

PURIFICATION OF CHOLERA TOXIN

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UNDER THE GUIDANCE OF
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A

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submitted to the

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in partial fulfilment for the Degree of

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' DEDICATED TO MY BELOVED PARENTS '

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CHAPTER I

Introduction

Introduction

Cholera is a disease of antiquity and has been the cause of untold sufferings and mortality. The earliest reference to cholera has been made in 'Shushruta Samhita' by Shushruta, the renowned Indian Physician of the 1st century A.D (Pollitzer, 1959). Cholera has remained endemic in the deltas of Ganges and Brahmaputra in Eastern India and in Bangla Desh, ever since the dawn of recorded history. But in 1817, a new epoch in the history of cholera began with the onset of the first pandemic of cholera, during which the infection gained impetus in India and then extended its sway over other parts of the world. From then to date, seven pandemics of cholera have annihilated vast populations of the world leaving behind an atmosphere of utter desolation, dismay and affliction in the affected areas. Even today, cholera has the connotation of disaster and creates panic even amongst sections of the medical profession.

The causative agent of cholera has been designated Vibrio cholerae, Paccini, 1854, in honour of the Italian scholar - Paccini, who first provided a vivid description of the organism. The involvement of this agent in cholera was, however, discovered only in 1883 by Robert Koch and Roux, who independently isolated this organism in cholera stools. Another type of choleraigenic vibrio, Vibrio El-Tor,

Pribram, 1933, appeared in the seventh pandemic of cholera (Gallut, 1971). Although this strain was considered to be of a separate species, the studies of Hugh (1965) on the characteristics of a number of vibrio strains indicated that the distinction between the neotypes - V.cholerae and V. El-Tor did not sufficiently justify the classification of the strains as separate species. Therefore, V.El-Tor was considered as a biotype of V.cholerae, with each having identical serotypes. Subtypes of V.cholerae and V.El-Tor are identified on the basis of differences in somatic antigens A, B and C. The subtypes are designated as Ogawa, Inaba and Hikojima. A fourth serotype carrying only the somatic factor A has been described recently (Bergey's Manual, 1974).

Besides the classical vibrios V.cholerae and V.El-Tor, many other vibrios differing with respect to their serologic character, habitat and pathogenicity have been encountered. Some of these that give rise to choleraform disease in man are termed as NCV (non cholera vibrios) or NAG (non-agglutinable with 'Group O' antisera).

Robert Koch, in 1884, expressed the view that cholera was essentially a toxicosis caused by a poison excreted by V.cholerae. But, in the ensuing decades the most research workers favoured the view that the organism does not produce a soluble exotoxin but owes its pathogenicity to the endotoxins liberated upon autolysis or disruption of the organism. Thus, ever since the

establishment of the etiology of cholera, the mechanisms underlying the pathogenicity in cholera remained a subject of great controversy. In the last decade, however, sufficient evidence was compiled to establish cholera firmly in the ranks of toxinoses (Craig, 1971).

The pathogenesis of cholera in man is relatively straightforward. The sequential events that lead to 'cholera' are -

1. Entry of viable V.cholerae into the body through the mouth
2. Delivery of the cells, that survive the gastric digestion in the stomach, to the small bowel
3. Attachment of the viable cells to the receptor sites on the gut epithelium
4. Proliferation of the organisms in the gut
5. Production of enterotoxin by the organism
6. Profuse secretion of isotonic fluid by the intestinal epithelial cells in response to the enterotoxin (Carpenter et al., 1974).

The Cholera Exo-enterotoxin

Kaur et al., (1969) described cholera toxin as an heterogenous toxic substance from which the lipid constituent could be eliminated without impairing the toxic activity. Finkelstein and LoSpalluto (1969, 1972) on the other hand, stated that purified toxin is free from lipid and carbohydrate

impurities. It is now established that the purified heat labile toxin (exo-enterotoxin) is proteinic in nature and is characterized by resistance to tryptic digestion and pronase inactivation. Heating at 56°C for 30 minutes inactivates the toxin (Rappaport, 1976). The relative charge of the toxin protein is positive (Coleman *et al.*, 1968). However, the molecule acquires a negative charge under certain conditions, especially when complexed with other charged substances (Kaur *et al.*, 1969).

The molecular weight of cholera toxin has been reported in the range of 3400 - 1,00,000 as listed in Chapter III.

