., 1974; Flesher and Insel, 1978; Issekutz and Biggar, 1978; Lerner et al., 1971; Niemetz, 1972) levels, and in the experimental (Corrigan and Bell, 1971 a, 1971 b; From et al., 1972; Gruninger and Howe, 1969; Rifkind and Palmer, 1966; Rifkind, 1967 a, 1967 b; Van Miert and Van Duin, 1977) levels are there. Bannatyne and Cheung (1979) used the mouse model of i.p. enterobacterial sepsis to evaluate the antiendotoxic effect of polymyxin B-sulfate and they observed that single or multiple therapeutic doses of polymyxin administered either before or after lethal challenge with Serratia marcescens produced statistically and clinically significant protective effects. Lipinska-Piotrowska (1979) reported that polymyxin, cholestyramine and hydrocortisone diminish liver cell injury in rats produced by P.mirabilis endotoxin. Palmer et al. (1974) observed that dogs exhibited marked hypotension, with a corresponding decline in cardiac index and calculated systemic vascular resistance after one and one-half hours treatment of endotoxin.

Lindocaine and Indomethacin were equally effective against the E.coli endotoxic shock in baboons (Fletcher and Remwell, 1978). Lindocaine alone clearly increased the mixed

venom PG (prostaglandin) F_2 levels but this was unrelated to the protective effect of lindocaine. Corrigan et al. (1974) studied that human neutrophils stimulated with endotoxins had an increased reduction of nitroblue tetrazolium dye. This stimulatory effect of the neutrophil was diminished or abolished by polymyxin B, suggesting protective effect of polymyxin B-sulfate. Szekely (1978) reported that indomethacin prevents the Ist phase febrile rise in the body temperature and also the consequent fall but not the IInd phase rise.

Chapter III

MATERIAL AND METHODS

1. Source and maintenance of culture

Throughout this study, the culture of Shigella dysenteriae was used. This culture was obtained from the Department of Microbiology, Post-graduate Institute of Medical Sciences and Research, Chandigarh, where this culture was found as an human enteropathogen. The culture was maintained on agar slants of following composition 2 per cent peptone, 0,05 per cent sodium taurocholate, 1 per cent lactose, 2 per cent agar and 1 ml solution of 2 per cent neutral red in 50 per cent alcohol. It was subcultured every month and stored at 4°C in refrigerator.

2. Enterotoxin

2.1. Production of enterotoxin :

2.1.1. Composition of medium : The medium employed for the production of enterotoxin was same as the one employed for the production of E.coli enterotoxin (Evans et al., 1973). The medium had the following ingredients :

Casamino acids (Difco)	:	20.00 g
Yeast extract (Difco)	:	1.50 g
Sodium chloride (AR)	:	2.50 g

Dipotassium hydrogen phosphate (AR)	:	8.71 g
Trace salt solution	:	1.50 ml

Distilled water to make 1 litre

The pH of the medium was adjusted to 8.5 and sterilized by autoclaving.

2.1.2. Composition of trace salt solution : Trace salt solution had the following composition :

Magnesium sulphate	:	5.0 g
Manganese chloride	:	0.5 g
Ferric chloride	:	0.5 g

Dissolved in 0.001 N H_2SO_4 and brought to a final volume of 1.0 litre and stored at 4°C.

2.1.3. Inoculation procedure : A loopful of 24 hr old slant culture of Sh.dysenteriae was inoculated into 50 ml broth in 250 ml Erlenmeyer flask. It was incubated for 18 hr at 37°C as shake culture (180 strokes per minute). The cells were harvested by centrifugation at 10,000 rpm for 15 minutes at 4°C. The cell free extract was tested for its entero-pathogenecity in rabbit ileal loop.

2.1.4. Precipitation of toxin by ammonium sulfate : Enterotoxin from the cell free supernatant was precipitated by slowly adding

solid ammonium sulfate to 90 per cent saturation at 4°C with constant stirring followed by 20 minutes without stirring. It was centrifuged at 10,000 rpm for 20 minutes at 4°C. The supernatant was discarded and pellet was dissolved in 0.2M tris buffer (pH 7.8; 25 ml per litre of culture supernatant extract) and dialyzed against double distilled water till it was free from $SO_4^{=}$ ions. An inactive residue in dialysand was removed by centrifugation at 10,000 rpm for 10 minutes at 4°C. In the final supernatant extract (approx. 30 times concentrated to original volume) neomycin sulfate 100 ug/ml was added to check bacterial growth. The enterotoxin preparation was stored at 4°C till further use.

2.2. Ligated rabbit intestinal loop technique :

The rabbit ileal loop method of Taylor et al.(1958) was employed for detection of enterotoxin. Healthy rabbits weighing 1.5 - 2.0 kg were fasted for 24 hr but allowed only water. Each rabbit was tranquilized by the intravenous injection of 3 per cent sodium pentabarbitone (1 ml/kg body weight). Anaesthesia was maintained by inhalation of ether while keeping a careful vigilence at the pupil of the animal.

2.2.1. Surgical procedure : After the rabbit has been secured in dorsal recumbency, the small intestine was brought

out of the peritoneal cavity through a mid-line incision. The intestine was gently flushed with sterile luke warm solution of 0.85 per cent sodium chloride or 0.1 M phosphate buffered saline (PBS pH 7.4) and ligated segments usually 20 averaging 4 to 5 cm long were prepared by employing a single tie of surgical silk between segments. Each segment received a 1.0 ml intraluminal injection consisting of enterotoxin dissolved in buffer. The control segments were injected with buffer alone (negative control). After injection of loops with the toxin, the abdomen was sutured and precautions were taken throughout to maintain asepsis. Animal was sacrificed at pre-determined time (usually between 6 to 12 hr) by intravenous injection of high dose of sodium pentabarbitone or air. The abdomen was opened again and the fluid in each segment was measured (by withdrawl into a syringe) the length of the empty segment was determined and volume per length ratio (ml/cm) was recorded. Results were considered valid only if the positive and negative controls gave appropriate results.

2.2.2. Interpretation : To minimise variations due to reactivity of different areas of the gut to toxin, the position of loops receiving a given preparation were rotated in different animals (Moon, 1971). This signifies the fact that animal was considered unreliable and the results discarded if the

negative control preparation produced significant fluid accumulation, i.e. more than two ml in rabbit gut loop. On the other hand, animals were considered poorly reactive and results discarded if the positive control preparations produced insignificant amount of fluid.

2.3. Effect of cultural conditions on the production of enterotoxin

2.3.1. Effect of different media on production of enterotoxin :

Eighteen hr old broth culture of Sh.dysenteriae was inoculated into flasks containing different types of broths (casamino acid, tryptone, Mac-Conkey's, nutrient). The flasks were incubated at 37°C for 24 hr as shake culture. The crude toxin was obtained and injected into rabbit intestinal segments according to the standard method described earlier.

2.3.2. Effect of temperature on the production of enterotoxin :

Eighteen hr old broth culture of Sh.dysenteriae was inoculated into flasks containing casamino acid broth and the flasks were incubated at different temperatures (15, 25, 37 and 45°C) for 24 hr. The broth was centrifuged at 4,000 rpm for 20 minutes at 4°C. The supernatant was lyophilized. The powdered toxin was dissolved in phosphate buffer pH 7.0 and samples were injected into rabbit intestinal segments according to the standard method described earlier.

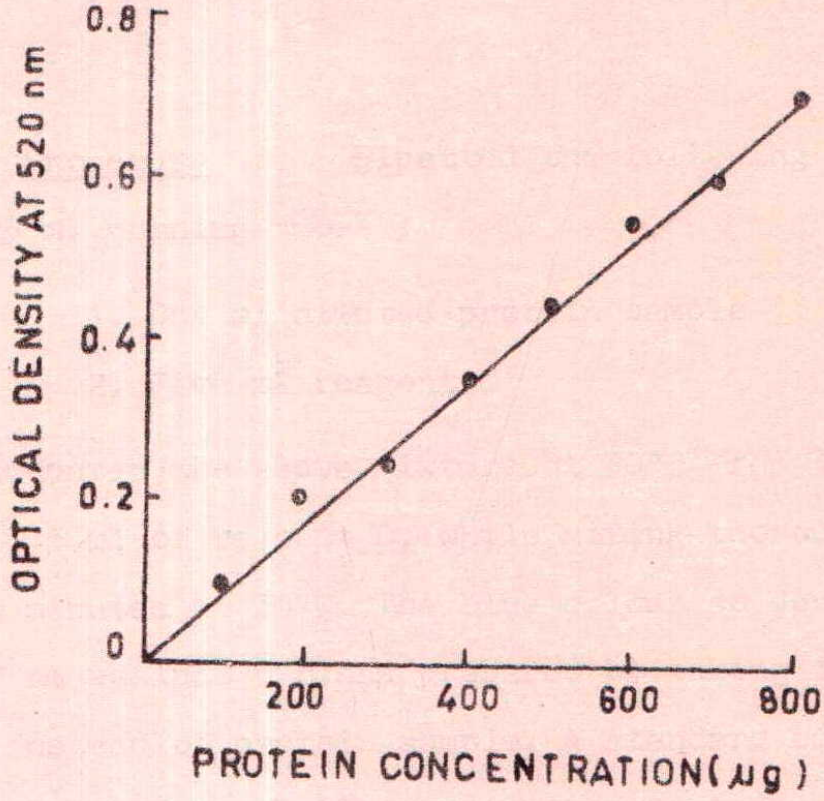
2.3.3. Effect of aeration on production of enterotoxin :

Eighteen hr old broth culture of Sh.dysenteriae was inoculated into flasks containing casamino acid broth and the flasks were incubated as shake culture (180 strokes per minute) at 37°C for 24 hr. Control flasks were incubated without shaking. The crude toxin samples were obtained as described earlier and injected into rabbit intestinal segments according to the standard method.