The toxic activity in cholera culture supernatant is known to have a half life of about one week, in neutral solution at 4°C . Aziz *et al.*, (1968) observed that, the cholera exo-enterotoxin can be stabilized upto four months by adding glycerine in the ratio of 2:1, to crude culture filtrate (100 fold concentrated) when stored at 4°C .

Analysis of the enterotoxin elaborated by *V.cholerae* revealed that two types of peptides, tentatively designated as H subunit (or cholerae) and L subunit (or choleraenoid or natural toxoid) constitute the toxin molecule. Each molecule comprises of a single cholerae subunit of molecular weight 28,000 daltons, noncovalently linked to 5 or 6 choleraenoid subunits of molecular weight 11,000 daltons each; while the

choleragen moiety is responsible for the toxigenicity of the cholera toxin, the cell binding capacity and antigenicity of the toxin is attributed to the choleragenoid component (Holmgren *et al.*, 1977).

Kinetic studies indicate that, when cholera toxin is introduced into the lumen of the small bowel of laboratory animals, it rapidly and irreversibly binds to its specific receptor, monosialosyl ganglioside GM1 (galactosyl-N-acetyl-galactosaminyl, sialosyl, lactosyl ceramide) on the gut epithelial cells (Pierce *et al.*, 1971, Van Heyningen, 1971 and Holmgren *et al.*, 1973). This is followed by a lag of about 30 minutes, after which secretion of isotonic fluid begins and reaches maximal rate within 3-4 hours. For 10-12 hours, the fluid output remains at maximal levels and then gradually decreases during the subsequent 12 hours (Barua and Burrows, 1974).

It has been suggested that the interaction of cholera enterotoxin with its specific receptor on the gut epithelial cells results in stimulation of cell bound adenylyl-cyclase, which in turn leads to increased levels of cyclic AMP in the gut epithelial cells. The increased intracellular cAMP level causes excessive movement of electrolytes and water from the plasma to the gut lumen (Banwell *et al.*, 1970) which ultimately results in the loss of isotonic fluid from the body (Love, 1965; Iber *et al.*, 1969).

It has been also observed that the enterotoxin moiety constitutes toxic factors that are responsible for -

1. the vascular permeability activity (FP; Craig, 1965),
2. the production of oedema in the rat food pad (Pinkelstein *et al.*, 1969),
3. the lipase activation in rat cells (Voughan *et al.*, 1970) and
4. the lethal activity in mice (Craig, 1971).

Besides enterotoxin (heat labile exotoxin, type 2) two other toxins viz. endotoxin (type 1) and heat stable exotoxin (type 3) are elaborated by *V. cholerae* (Burrows, 1968).

The endotoxin is the somatic antigen, which consists of a lipopolysaccharide-protein complex.

The heat stable exotoxin (Furhman and Furhman, 1960) is similar in action to the Receptor Destroying Enzyme (RDE) of the vibrio culture.

Experimental models

The experimental models in which the pathogenesis of cholera has been studied can be grouped into two types; one employed to study the generalized infection produced by parenteral inoculation of the animal with *V. cholerae*, and the other for studying localised infection. The different models used by different investigators are listed overleaf.

<u>Experimental models</u>	<u>Investigators</u>
<u>Generalized infection</u>	
1. Mouse	Ujiye <u>et al.</u> , 1968 Chalcumpa <u>et al.</u> , 1972 Basu and Picket, 1969
<u>Localized infection</u>	
1. Rat	Basu and Picket, 1969
2. Guinea pig	De and Chatterjee, 1953 Nikonor <u>et al.</u> , 1958 Larvovskaya and Blant, 1964 Basu and Picket, 1969
3. Infant rabbit	De and Chatterjee, 1953 Datta and Habbu, 1955
4. Adult rabbit by ligated ileal loop technique	De and Chatterjee, 1953 De and Chatterjee, 1959 De <u>et al.</u> , 1960
5. Adult rabbit by multiple loop technique	Jenkin and Rowley, 1959
6. Dog intestinal ileal loop technique	Swallow <u>et al.</u> , 1965 Sack and Carpenter, 1969 Curlin <u>et al.</u> , 1970
7. Patas monkeys	Greer <u>et al.</u> , 1968
8. Rhesus monkeys	Violle and Hansen <u>et al.</u> , 1965

Besides these models, chick embryo was used to study the effect of cholera enterotoxin by various investigators (Wilson, 1946; Gardner et al., 1963 and Finkelstein et al., 1964).