2.3.4. Effect of pH on the production of enterotoxin : Eighteen hr old broth culture of Sh.dysenteriae was inoculated into flasks containing casamino acid broth adjusted at different pH, viz. 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 using 1N NaOH and 1N HCl. The flasks were incubated at 37°C for 24 hr. The broth was centrifuged at 4,000 rpm for 20 minutes at 4°C. The supernatant was lyophilized. It was dissolved in phosphate buffer pH 7.0 and samples were injected into rabbit intestinal segments according to the standard method described earlier.

2.4. Effect of polymyxin B sulfate on the release of enterotoxin :

Twelve flasks each containing 100 ml of nutrient broth were inoculated with 5 ml of 18 hr Sh.dysenteriae culture. These were then incubated for 6 hr at 37°C. Cells were harvested from each flask by centrifugation at 6,000 rpm



STANDARD CURVE FOR ESTIMATION OF PROTEIN CONCENTRATION

4. Reagent D : This reagent was prepared by mixing one part of Folin Ciocalteus phenol reagent and one part of water.

5. Solution of bovine serum albumen (BSA)(1000 ug/ml).

2.5.2. Procedure : Pipetted the following successively into 25 ml corning tubes :

1. One ml diluted protein sample
2. Five ml reagent C

and incubated the above mixture at 30°C for 30 minutes and added 0.5 ml of reagent D, while mixing thoroughly. Left it for 30 minutes at 30°C. The blue colour so developed was read at 520 nm against a blank prepared by taking 1 ml distilled water instead of protein sample. A standard curve of bovine serum albumen was plotted against optical density at 520 nm by taking 0.1 to 1.0 ml of standard solution of BSA and proceeding the same way thereafter.

2.6. Immunodiffusion :

2.6.1. Antisera production :

2.6.1.1. Selection of animal : Healthy rabbits weighing 1 to 2 kg were selected for antisera production. They were kept in airy room and were fed on good diet (Hind Lever rabbit feed).

2.6.1.2. Preparation of toxoid : The crude toxin was heated in waterbath at 60°C for 30 minutes. This resulted in the formation of toxoid which retained the immunologic property but had no pathogenecity.

2.6.1.3. Production schedule : Weekly 1/m injection of toxoid with an adjuvant was given to rabbits for a period of 4 weeks. Blood was collected, from ear vein and serum removed and stored at -4°C.

2.6.2. Double diffusion agar gel for immunoprecipitation test:

2.6.2.1. Preparation of buffer (pH 8.35) :

Diethyl barbituric acid	:	1.4 g
Sodium barbiturate	:	5.0 g
Sodium chloride	:	1.0 g

All these chemicals were dissolved in one litre of glass distilled water and pH was adjusted to 8.35.

2.6.2.2. Preparation of agar gel : One per cent noble agar was made in the above buffer. A glass plate (9.1 cm x 7.5 cm) was taken and four glass strips (made by cutting the glass slide lengthwise into three) were placed on its four edges. Melted noble agar was put on the single strip plate and let it set

for two hours and then it was kept in the refrigerator at 4°C. After taking it out, the surface of the gel was dried using filter paper. Two more sets of glass strips were placed on the edges of the plate. Agar gel (0.8%) in the same buffer was put on the three strip plate and let it set, and was then kept in the refrigerator for three hours at 4°C.

2.6.2.3. Standardisation of distance between wells to produce precipitation line : After taking out the agar gel plate from the refrigerator, its surface was dried with filter paper. Wells were made at different distances. Antibody being a bigger molecule was added 2-3 hours prior to antigen. The suitable distance for the production of precipitation line was found to be between 1.25 to 1.50 cm.

2.6.2.4. Cross reactions by gel diffusion : Wells were made on the agar gel plates. In the central well, antiserum (antitoxin) was poured and in the other wells, different enterotoxins were put. The plates were kept at room temperature in the petri plate having 2 per cent phenol solution to provide saturated (humid) atmosphere and to check growth of fungus. The plates were examined daily for precipitation lines.

2.6.2.5. Washing and drying of agar gel slides : After, the development of arcs or lines was complete, the slides were

washed with saline solution for two or three days with frequent changes of the saline solution. The washings eliminated from the gel, all substances, which had not precipitated in the immune reaction. After, washing a wet smooth sheet of filter paper No.1 was applied on the gel and allowed to dry at 37°C. Use of filter paper allows transformation of gel into a perfectly transparent film. After drying, the gel became firmly attached to the plates. The plate was washed with distilled water to make it transparent.

2.6.2.6. Staining of gel plate : The plate was placed for about 20 minutes in 1 per cent solution of amido black in water : methyl alcohol : acetic acid (50 : 50 : 10), and then washed with washing solution (water : methyl alcohol : acetic acid, 50 : 50 : 10) until the back ground became colourless.

3. Endotoxin

3.1. Collection of bacterial cell mass :

Nutrient medium (with slight variation) was used for the growth of Sh.dysenteriae cells. The composition of the medium was as follows :

Peptone	:	10.0 g
NaCl	:	5.0 g

Beef extract	:	1.0 g
pH	:	7.4
Distilled water	:	1000 ml

Fifty ml of medium was inoculated with a loopful from slant culture and incubated as shake culture at 37°C for 24 hours. The cells were harvested by centrifugation at 6,000 rpm for 20 minutes. The cells were washed twice with normal (0.85%) saline, centrifuged and stored at -4°C in a screw capped container.

3.2. Extraction of lipopolysaccharide :

Phenol water method of Westphal and Jann (1965) was employed for the extraction of lipopolysaccharide of Sh. dysenteriae.

Dried bacterial mass was treated with a mixture of 90 per cent phenol and water (1 : 1 v/v) at 65°-68°C for 10-15 minutes with vigorous shaking. After cooling in a water bath to 10°C, it resulted in the formation of three layers : a water layer, phenol layer and an insoluble residue at the phenol, water interphase. The water phase was siphoned off, and the phenol layer and insoluble residue were treated in the same way as described earlier with another volume of water. The pooled water extracts were dialysed for 3-4 days against glass distilled water to remove phenol and small amounts of a

low molecular weight bacterial substances. The dialysate was a slightly opalescent solution containing lipopolysaccharide. It was then concentrated under reduced pressure at 35°-40°C to about half the volume. It was then centrifuged to remove traces of insoluble materials and was freeze dried to give a fluffy substance. The crude extract was composed of about 40-50 per cent of LPS and 50-60 per cent of bacterial ribonucleic acid.

3.3. Purification of lipopolysaccharide :

One gram of crude LPS containing RNA was dissolved in about 150 ml of water. To this 15 ml of 2 per cent aqueous solution of "cetavlon" was added and the mixture was stirred for about 15 minutes at room temperature. The turbid mixture was then centrifuged for 20 minutes at 3,000 rpm to remove the precipitated RNA. The opalescent supernatant was lyophilized, and the fluffy residue was dissolved in 50-60 ml of 0.5M NaCl solution. The solution was poured into a ten-fold volume of ethanol to precipitate the LPS-excess-"cetavlon" remaining in solution. After standing for 1-2 hours at 0-4°C the precipitate was centrifuged and dissolved in water. After dialysis for 2 days against deionised water to remove NaCl the solution was freeze dried to yield RNA free LPS.

3.4. Analysis of lipopolysaccharide :

3.4.1. Determination of total sugars in lipopolysaccharide :

Total sugars were determined by the method of Yemm and Willis (1954).

3.4.1.1. Anthrone reagent : Two concentrations of anthrone reagent 0.02 and 0.2 per cent (v/v) in 70 per cent H_2SO_4 (AR) were tried. The reagents were freshly prepared each day and allowed to stand for 30-40 minutes before use.

3.4.1.2. Procedure : Ten ml of anthrone reagent was pipetted in each of the test tubes (150 x 25 mm) and chilled in ice cold water. The solution, under test (1 ml) was layered on the acidic reagent, cooled for further 3-5 minutes and then thoroughly mixed while still immersed in ice cold water. The percentage transmission of the coloured solution was read at 620 nm. The standard curve of glucose in the range of 10-100 ug/ml was drawn. In order to measure the error, if any arising from variation in heating time and anthrone reagent used, standard sugar solutions (10-100 ug/ml) were used, as an internal standard, with every lot of the test solution.

3.4.2. Determination of sugars in lipopolysaccharide of Sh. dysenteriae :

In order to ascertain the qualitative make up of various sugars in lipopolysaccharide, 5-10 mg of it was

sealed alongwith 1N H_2SO_4 in soft glass tube. The sealed tube was heated in boiling water for 4 hr. The tube was broken and excess of acid was neutralised with $BaCO_3$ while using Congo red as an internal indicator. It was filtered through Whatman No.1 filter paper and the filtrate was concentrated over boiling water bath.

3.4.2.1. Chromatographic procedure : The filtrate was analysed by descending type paper partition chromatography using Whatman No.1 filter paper. The test solution alongwith a mixture (1%) of reference sugars was spotted. The paper was first saturated and then irrigated with n-butanol : acetic acid : water (4 : 1 : 5) solvent. After making the position of the solvent front, the paper was air dried and sprayed with benzidine T.C.A. reagent. The spots were developed by heating the sprayed chromatogram, in hot air oven at 100°-105°C for 4-5 minutes. The constituents on the chromatogram were identified by comparing the position of the spots with those of the standards and also from the Rf values given in standard tables.

3.4.2.2. Spray reagents : Benzedine trichloroacetic acid the spray reagent of Bacon and Edelman (1951) was employed. It was prepared by dissolving 0.5 g benzedine, 10 ml of glacial acetic

acid, 10 ml of 40 per cent (v/v) aqueous T.C.A. and 80 ml of ethanol. After spraying, the chromatogram was developed by heating at 105-110°C for 3-5 minutes.

3.4.3. Detection of phospholipids in Lipid-A of endotoxin :

3.4.3.1. Production of Lipid-A : The endotoxin was hydrolysed with 0.5N H_2SO_4 for 3 hours at 100°C. The hydrolysate was centrifuged and precipitated. The precipitates were washed with deionized water and lyophilized. The dry content was dissolved in chloroform/methanol.

3.4.3.2. Preparation of thin-layer plates and application of the sample : Silica gel G plates were prepared by the method of Lees and de Muria (1962). Eight grams of silica gel G was required to make four plates of 5 x 20 cm size with 250 u layer thickness. The plates were left in position until their surface was completely set (about 10 minutes). After drying in air, thin-layer plates were activated in a hot air oven at 100°C for 45 minutes before use. Ten ul of the sample was applied with the help of a Lambda-pipette, about 3 cm above the lower edge of the plate.

3.4.3.3. Thin-layer chromatography : Rectangular glass vessels (24 x 12 x 24 cm) were used to develop the thin-layer plates. To ensure solvent saturated atmosphere, the inside of

the chamber was lined with solvent soaked filter paper and a sufficient amount of the solvent mixture was added to it. The neutral solvent system consisting of chloroform : methanol : water (65 : 25 : 4, v/v) was used.

Thin-layer plates were developed by ascending chromatography upto 13-15 cm from the origin. The developed chromatoplates were air dried for 15-20 minutes.

3.4.3.4. Spray :

(i) Concentrated sulphuric acid (50%) : The destructive spray reagent, 50 per cent H_2SO_4, was used (Heftmann et al., 1966). Lipids were detected by charring in an oven at 220°C for 20 minutes after spraying.

(ii) Modified spray reagent for the detection of phospholipids : (Voskovsky and Kostetsky, 1968) : This reagent was prepared by dissolving 16 g of ammonium molybdate in 120 ml water to give solution (a). Forty ml of concentrated HCl and 10 ml of mercury were shaken with 80 ml of solution (a) for half an hour to give after filtration solution (b). Two hundred ml of concentrated H_2SO_4 followed by solution (b) were added carefully to the remainder of solution (a). The cooled mixture was diluted to one litre with water.

Phospholipids immediately gave blue colour when the dried chromatoplates were sprayed with this reagent. The

intensity of the colour increased on keeping the sprayed plate for several minutes.

3.4.4. Ultra-violet spectrophotometric assay of endotoxin :

Lipopolysaccharide (12 µg/ml) of Sh.dysenteriae dissolved in phosphate buffer (pH 7.0) was subjected to an U.V. spectrophotometric assay, blank being phosphate buffer (pH 7.0).

The readings were taken at different wavelengths, viz. 220, 240, 258, 259, 260, 280, 300, 350 and each reading was taken three times with refilling the cell.

3.5. Pyrogenecity test :

Healthy rabbits weighing 2-3 kg were fed on feed and water ad libitum in animal quarters at 70 ± 2°F . The animals were conditioned for 24 hr before injection of the endotoxin material to establish a base line temperature. Sh. dysenteriae endotoxin, 12 µg/ml was injected i/v in 4 rabbits. A thermometer was inserted approximately 1 cm into the rectum of rabbits and held there for 1 minute to record temperature. The rectal temperature was recorded at 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 26 and 30 hr intervals.

3.6. Analysis of blood :

3.6.1. Collection of blood : Blood (control) was collected from the ear vein of healthy rabbits. Test samples were drawn from each of the animals after injecting 12 µg/kg of *Sh. dysenteriae* endotoxin intravenously, at 1, 2.5, 5, 7.5 and 10 hr intervals after administration. TLC (total leukocytic count) and DLC (differential leukocytic count) were conducted.

3.6.2. Total leukocytic count (TLC) : Manual leukocyte counting method was used for TLC.

3.6.2.1. Equipment :

1. White blood cell diluting pipette with white bead in bulb (Thoma pipette).
2. Hemocytometer
3. Hemocytometer cover glass

3.6.2.2. Reagent :

Acetic acid (0.5%)	:	100 ml
Crystal violet (0.1%)	:	0.5 ml

3.6.2.3. Method : EDTA (ethylene diaminetetra-acetic acid) anticoagulated blood was drawn to the mark 0.5 in the capillary end of the pipette and then counting solution was drawn to the mark 11. It was shaken for 3 minutes and both sides of the

counting chamber was filled with it. The count was conducted under low power.

3.6.2.4. Calculations :

$$\text{Number of cells counted} \times \frac{1}{V} \times \text{dilution factor}$$

In case dilution factor = 20

Volume (V) = 0.4

$$\text{Number of leukocytes/mm}^3 = \text{Number of cells counted} \times \frac{1}{0.4} \times 20$$

3.6.3. Differential leukocytic count (DLC) : Blood smears were made and stained with Giemsa's stain and leukocytes were counted.

3.6.4. Procedure : A cleaned glass slide was used to make a thin blood smear. The film was fixed in methyl alcohol for 3 minutes. It was stained with a mixture of 1 part stain and 10 parts buffer solution pH 7.0 for 1 hr. The film was then washed with buffer solution. The preparation was kept in buffer for about 30 seconds to differentiate different types of leukocytes. After drying, the cells were observed under low power and oil immersion.

3.7. Systematic effect on serum constituents and enzymes :

3.7.1. Collection of serum after i/v injection of endotoxin :

The rabbits were kept fasting for 24 hr prior to injection of endotoxin. Intravenous injection of 12.0 µg/kg body weight of Sh.dysenteriae endotoxin preparations were given in adult rabbits weighing 1-2 kg. Blood was collected from the ear vein at intervals of 0, 2.5 and 5 hr and serum was separated and stored at -4°C till further use.

3.7.2. Systematic effect on serum constituents :

3.7.2.1. Glucose estimation : Glucose was estimated by the method of Folin-Wu (1920). To 0.1 ml of serum added 1.9 ml tungstic acid and mixed well. The mixture was centrifuged. To 1 ml aliquot of supernatant in a 13 x 100 m test tube, added 1 ml of alkaline copper reagent. The mixture was mixed well and heated at 100°C for 10 minutes and cooled to room temperature. Two ml of phosphomolybdic acid was added and was mixed. It was allowed to stand for 3 minutes and read at 420 nm.

Blank had 1 ml tungstic acid and 1 ml alkaline copper reagent. The standard had 100 mg of glucose, dissolved and made 1 litre with 0.1 per cent benzoic acid.

Calculations :

$$\text{mg of glucose/dl of serum} = \frac{\text{absorbance unknown}}{\text{absorbance standard}} \times 100$$

3.7.2.2. Urea estimation : Urea in the serum was estimated by diacetyl method of Wootton (1964). Tests were made by taking 4.6 ml of isotonic diluent, 0.2 ml of serum and 0.2 ml of 0.5N, sodium hydroxide. These were thoroughly mixed and centrifuged. Two ml of supernatant was taken, standard being 2.0 ml of urea solution (5 mg per 100 ml) and blank 2.0 ml of water.

To blank, standard and test 2.0 ml of diacetyl reagent and 1.0 ml of phospho-nitric acid were added. The tubes were heated in a boiling water bath for 30 minutes and then cooled rapidly in water. The colors were compared at 480 nm.

The calculations were done as follows :

$$\text{Serum urea (mg/dl)} = \frac{T-B}{S-B} \times 5 \times 25 = \frac{T-B}{S-B} \times 125$$

3.7.2.3. Total lipids estimation : Total lipids were estimated by the method of Wootton (1964).

One ml of fasting serum or plasma was introduced into a 100 ml Erlenmeyer flask fitted with a ground glass stopper, 20 ml of chloroform and 20 ml of 1N sulfuric acid were added. The mixture was shaken for 30 minutes. It was transferred to a 60 ml test tube, and then centrifuged for 15 minutes at 2000 rpm. The aqueous layer was aspirated off and discarded. The protein button was pushed aside and a 10 ml

aliquot of the chloroform layer was placed in a weighed glass dish, evaporated to dryness on a steam bath, and then dried in a 70°C oven to constant weight. The glass dish was weighed.

mgs of total lipids/dl of serum = mgs of lipids found x 200

3.7.2.4. Cholesterol estimation : Total cholesterol in serum was estimated by the method of Wootton (1964). Certain sterols in acetic acid solution give a red color with ferric chloride and sulfuric acid.

Test was made by using 6.0 ml of aldehyde-free glacial acetic acid and 0.1 ml of serum, standard by using 5.9 ml of acetic acid, 0.1 ml of standard solution (200 mg per 100 ml) and 0.1 ml of water and blank being 6.0 ml of glacial acetic acid plus 0.1 ml of water.

Into test, standard and blank, 4.0 ml of the freshly prepared mixed color reagent was added, carefully down the side of the tube to form a layer under the acetic acid. It was then mixed to ensure even heat distribution. It was left for 20 minutes to cool and air bubbles to disperse. The colors were compared at 570 nm. Calculations were done as under :

$$\text{mgs of total cholesterol/dl of serum} = \frac{T-B}{S-B} \times 200$$

3.7.2.5. Color reagent : Color reagent was prepared by adding 1.0 ml of the ferric chloride solution to 15 ml of

concentrated sulfuric acid. After agitating well, it was made to 100 ml with concentrated sulfuric acid.