The validity of experimental models is based on the essential similarities between the pathophysiological changes produced in the experimental disease and the clinical

manifestations in human disease. In view of this, the rabbit ligated ileal loop model is most appropriate for laboratory studies on cholera (Burrows and Sack, 1974).

Toxin production in vitro

In view of its relatively high toxigenicity, methods of production of heat labile cholera toxin have been largely applied to the *V.cholerae* 569 B Inaba strain. Various media have been devised for this purpose.

Peptone media

For the production of crude cholera toxin, simple peptone media have been employed successfully. The toxin yield in 2% peptone medium has been established to be much higher than in media prepared from 3-5% peptone (Craig, 1965; Kaur *et al.*, 1969 and Burrows *et al.*, 1965). Moreover, a remarkable improvement in toxin production is achieved by the inclusion of 0.5% Sodium chloride in the peptone media.

Surprisingly enough, other richer media such as brain heart infusion, tryptose trypticase soy broth and thioglycolate broth are incapable of supporting the production of cholera toxin.

However, peptides of the peptone media complicate toxin purification procedures and hence, semisynthetic media devoid of any proteins and peptides are employed when purified enterotoxin is desired.

Semisynthetic media

Finkelstein et al., (1966a) modified the Sucrose-inorganic salt medium of Finkelstein and Lankford (1955) consisting of phosphates, ammonium chloride, sodium sulphate, Manganese chloride and Ferric chloride by supplementing it with 1% Caseinhydrolysate (Casamino acids, Difco). This medium was named 'Syncase' medium. Richardson (1968,1969) later improved the Syncase medium and termed it as TCY medium.

Synthetic media

The first synthetic, i.e. chemically defined medium, for the production of heat labile cholera toxin was devised by Craig (1966). This was a modification of the semi-synthetic medium of Finkelstein and Lankford (1955) and consisted of Ammonium chloride, Magnesium chloride, Manganese chloride and Ferric chloride with sucrose as the carbon source in a phosphate buffered medium.

Richardson et al., 1970 developed another synthetic medium. They replaced the Casamino acids of their TCY medium with Glutamate, Serine, Aspartate and Arginine and termed it as THY medium.

Besides, the ingredients of the medium employed, in vitro toxin production is influenced by several cultural factors such as aeration, temperature, period of incubation and the presence of bile salt (described in Chapter III).

Purification of the toxin

The heat labile and non-dialyzable nature of the enterotoxin, evident in initial studies with crude preparations, forms the basis for various procedures employed in the purification of cholera toxin.

An initial concentration of the toxic activity is advantageous as a primary step in purification procedures. Several methods have been employed to achieve this.

Kaur *et al.*, (1969) have used 90% saturation with Ammonium sulphate to precipitate the toxic activity from supernatant.

Richardson and Evans (1968) described a double concentration method involving dialysis against EDTA followed by precipitation with dextran sulphate.

It has been shown by Spyrides and Feeley (1970) that toxin activity can be quantitatively adsorbed on Aluminium hydroxide or Aluminium phosphate gel at pH 6.4 and then recovered in citrate on solubilising the gels.

The enterotoxin was shown to be almost completely precipitated from crude preparations by ethanol or ethanol-ether at 10°C by Heckly and Wolochow (1970). All the toxic activity was precipitated with 44% ethanol in the presence of Sodium chloride at pH 7.5.

Recently, ultrafiltration using polymeric membranes or other methods appear to be the most widely used methods for primary purification. Number of workers (Grady *et al.*, 1968, Kaur *et al.*, 1969, Finkelstein and LoSpalluto, 1970 and Duhamel *et al.*, 1970) have concentrated cell free supernatant by filtering it through polymeric membranes like UM 10 and PM 10.

Further purification of the concentrated toxin is achieved by chromatographic techniques employing various grades of Sephadex. Finkelstein *et al.*, (1970) used Sephadex G-100 and Sephadex G-200 columns arranged sequentially to get purified cholera toxin. Finkelstein and LoSpalluto (1970) used a single Sephadex G 75 column followed by a pair of Sephadex G 75 columns to separate cholera toxin from cholera toxinoid.

Grady and Chang (1970) purified the toxin with Sephadex QAE ion exchange chromatography. Ion exchange chromatography was also recommended by Lewis and Freeman (1969) and Coleman *et al.*, 1969. ^D

DEAE sephadex ion exchange chromatography was recommended by Kaur *et al.*, 1969.