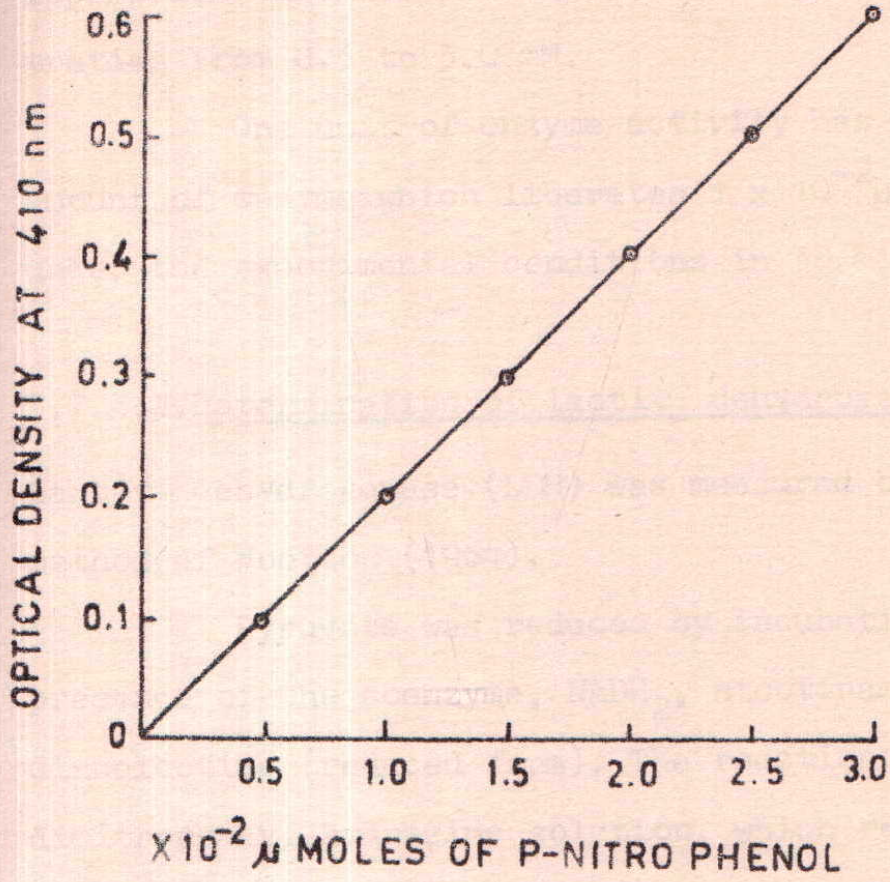
3.7.3. Systematic effect on serum enzymes :

3.7.3.1. Determination of serum alkaline phosphatases : The serum alkaline phosphatase activity was determined by method of King and Delory (1939) using p-nitrophenyl phosphate as the substrate.

The reaction mixture for the assay of alkaline phosphatase activity consisted of : (i) 0.1 ml of 25 mM p-nitrophenyl phosphate, (ii) 0.8 ml of glycine NaOH buffer (0.2M) pH 10 containing 0.001 M $MgCl_2$, (iii) 0.1 ml of the serum.

The reaction mixture was incubated for 30 minutes at 37°C and the reaction was stopped by adding 2 ml of 0.2M NaOH. The extinction of p-nitrophenol produced as a result of the reaction was read at 410 nm and its amount was determined from a suitable calibration curve.

3.7.3.2. Determination of serum acid phosphatase activity: The serum acid phosphatase activity was determined by the same method as of alkaline phosphatase except that here sodium acetate buffer pH 5.0 was used instead of glycine NaOH buffer pH 10.0



STANDARD CURVE FOR ESTIMATION OF P-NITRO PHENOL

Expression of enzyme activity :

The standard curve for p-nitrophenol was prepared by diluting a stock solution (100 μ moles/0.1 ml) with appropriate amounts of 0.2 M NaOH to give the final concentration from 0.5 to 3.0 mM.

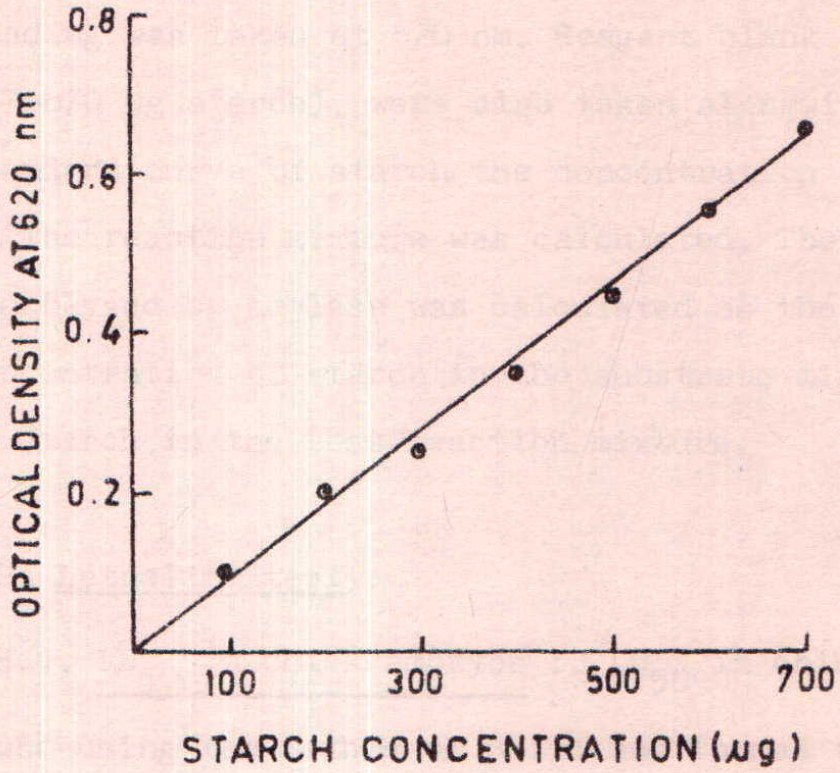
One unit of enzyme activity has been defined as the amount of enzyme which liberates 1 x 10^{-2} μ moles of p-nitrophenol under the experimental conditions in 30 minutes.

3.7.3.3.Determination of lactic dehydrogenase activity (LDH):

Lactic dehydrogenase (LDH) was measured by colorimetric method of Wootton (1964).

Pyruvate was reduced by incubation with serum in the presence of the coenzyme, $NADH_2$, nicotinamide adenine dinucleotide (reduced form). The reaction was stopped by adding dinitrophenyl-hydrazine solution, which reacted with the remaining pyruvate forming a hydrazone. The amount of unreacted pyruvate was found by measuring the brown colour produced when the hydrazone was made alkaline. The determination was performed at 25°C because of the sensitivity of LDH to heat.

Test consisted of 1 ml of buffered substrate (23 mM) and 0.1 ml of serum. The mixture was placed in a water bath at 25°C. After a few minutes, the reaction was started by adding 0.1 ml of $NADH_2$ solution (2.5 mg/ml). The mixture was



STANDARD CURVE FOR ESTIMATION OF STARCH CONCENTRATION

In case of blank, the reaction mixture contained 2.0 ml of sodium acetate buffer. The above mixture was incubated at 25°C for 15 minutes and the reaction was stopped by adding 1.0 ml of iodine-HCl solution (60 mg KI and 6 mg I_2, in 100 ml of 0.05N HCl). After the development of the colour, the reading was taken at 620 nm. Reagent blank and the standard (67-670 ug starch), were also taken alongwith them. From the standard curve of starch the concentration of the starch left in the reaction mixture was calculated. The amount of starch hydrolysed by amylase was calculated as the value of the concentration of starch in the substrate minus the concentration of starch in the test reaction mixture.

3.8. Lethality test :

3.8.1. LD_{50} in chick embryos : LD_{50} in chick embryos was found using the method of Smith and Thomas (1956). Fertile eggs were candled and healthy eggs were incubated at 37° ± 0.5°C with proper humidity. The eggs were turned twice a day. On the 8th day the eggs were candled and living embryos were employed for the test. Chick embryos were divided into 9 groups, each group having 12 eggs. Endotoxin samples of different concentrations,viz. 0.24, 0.60, 2.4, 4.8, 6.0, 8.0, 9.6, and 12 ng in 0.2 ml of normal saline were injected by a 26-gauge needle into chorio-allantoic membrane of the embryos of each group. In the group (control), saline (0.85%)

was injected. The treated embryos were incubated at 37°C ± 0.5°C temperature for 24 hr and scored for viability. The embryos were also candled after 8 hr and non-specific deaths were noted. A graph was plotted in mortality and concentration of endotoxin and fifty per cent chick embryo lethal dose ($CELD_{50}$) was determined.

3.8.2. Effect of antibiotics on the detoxification of endotoxin in chick embryo lethality test : Five drugs, tetracycline, streptomycin, benzyl penicillin, neomycin, polymyxin B sulfate were tested for the detoxification effect on the endotoxin.

In all 100 chick embryos were taken in 10 groups of 10 embryos each. In five groups, the antibiotics : tetracycline (50 µg), streptomycin (1000 µg), benzyl penicillin (100 µg), neomycin (1500 µg), polymyxin B sulfate (25 µg) dissolved in 0.2 ml of pyrogen free normal saline were injected, these being control. In the other five groups test antibiotic and endotoxin were injected. The test antibiotic and endotoxin were mixed and incubated at room temperature for 30 minutes prior to injection into chick embryos. The concentration were adjusted so that the 0.2 ml inoculum contained the selected amount of antibiotic plus 4.8 µg (LD_{50}) of endotoxin.

The results were expressed in the same way as for 50 per cent chick embryo lethality test ($CELD_{50}$).

Chapter IV

RESULTS AND DISCUSSION

An essential step in the pathogenesis of bacillary dysentery is the penetration of host intestinal epithelial cells by the pathogens while the mutant strains of dysentery bacilli, which lack the ability to penetrate intestinal epithelial cells are avirulent. The pathogen penetrates and replicates within the intestinal mucosa, produces toxin and results in tissue damage and abnormal fluid accumulation which is not reabsorbed by the colon leading to diarrhoea.

Lipopolysaccharides are the endotoxic principle of Gram-negative bacteria. The cell wall lipopolysaccharides are acidic amphipathic macromolecules. They contain a heteropolysaccharide linked to lipid A moiety. They are responsible for many physiopathological reactions expressed during infection. Lipopolysaccharides, being anchored on the surface of the bacterial cell through the lipid A component and thus occupying an exposed position certainly, play a significant role in the interaction of bacteria with the host during infection because of their unique biological properties.

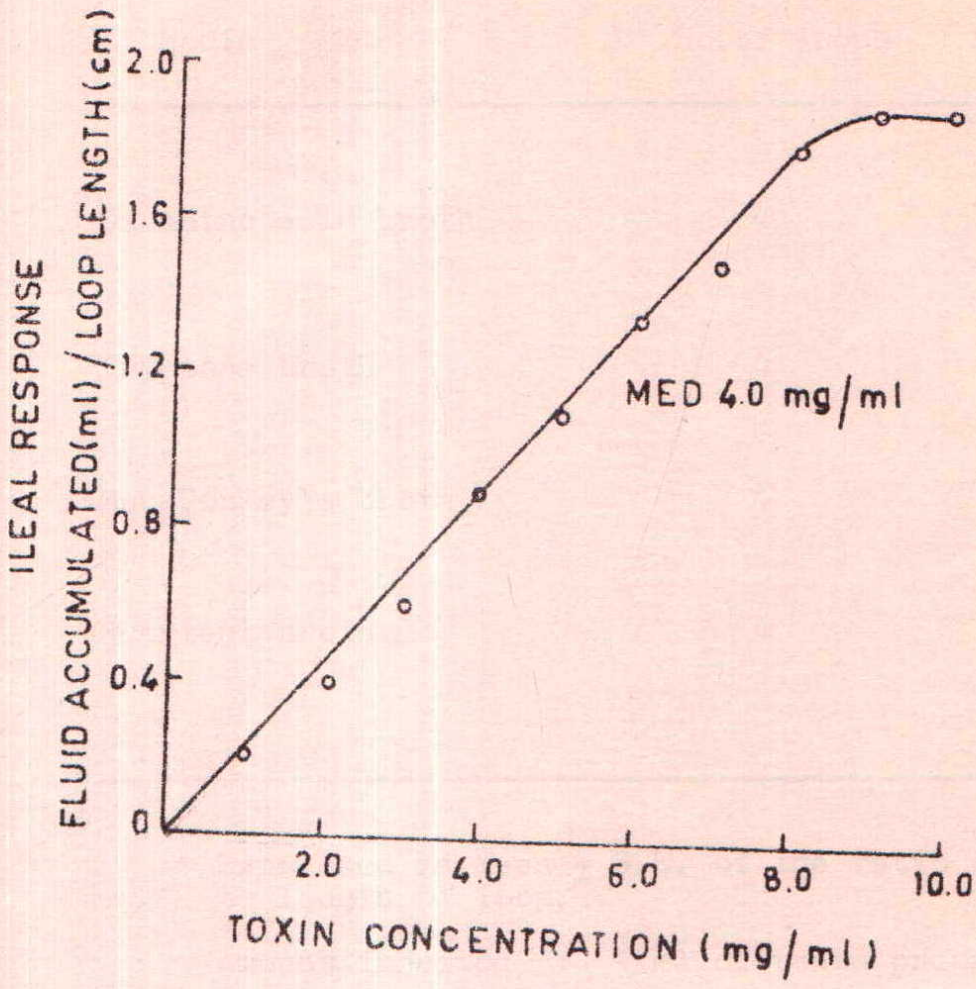


Fig.1 DOSE RESPONSE CURVE FOR Sh. dysenteriae ENTEROTOXIN IN RABBIT ILEAL LOOP

Table 1. Effect of use of different media on the production of enterotoxin by Shigella dysenteriae

Media	No. of loops	Loop response*
Casamino acid broth	4	1.85 ± 0.06
Tryptone broth	4	1.60 ± 0.00
Mac-Conkey's broth	4	1.55 ± 0.40
Nutrient broth	4	1.35 ± 0.47

* Expressed as mean ± S.E. of the ratio of fluid to length of loop.

Amount injected : 1 ml of toxin preparation

Incubation time : 24 hr.

Effect of temperature on the production of enterotoxin

Eighteen hr old broth culture of Sh. dysenteriae was inoculated into casamino acid medium and flasks were incubated at different temperatures (15, 25, 37 and 45°C). The production of enterotoxin was tested by rabbit ileal loop technique and is shown in Table 2. Maximum enterotoxin production was observed at 37°C incubation (mean volume per length ratio 1.50 ± 0.01). At 25°C, enterotoxin production was better (1.25 ± 0.09) than at 45°C (0.95 ± 0.58), however, at 15°C it was very less (0.27 ± 0.02). At higher temperature of incubation, the bacterial enzymes might have been inactivated or part of the toxin denatured as the incubation time was 24 hr. At 15°C the lag phase was prolonged, hence there was less toxin production as shown by very less fluid accumulation (0.27 ± 0.02). These findings are corroborated by the observations of Keusch et al. (1972); McIver et al. (1975); and Ketyi et al.(1978) who reported 37°C to be the best temperature for toxin production. They observed partial inhibition of toxin production at 60°C while at 90°C inhibition was 85 to 100 per cent.

Effect of aeration on the production of enterotoxin

Aeration was found to have profound effect on the production of enterotoxin (Table 3) as the fluid accumulated in the aerated sample was 1.70 ± 0.03 as compared to the 1.40 ± 0.01 of

Table 2. Effect of temperature on production of enterotoxin by *Shigella dysenteriae*

Sr No.	Temperature (°C)	No. of loops	Ileal response*
1.	15	4	0.27 ± 0.02
2.	25	4	1.25 ± 0.09
3.	37	4	1.50 ± 0.01
4.	45	4	0.95 ± 0.58

* Expressed as mean ± S.E. of the ratio of fluid accumulation (ml) to length of loop (cm)

Amount injected : 1 ml of toxin preparation

Incubation time : 24 hr.

Table 3. Effect of aeration** on the production of enterotoxin by Shigella dysenteriae

Sr. No.		No. of loops	Ileal response*
1.	Aerated	4	1.70 ± 0.03
2.	Non-aerated	4	1.40 ± 0.01

* expressed as mean ± S.E. of the ratio of fluid accumulation (ml) to length of loop (cm)

Amount injected : 1 ml of toxin preparation

Incubation time : 24 hr.

** 180 strokes per minute.

still sample, all other conditions being same. Aeration of culture resulted in more number of cells and this indirectly lead to more toxin production. Keusch et al. (1970), McIver et al. (1975), Ketyi et al. (1978) and Van Heyningen and Gladstone (1953) had corroborated these findings.

Effect of pH on the production of enterotoxin

pH of the medium plays a very important role in the production of enterotoxin. The effect of pH on production of enterotoxin was studied by employing ileal loop technique (Table 4). The enterotoxin production was found to be maximum at pH 8.0 (mean volume per length ratio 1.51 ± 0.00). However, the enterotoxin production at the alkaline pH (pH 9.0 and 10.0) was more (mean volume per length ratio 1.10 ± 0.06 and 0.78 ± 0.41) as compared to the acidic pH.4.0, 5.0 and 6.0. At acidic pH, less production of enterotoxin was observed. Minimum enterotoxin production was at pH 4.0 (mean volume per length ratio 0.45 ± 0.05). pH might have some inhibitory effect on the release of toxin from Sh. dysenteriae cells. Another plausible explanation is, toxin was released normally but was detoxified at unfavourable pH or the growth of organisms was less due to long lag phase. As this toxin is protein in nature (Sahney, 1979), pH had significant effect on unfolding of the toxin. Moreover solublization of toxin at pH 8.0 might have been the reason

Table 4. Effect of pH on the production of enterotoxin by *Shigella dysenteriae*

Sr. No.	pH	No. of loops tested	Ileal response*
1.	4.0	4	0.45 ± 0.05
2.	5.0	4	0.60 ± 0.06
3.	6.0	4	0.75 ± 0.02
4.	7.0	4	1.00 ± 0.00
5.	8.0	4	1.51 ± 0.00
6.	9.0	4	1.10 ± 0.06
7.	10.0	4	0.78 ± 0.41

* Expressed as mean ± S.E. of the ratio of fluid accumulation (ml) to length of loop (cm).

Amount injected : 1 ml of toxin preparation

Incubation time : 24 hr.

for maximum fluid accumulation in the gut. These observations are in part agreement with those of Keusch et al. (1972) who reported that maximum enterotoxin was produced at pH 8.0. However, Van Heyningen and Gladstone (1953) reported pH 11 as the best pH for extraction of toxin from cells.

Effect of polymyxin B sulfate on the release of enterotoxin

Sh. dysenteriae cells were grown, washed, resuspended in toxin-release buffer (containing 2.0 mg of polymyxin B sulfate per ml) and incubated for 1, 2 and 3 hr at 37°C as well as at 4°C. The supernatant fluid recovered after high speed centrifugation (10,000 rpm) was tested for enterotoxic activity by rabbit ileal loop technique as shown in Table 5. The enterotoxic activity was significant (mean volume per length ratio 1.45 ± 0.06) after 1 hr of incubation at 37°C in toxin release buffer and was found to increase on extending the incubation time to 2 hr (1.53 ± 0.08) and 3 hr (1.65 ± 0.06). However, there was no enterotoxic activity at 4°C even after 3 hr of incubation in toxin release buffer.