Lewis *et al* (1976) have efficiently employed cation exchange chromatography in purification of cholera toxin. They also reported that secondary purification using preparative

acrylamide gel electrophoresis was an efficient method for large scale purification.

Recently, Holmgren *et al.*, (1977) have recommended a new method, wherein H and L subunits of cholera toxin were isolated by formic acid treatment followed by Sephadex G-100 column chromatography.

Toxoidation

For the purpose of immunization, it is essential to convert cholera exo-enterotoxin into a stable, nontoxic and antigenically competent form i.e. toxoid, which can induce the production of specific antibodies in experimental animals as well as in man. Formic acid, Glutaraldehyde and heat treatment have been employed thus far for the toxoidation of cholera toxin.

Detoxification with formaldehyde does not yield a stable toxoid (Linggood *et al.*, 1963; Relyveld 1969; Scheibel *et al.*, 1969 and Stainer 1969). Studies in Rhesus monkeys showed that the toxoid reverts to partially toxic material (Northrup *et al.*, 1972).

The toxoid obtained by Glutaraldehyde treatment was stable but its immunogenicity was significantly diminished (Rappaport *et al.*, 1974a, 1974b).

Germanier *et al.*, (1977) developed a cholera toxoid by first exposing the toxin to heat and then treating it with

formaldehyde. This toxoid has been found to be stable in vitro as well as in vivo studies.

The recent insight into the relationships between the structure, biological activity and antigenicity of the enterotoxin molecule suggests another possibility for more specific inactivation of cholera toxin. This involves preparation of pure subunit L (choleragenoid) and appropriate reaggregation of this subunit to yield an almost optimal toxoid, since the elimination of subunit H excludes reversion to toxicity without any loss of protective antigenic determinants (Holmgren *et al.*, 1970, 1977). They treated cholera toxin with formic acid and then subjected it to Sephadex G 100 column chromatography so as to obtain the choleragenoid moiety that was permanently non-toxic and antigenically complete.

Immunological activity of cholera enterotoxin

The heat labile toxin, choleragenoid (natural toxoid) as well as artificially prepared toxoids administered with adjuvants such as Freund's adjuvant, Adjuvant 65 (Vitakar, 1973) are capable of inducing the formation of specific antitoxic antibodies.

Using the rabbit ligated ileal loop model, Kasai and Burrows (1966) established that the antitoxic antibody produced in response to immunisation with crude cholera toxin was capable of neutralising the toxin. A similar observation

in the canine model was reported by Curlin *et al.*, 1970. Finkelstein (1970) demonstrated that immunity to cholera toxin or live vibrio could be achieved in rabbits by immunization with highly purified toxoid. Moreover, in Finkelstein's experiments only antitoxic antibodies were induced, indicating that protection against the disease was due to antitoxic and not due to antibacterial immunity.

Holmgren *et al.*, 1977 observed that antibodies to the L subunit of the toxin molecule play the major role in protection against the disease and suggested that the protective antitoxic antibodies act by preventing binding of toxin to the GM 1 ganglioside receptor in the intestinal epithelium rather than by interaction with the 'toxic' site of the H subunit. Immunological studies have shown that IgG and IgA antibodies are effective in neutralising the activity of the enterotoxin. As the site of the enterotoxin action is the mucosal surface of the intestinal lumen, gut associated antitoxic immunity could be most efficient in affording protection against the disease.

Aims and objects

The systematic mechanisms directed against both - the vibrios and their enterotoxin, are of potential significance in the immunology of cholera. In order to assess the role of antitoxic immunity in protection against 'cholera', it is indispensable to have in hand highly purified cholera exo-enterotoxin. However, the most modern methods for purifying cholera toxin involve sophisticated procedures that prove expensive. It was, therefore, decided to compare different methods and formulate a simple and economic purification procedure based on the properties of cholera exo-enterotoxin.

Moreover, attempts were made to improve the existing Syncase medium to obtain higher yields of cholera toxin so as to initiate the purification process with high titre crude toxin.

In vitro production of NAQ vibrio toxin using Syncase medium was also undertaken.

The antigenic relationship of the toxin elaborated by NAQ vibrios and that of the classical Vibrio cholerae was studied.