The rapidity of the release of enterotoxin indicates that it probably resides in the periplasmic space of the cell. Initial enterotoxic activity might have been due to release of periplasmic proteins and then further increase was due to slow release of cytoplasmic proteins during subsequent incubation, presumably by lysis. Evans et al. (1974) also reported that

Table 5. Effect of polymyxin B sulfate on the release of enterotoxin from Shigella dysenteriae

Sr. No.	Sample (hr)	Number of loops	Ileal response*	
			37°C	4°C
1.	1	4	1.45 ± 0.06	0.00 ± 0.00
2.	2	4	1.53 ± 0.08	0.00 ± 0.00
3.	3	4	1.65 ± 0.06	0.00 ± 0.00

* Expressed as mean ± S.E. of the ratio of fluid to length of loop.

Toxin-release buffer : 2.0 mg of polymyxin B sulfate per ml dissolved in 0.15M Tris-chloride, pH 6.6, containing 0.9 per cent NaCl.

polymyxin B-induced release of enterotoxin did not occur at temperature below 15°C but significant enterotoxin release was recorded at 37°C, even after incubation for 2, 5 and 7 minutes.

Immunological cross reactions

The immunological relationship between enterotoxin of Sh.dysenteriae, E.coli and Pseudomonas aeruginosa were studied by cross reaction experiments. There was precipitation line formation between the homologous antiserum and enterotoxin of Sh.dysenteriae. However, no precipitation line was observed between Sh.dysenteriae enterotoxin antiserum and E.coli and Ps.aeruginosa enterotoxins. It indicated that these three enterotoxins were heterologous in nature. In a similar study, Sahney (1979) established that there was no cross reaction between antiserum of Sh.dysenteriae enterotoxin and E.coli enterotoxin.

Endotoxin

The purified endotoxin of Sh.dysenteriae at a concentration of 1.2 mg/ml was produced by the given procedure and used in the present study. A concentration of 12.0 µg/kg was used as the injecting dose throughout the research experiments.

Chemical analysis of endotoxin

Total sugars in lipopolysaccharide, as determined by the method of Yemm and Willis (1954) were found to be 32.319 per cent. On chromatography, glucose, galactose and rhamnose (neutral sugars), glucosamine (amino sugar), and 2-keto-3 Deoxy octonate (keto-sugar) were identified (Table 6). On performing thin layer chromatography of lipid A moiety of LPS, a spot of phosphatdylethanolamine was observed. However, Benerrji et al. (1979) reported that lipid A fraction had phosphate, carbohydrate and esterified fatty acids. The presence of phosphatdylethanolamine in LPS hydrolysate might be due to difference in the extraction procedure of lipid A.

Ultra-violet spectrophotometric assay of endotoxin

The ultraviolet absorption curve of Sh.dysenteriae endotoxin at a concentration of 12.0 µg/ml is shown in Fig.2. Each point represents the mean of two measurements made on a single solution with refilling the cell. Fresh solution of endotoxin was prepared and the absorbance measurements(Table 7) w recorded. Scruplous care was excercised in handling glassware, cells and solution to achieve the precision reported. The absorption peak was at 260 nm and approximate similar absorption was recorded at 259 nm. The absorption maxima for nucleic acid is also at 260 nm. The presence of nucleic acids acts as an interfering substance. To avoid this confusion,

Table 6. Chromatographic identification of sugars in Shigella dysenteriae endotoxin

Sr. No.	Neutral sugars	Amino sugars	Keto sugars
1.	Glucose	Glucosamine	2-keto-3 Deoxy octonate (KDO)
2.	Galactose		
3.	Rhamnose		

Unidimentional paper chromatography was employed.

Table 7. U.V.Spectrophotometric assay of Shigella dysenteriae endotoxin*

Sr. No.	Wave length (nm)	Absorbance
1.	220	0.142
2.	240	0.082
3.	258	0.097
4.	259	0.113
5.	260	0.115
6.	280	0.075
7.	300	0.062
8.	350	0.015

* Dose of endotoxin : 12.0 ug/ml

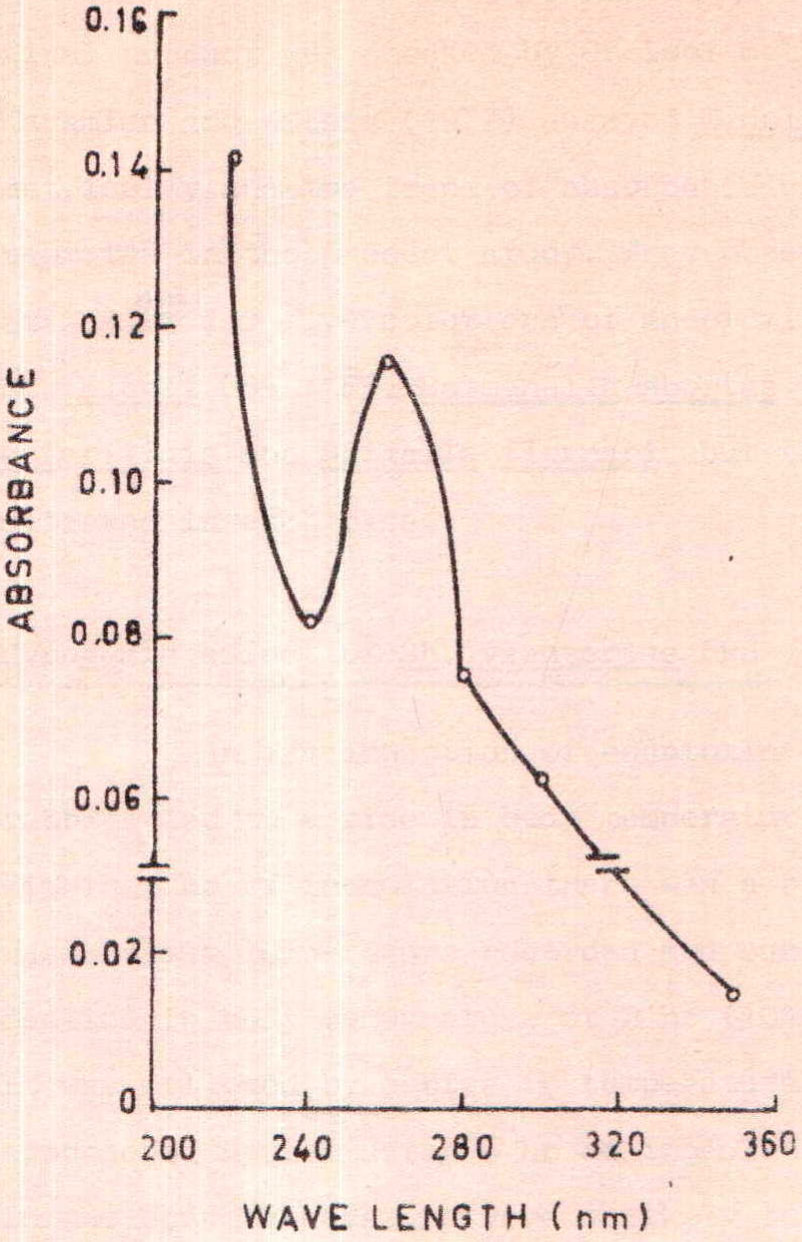


Fig.2 ULTRAVIOLET SPECTRA OF ENDOTOXIN (12 µg/ml) OF Sh. dysenteriae

the nucleic acids were removed as already mentioned in Material and Methods. The absence of nucleic acids in the final product was checked by Orcinol method (Schneider, 1957). Karamian and Waters (1977) assayed E.coli endotoxin spectrophotometrically and the trend of absorbance curve was similar as reported in the present study. They observed absorption maxima at 259 nm for five solutions of endotoxin, viz. E.coli 0127 : B8, E.coli 055 : B5, Salmonella abortus equi, Salmonella enteritidis and Shigella flexneri, but the absorptivities differed in each case.

Pyrogenic effect of Sh.dysenteriae LPS

An i/v injection of endotoxin (12 µg/ml) into 4 rabbits led to a rise in body temperature as shown in Table 8. Within 1 hr of inoculation there was a rise of 1.6°F and this was maximum temperature recorded and subsequently there was a decline in body temperature at 2 hr (104.8°F) and 3 hr (103.6°F). It was followed by a rise in temperature. The temperature approached normal after 6 hr of inoculation. The phasic changes in the body temperature were unlikely to be mediated by a single central factor, and a sequence of several factors could be postulated. Prostaglandins and their derivatives of endogenous arachidonic acid in the brain might be responsible for the first rising phase of fever and this might also initiate

Table 8. Effect of Shigella dysenteriae endotoxin* on body temperature in rabbits

Sr. No.	Sampling time (hr)	Body temperature (°F)
1.	0.0	103.6
2.	0.5	103.8
3.	1.0	105.2
4.	2.0	104.8
5.	3.0	103.6
6.	4.0	104.4
7.	5.0	103.8
8.	6.0	103.6
9.	7.0	103.6
10.	26.0	103.6
11.	30.0	103.6

The endotoxin was administered by i/v route.

* Dose of endotoxin : 12 µg/ml.

** Average of three readings of temperature.

a mechanism which in turn could lead to the transient falling phase between the two rising phases of fever. It could also be associated with a disaggregation of brain polysomes to monosomes. This rise in temperature was not due to RNA ase activation but was associated with a relocalization of poly-adenylated mRNA from polysomes to monosomes. Since these observations have been recorded in the brain, it can be stated that this could be the most sensitive organ to rise in temperature. The mechanism awaits some more explanation. Szekely (1978) while studying E.coli endotoxin also recorded biphasic rise in temperature, as has been recorded in the present study. According to Cooper et al. (1971) if the rise in body temperature is 0.5°F or more than that, pyrogenic response by the animals is taken as positive.

The endotoxins, when injected, are absorbed on the surface of macrophages and the latter release a soluble protein, which reaches the hypothalamus via blood and binds with temperature regulating cells of hypothalamus. Pyrogenic effects of endotoxin had been reported in E.coli by Philipp-Dormston and Siegert (1975) and Sh.dysenteriae (Sukhjit, 1980). They observed that in addition to prostaglandins of the E.series, cyclic AMP (adenosine monophosphate) might act as another mediator in the genesis of fever during infection. Heikkila and Brown (1979) recorded similar observations following injection of Salmonella abortus equi endotoxin in rabbits.

Leukocytic response to Sh.dysenteriae endotoxin

The hematological changes observed in response to Sh.dysenteriae endotoxin are exhibited in Table 9. Within 1 hr there was a marked decrease in the total number of leukocytes. At 5 hr, the total leukocytic count was double (12,000) against the control (6,000) showing leukocytosis. The differential leukocytic count showed lymphocytosis (61%) upto 2.5 hr. The peak of neutrophilia occurred at 7.5 hr interval. The increase in total leukocytic count started after 5 hr and continued till 10 hr. The granulocytes were vanished after endotoxin injection but reappeared after 7.5 hr. The changes in total and differential leukocytic count were typical of endotoxicosis. Leukocytosis occurred due to mobilization of the bone marrow granulocyte reserve. Administration of endotoxin was associated with marked increase in granulocyte colony stimulating factor (CSF) which accounts for granulocytosis. Rahaman et al.(1974) have reported Shiga's bacillus dysentery associated with marked leukocytosis.

Jain and Lasmanis (1978) and Konickova et al.(1980) have reported that 5-20 ug of E.coli endotoxin induce a neutrophilia between 4-8 hr, whereas 50-500 µg produce a severe neutropenia for 4 to 6 hr followed by a gradual increase in neutrophils.

Table 9. Leukocytic response in rabbits to i/v injection of Shigella dysenteriae endotoxin**

Sampling time (hr)	Total leukocytic count (TLC) (per cu.mm. of blood)	*Differential leukocytic count (DLC)				
		Lymphocytes	Neutrophils	Monocytes	Basophils	Eosinophils
0.0	6000	43	53	2	1	1
1.0	1200	55	45	-	-	-
2.5	4200	61	38	1	-	-
5.0	12,000	36	63	1	-	-
7.5	15,000	30	64	2	1	3
10.0	16,000	32	58	0	1	9

Values are expressed as an average of three trials.

* Expressed as percentage

** Dose of toxin : 12 µg/ml

Effect of Sh.dysenteriae endotoxin on serum glucose

Effect of the endotoxin on serum glucose is given in Table 10. When 12.0 µg/kg of endotoxin was injected intravenously, there was a steep rise in glucose level in serum. Two and a half hr after injection of endotoxin, glucose concentration in experimental serum was 367.85 mg/dl as compared to control of 46.70 mg/dl. There was approximately nine-fold increase of glucose level in serum. After 5 hr, there was decline in glucose concentration in the serum. The increase in glucose level after injection of endotoxin results from augmented degradation or depressed synthesis of glycogen or both. A decrease in the catalytic activity of phosphoenol pyruvate (PEP) carboxykinase accounts for gluconeogenesis. The loss of glycogen results, therefore, from impaired enzymatic activity, an effect, thatprecedes the decrease in carbohydrate. The mode of action of endotoxin was to impair synthesis. Merrill and Spitzer (1978) and Nagaraja et al. (1979) also reported that when endotoxin was injected i/v in rabbits there was hyperglycemia followed subsequently by a fall in glucose level.

Effect of Sh.dysenteriae endotoxin on serum urea

The effect of endotoxin on serum urea concentration is shown in Table 11. There was increase in the concentration of serum urea after 2.5 hr and this increase was continued

Table 10. Effect of Shigella dysenteriae endotoxin* on serum glucose in rabbits

Sr. No.	Sampling time (hr)	Glucose mg/dl**
1.	0.0	46.70
2.	2.5	367.85
3.	5.0	260.50

* i/v injection of 12 µg of toxin.

$$\text{Mg of glucose dl/ of serum} = \frac{\text{Absorbance unknown}}{\text{Absorbance standard}} \times 100$$

** Average of 3 readings.

Table 11. Effect of *Shigella dysenteriae* endotoxin* on serum urea in rabbits

Sr. No.	Sampling time (hr)	Serum urea** (mg/dl)
1.	0.0	34.09
2.	2.5	53.03
3.	5.0	62.50

* i/v injection of 12.0 µg of toxin

** Average of three readings

$$\text{mg of urea/dl of serum} = \frac{\text{Absorbance unknown}}{\text{Absorbance known}} \times 5 \times 125$$

after 5 hr. Weil (1963) also found elevated levels of serum urea. He attributed this change for the most part to circulatory derangements secondary to endotoxemia, rather than a direct effect of endotoxin on the cells.

Effect of Sh.dysenteriae endotoxin on serum total lipids and cholesterol

When Sh.dysenteriae endotoxin (12 µg/kg) was injected i/v in rabbits, there was increase in the serum lipids after 2.5 hr. This increase was continued uptil 5 hr (Table 12). This increase could be attributed to the fact that endotoxins act as stressors and this increase probably resulted from release of pancreatic glucagon. It might be that injection of endotoxin resulted in deficiency in lipid utilization. The cholesterol levels remained approximately same after 2.5 hr of endotoxin injection but there was an elevation after 5 hr (Table 13). This increase in cholesterol level could be attributed to induction of tissue anoxia by endotoxin which could subsequently stimulate the liver to increase cholesterol production. Butler et al. (1977) gave clear cut evidence of the behaviour of E.coli 078 endotoxin as a stressor by the increase in plasma free fatty acid levels. Le Quire et al.(1959) suggested three possible mechanisms leading to endotoxin-induced hyperlipemia : (1) reflex, release of epinephrine, with

Table 12. Effect of *Shigella dysenteriae* endotoxin* on serum total lipids in rabbits

Sr. No.	Sampling time (hr)	Mgs of total lipids/ dl of serum**
1.	0.0	120
2.	2.5	160
3.	5.0	200

* i/v injection of 12 µg of toxin.

Mgs of total lipids/dl of serum = mgs of lipids found x 200

** Average of 3 readings.

Table 13. Effect of Shigella dysenteriae endotoxin* on serum cholesterol levels

Sr. No.	Sampling time (hr)	**Concentration of cholesterol (mg/dl).
1.	0.0	100.70
2.	2.5	105.88
3.	5.0	200.00

* i/v injection of 12 µg/ml of endotoxin

** Average of three trials

$$\text{mg of cholesterol/dl of serum} = \frac{\text{Absorbance of unknown}}{\text{Absorbance of known}} \times 20$$

epinephrine accelerating the mobilization of depot fat, (2) induced tissue anoxia and subsequent anoxic stimulation of the liver to increase cholesterol production, or (3) a deficiency in lipid utilization.

Effect of Shigella dysenteriae endotoxin on enzymes

The endotoxin of Sh. dysenteriae when administered intravenously at a concentration of 12 µg/ml showed an increase in the levels of serum enzymes upto 5 hr of injection of endotoxin as shown in Table 14. This increase in the circulating levels of acid phosphatase could be due to disruption of lysosomes and release of contained enzymes in free active forms. There was slow but continued increase in the value of serum alkaline phosphatase (SALP), however, there was no significant difference in the values at different time intervals. When the toxin was injected i/v in the rabbit, part of the toxin was detoxified or modified in the liver and these enzymes might be involved in the modification of toxin. The endotoxin also acts upon macrophages and a few of them are partially lysed to release alkaline phosphatase.

There was abrupt elevation in the serum LDH level after endotoxin injection and the values were 50 I.U. at zero hr and 107.14 I.U. at 2.5 hr(Table 15) thus there was about two-fold increase in the activity of lactic dehydrogenase. Even after 5 hr of injection of toxin, the level of LDH continued

Table 14. Effect of Shigella dysenteriae endotoxin on serum enzymes

Sr. No.	Sampling time (hr)	SAP units	SALP units
1.	0.0	2.25	1.40
2.	2.5	2.45	1.55
3.	5.0	2.60	1.75

SAP = Serum acid phosphatase
SALP = Serum alkaline phosphatase

1 unit is the amount of enzyme which liberates 1×10^{-2} micro moles of p-nitrophenol in 30 minutes at 37°C pH 10.0 for alkaline phosphatase and pH 5.0 for acid phosphatase.

Table 15. Effect of Shigella dysenteriae endotoxin* in serum lactate dehydrogenase

Sr. No.	Sampling time (hr)	Lactate dehydrogenase (I.U.)
1.	0.0	50.00
2.	2.5	107.14
3.	5.0	142.86

* 12 µg of endotoxin

$$\text{Serum lactate dehydrogenase} = \frac{\text{Absorbance unknown}}{\text{Absorbance known}} \times 500$$

increasing. This increase might be due to tissue damage or injury to macrophages. Liver might have been a likely source of leakage of this enzyme. The above findings were corroborated by Sleeman et al. (1971) and Nagaraja et al. (1979). However, Sakaguchi et al. (1979) reported 1.75 fold increase in serum LDH activity by 16 hr post-injection.

Effect of Shigella dysenteriae endotoxin on serum amylase

Effect of Sh.dysenteriae endotoxin (12 µg/ml) is shown in Table 16. There was decrease in the amylase activity after two and a half hr from 0.109 units at zero hour to 0.097 units. This decrease continued even after this period and after five hr amylase activity was 0.093 units. However, Weil (1963) reported increase in the serum amylase activity after endotoxin administration and attributed this change to circulatory derangements secondary to endotoxemia, rather than a direct effect of endotoxin on the cells. Generally, serum amylase activity increases due to pancreatitis or mild pancreatitis.