CHAPTER II

Material and Methods

Material and Methods

2.1. Production of Cholera Toxin

2.1.1. The Vibrios

Rabbit passaged 569 B Inaba strain of Vibrio cholerae (Haffkine strain) was used for the production of cholera toxin.

For 'HAG toxin' production five different locally isolated strains of HAG vibrios were employed.

All the above strains were preserved in lyophilized state in ampoules at 4°C.

2.1.2. Preparation of the Inoculum

After opening the ampoule aseptically, the lyophilized culture (to be employed for toxin production), was emulsified with 3-4 drops of peptone water and transferred to about 1.0 ml. of peptone water in a small test tube (4" x 3/8"), with a sterile capillary pipette. The culture was incubated at 37°C for 3-4 hours and then streaked on nutrient agar slants. After overnight incubation at 37°C the purity of the culture was confirmed. A loopful (diameter 4 mm) of the growth was then uniformly suspended in 10 ml. of syncase medium (Appendix I₃₁). On incubation

at 37°C for 3½ hours, logarithmic phase culture containing approximately 10^8 cells/ml. (compared with Brown's standard opacity tubes) was obtained. This standard inoculum was employed in subsequent experiments.

2.1.3. Preparation of Crude Cholera Toxin

Flasks containing standard syncase medium and the modified media (given in Appendix I 1 and 2) were aseptically inoculated with 0.1 ml. of standard inoculum (Preparation described in 2.1.2.) and incubated for 12-16 hours at 37°C on a rotary shaker at 180 rev./mt. Merthiolate solution (Appendix II 1) was then added to each flask to achieve a final concentration of 0.0001 gm/ml. in the culture and further shaken for 1 hour to kill the vibrios. The flasks were then removed from the shaker and the contents were centrifuged at 6000 r.p.m. at 4°C for 4½ minutes. The supernatant, which constituted the crude toxin was separated in 2.0 litre flasks and stored at 4°C.

2.2. Protein estimation

Protein content of the toxin was estimated by Lowry's modification of Folin's method of protein estimation (Lowry et al., 1951).

To a series of clean dry test tubes, different quantities of standard protein and reagents were added in the following order -

Tube No.	Standard protein (120 µg/ml) added in ml.	Conc. of protein in µg.	0.1 N NaOH added in ml.	Alk. CaSO ₄ solution added in ml.		'Working' Phenol Polin Ciocalteu reagent added in ml.
1	0.1	10	1.9	1.0		0.1
2	0.2	20	1.8	1.0		0.1
3	0.3	30	1.7	1.0	Incubated	0.1
4	0.4	40	1.6	1.0	at room	0.1
5	0.5	50	1.5	1.0	temperature	0.1
6	0.6	60	1.4	1.0	for	0.1
7	0.7	70	1.3	1.0	15 minutes	0.1
8	0.8	80	1.2	1.0		0.1
9	0.9	90	1.1	1.0		0.1
10	1.0	100	1.0	1.0		0.1
11	test sample 0.1 ml.	-	1.9	1.0		0.1

After addition of the Polin Phenol reagent the contents were mixed and incubated at room temperature for 30 minutes. The intensity of the blue colour developed as a result of reduction of phosphomolybdic acid (Polin Phenol Ciocalteu reagent) by amino acids such as tyrosine, tryptophan, phenylalanine was measured at 660 nm wavelength in a

spectrophotometer. A 'standard graph' showing protein content verses optical density was plotted and the protein content of the test sample was estimated from the graph.

The reagents employed in this estimation are listed in Appendix II 2.

2.3. Purification of Crude Toxin

2.3.1. Primary purification

2.3.1.1. Ammonium sulphate precipitation method

To 100 ml. of the crude extract, 65 grams of Ammonium sulphate (a modified method of Kaur et al., 1969) were added (to saturation). The contents were thoroughly mixed and left overnight at room temperature for complete precipitation of the toxin. The precipitate was then separated by centrifuging the contents at 6000 r.p.m. at 4°C for 45 minutes. The supernatant was then separated and examined for its PF activity, in order to determine the amount of toxin lost in the supernatant. The precipitate was suspended in 10 ml. of distilled water and dialysed against several changes of distilled water until all the sulphate ions were removed from the toxin (The complete removal of sulphate ions was ensured by adding 2% BaCl_2 solution, Appendix II 3, to the dialysing distilled water). The quantity of toxin was measured after dialysis. The toxin was then concentrated at $4^{\circ} - 8^{\circ}\text{C}$, by 'per evaporation' or by 'vacuum dialysis'. The toxin thus purified was freeze dried under vacuum and stored at 4°C .