LD_{50} in chick embryos

Different concentrations of endotoxin were injected into 7-day-old chick embryos and the mortality of embryos was observed as shown in Table 17. The 50 per cent lethal dose (LD_{50})

Table 16. Effect of Shigella dysenteriae endotoxin* on serum amylase activity

Sr. No.	Sampling time (hr)	Amylase units**
1.	0.0	0.109
2.	2.5	0.097
3.	5.0	0.093

* i/v injection of 12 µg of toxin

** Average of three trials

One amylase units is the amount of enzyme which digests 5 mgs of starch.

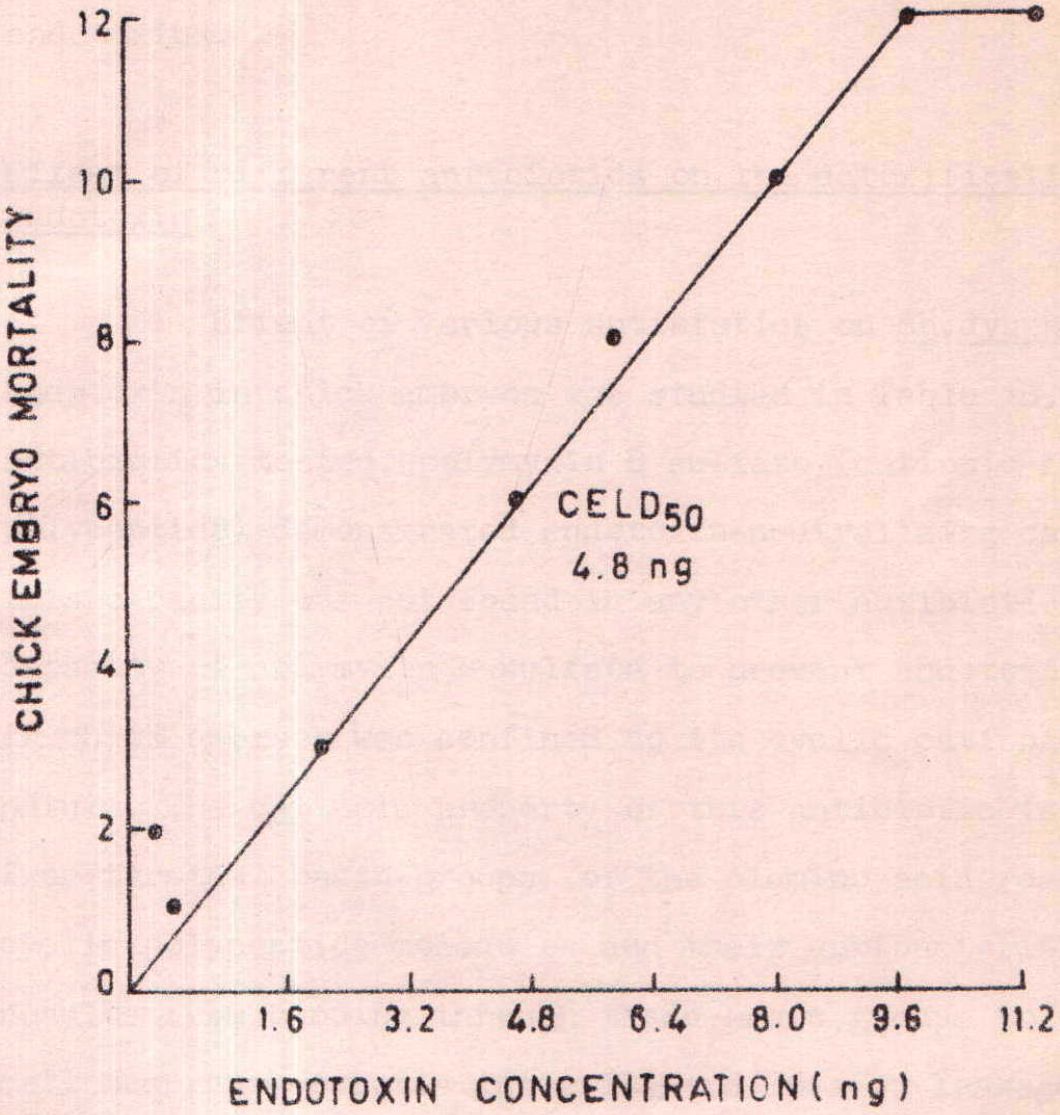


Fig.3 CHICK EMBRYO MORTALITY TO ENDOTOXIN

for chick embryos was calculated from the graph plotted between dose of toxin and mortality of chick embryos and was found to be 4.8 ng of endotoxin. Similar results have been obtained by Konno and Yoshioka (1976) with Salmonella typhosa endotoxins.

Effect of different antibiotics on the detoxification of endotoxin

Effect of various antibiotics on Sh.dysenteriae endotoxin in chick embryos was studied in Table 18. Of all the antibiotics tested, polymyxin B sulfate (cationic cyclic polypeptide) demonstrated endotoxin-neutralizing capacity. This capacity was not found in any other antibiotic. The capacity of polymyxin B sulfate to prevent endotoxin lethality in chick embryos was confined to its cyclic cationic polypeptide nature. The cationic property of this antibiotic is derived from terminal basic groups of the diamino acid residues in the cyclic polypeptide molecules and their antibacterial activity results from binding through these basic groups to the microbial cell membrane, osmotic integrity and loss by leakage of intracellular constituents. Osborn et al. (1964) suggested that specific receptor site for polymyxin B on the bacterial cell membrane was polyphosphate groups of phospholipids.

Table 18. Effect of antibiotics on the toxicity of *Shigella dysenteriae* endotoxin in chick embryos

Sr. No.	Antibiotic	Amount of antibiotic (µg)	Lethality (dead/total)	
			Antibiotic	Antibiotic + endotoxin (4.8 ng)
1.	Benzyl penicillin	1000	3/9	6/8
2.	Streptomycin	1000	4/10	6/8
3.	Neomycin	500	2/10	5/8
4.	Tetracycline	50	3/9	5/7
5.	Polymyxin B sulfate	25	1/7	2/8

The type of binding which polymyxin B demonstrated with cell wall and cell membrane preparations may also account for the antiendotoxin activity of this class of antibiotics. The cationic polypeptide antibiotic may attach to the phospholipid moiety of the endotoxin molecule. This observation of antiendotoxins effect in cyclic cationic polypeptide antibiotic supports the role of the positively charged groups in this phenomenon.

Chapter V

SUMMARY

Four media were compared for enterotoxin production. Casamino acid medium supported maximum production of enterotoxin (1.85 ± 0.06). Maximum production of enterotoxin was at pH 8.0 (1.51 ± 0.00), however, production was considerably diminished at pH 4.0 or 5.0. Optimum temperature for enterotoxin production was 37°C (1.50 ± 0.01). Aeration had pronounced effect on toxin production.

Polymyxin B sulfate increased the release of enterotoxin from Shigella dysenteriae cells. Cross reactions by gel diffusion revealed no similarities between Sh.dysenteriae, E.coli and Pseudomonas aeruginosa enterotoxins.

Lipopolysaccharide was extracted from Sh.dysenteriae cells by phenol-water and its purification was tested spectrophotometrically. The chemical profile of the endotoxin showed that it contained 32.319 per cent total carbohydrates and sugars were identified as glucose, galactose, rhamnose glucosamine and 2-keto-3 deoxyoctonate (KDO). Phosphatdylethanolamine was also detected.

Intravenous administration of the endotoxin lead to a biphasic rise in body temperature. Maximum temperature was

recorded at 1 hr and approached normal after 6 hr. The hematological changes showed that within 1 hr there was a decrease in the total number of leukocytes followed by leukocytosis. The differential leukocytic count exhibited marked lymphocytosis (85%) upto 2.5 hr. The peak of neutrophilia occurred at 7.5 hr interval. The granulocytes vanished after endotoxin injection but reappeared after 7.5 hr.

Intravenous injection of endotoxin, showed a nine-fold rise in serum glucose (367.85 mg) against the control value (46.70 mg). Serum urea, total lipids and cholesterol were also found to increase after i/v injection of endotoxin. Acid phosphatase was found to increase after 5 hr from 2.25 units to 2.60 units. A gradient increase was observed in the level of alkaline phosphatase from 1.40 units to 1.75 units in 5 hr. The level of serum lactic dehydrogenase was 142.86 I.U. at 5 hr as compared to control (50.00 I.U.). The serum amylase was found to decrease from 0.109 units to 0.093 units in 5 hr.

The LD_{50} for chick embryos was 4.8 ng. Polymyxin B sulfate detoxified endotoxin in chick embryo lethality test.

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* Original not seen

"Biological Studies on *Shigella dysenteriae* Toxins"

by

Neelam Kumari
(L-79-BS-132-M)

Major Advisor : Dr Ajit Singh, Microbiologist,
Department of Microbiology,
Punjab Agricultural University,
Ludhiana

ABSTRACT

Shigella dysenteriae produced maximum amount of enterotoxin when grown in casamino acid medium. Maximum yields of enterotoxin was recorded at pH 8.0 when cultures were grown at 37°C with constant aeration. Polymyxin B sulfate induced release of enterotoxin from *Sh.dysenteriae* cells. Heterogenecity of the enterotoxins of *Sh.dysenteriae*, *E.coli* and *Ps.aeruginosa* was observed by cross reactions. The purified LPS comprised of 32.319 per cent carbohydrates. Glucose, galactose, rhamnose, glucosamine and 2-keto-3-deoxyoctonate were detected by paper chromatography. Phosphatdylethanolamine was detected by TLC. Pyrogenecity and leukocytic changes were observed after endotoxin injection to rabbit. The toxin had a marked effect on serum, glucose, urea, total lipids, cholesterol, SAP, SALP, LDH and amylase. Polymyxin B sulfate was found to detoxify the endotoxin.