2.3.1.2. Sodium hexametaphosphate precipitation method

This method was recommended by Spyrides and Feeley (1970).

To 100 ml. of crude extract (at 4°C), 0.255 gms of Sodium hexametaphosphate (BDH, LR) were added and dissolved by constant stirring. The pH was adjusted to 4.6 with concentrated HCl and the contents were stirred further on a magnetic stirrer at 4°C , to allow complete precipitation of toxin. Completion of the precipitation reaction was ensured by the constant optical density on Spectronic-20. The pH was maintained at 4.6 throughout the experiment. The precipitate was separated by centrifugation at 4°C for 60 minutes at 6000 r.p.m. The supernatant was subjected to rabbit skin test to check whether PF activity was lost in it. The precipitate was then washed thrice with a volume of cold 0.2 M Sodium chloride (pH 4.5), equal to 1/5th of the original volume of the crude extract. The Sodium chloride used in the three washings was checked by skin test to determine whether PF activity was lost in it. The washed precipitate was then suspended in minimum quantity of 0.15 M Na_2HPO_4 buffer (pH 8.0) and dissolved by stirring for 1 hour. The toxin was further concentrated by vacuum dialysis and freeze dried under vacuum, and stored at 4°C .

The reagents employed in this method are listed in Appendix II 4.

2.3.1.3. Aluminium phosphate gel adsorption method

This method was recommended by Rappaport et al., (1970) to achieve primary purification of cholera toxin.

Using concentrated sulphuric acid the pH of 100 ml. of crude toxin was adjusted to 6.4 and 120 ml. of freshly prepared 20% w/v Alum potash was added to the crude toxin. The gel thus formed, was stirred on a magnetic stirrer at 4°C for 30 minutes so as to achieve proper adsorption of the toxin on the gel. The gel was then separated by centrifugation at 6000 r.p.m. for 45 minutes at 4°C. The supernatant was checked for its PF activity to determine if toxin was lost in it. The gel was then washed thrice with 0.1 M phosphate buffer, pH 6.4. Loss of PF activity in the buffer washings was checked by rabbit skin test. The 'washed' gel was dissolved in 0.1 M Sodium citrate, pH 8.5, to elute the toxin. The phosphates in 'eluted' toxin were then eliminated by dialysing the dissolved toxin against several changes of 0.1 M Sodium citrate, pH 8.5. The quantity of toxin was measured, and then concentrated by 'per evaporation' and further freeze dried under vacuum and stored at 4°C.

The reagents used in this method are listed in Appendix II 5.

2.3.2. Secondary Purification

Sephadex G 75 Column Chromatography

Gel filtration is one of the most modern and efficient method used for separation of macromolecules. In this method, separation of molecules is effected solely on the basis of molecular size. Various types of gels are known of which Sephadexes are very convenient. Sephadexes are obtained as beads of different porosity. Thus, Sephadex G 75 has an exclusion limit of 1,00,000 meaning thereby that molecules with a molecular weight of less than 1,00,000 will be able to enter the beads and will be eluted in a gradient of decreasing molecular weight.

Sephadex G 75 was therefore, used in the present study to separate cholera toxin (molecular weight approximately 84,000) from other impurities.

Ten grams of Sephadex G 75 were accurately weighed and suspended in 500 ml. of 0.4 M Ammonium formate buffer containing 0.02% Sodium azide as a preservative (Appendix II 6), in a 2 litre flask fitted with a side arm. This suspension was then stored at 4°C for 24 hours to allow the beads to swell to their maximum size (200 ml). After degassing the gel for $\frac{1}{2}$ an hour by evacuation, it was carefully packed (without trapping any air bubble), in a 125 cm. burette having wetted glass wool at the bottom, and equilibrated at 4°C for 48 hours

by the continuous flow of the Ammonium formate buffer. The packing of the gel was carried out as per the instructions of H. Determann (1969). Extra buffer on the gel was removed by a Pasteur pipette and the sample was loaded on the gel from the side of the burette, care being taken not to disturb the edge of the gel. After the sample entered the gel, the column was refilled with the buffer upto the top end and a continuous flow of Ammonium formate buffer was maintained so as to regulate the outflow at 10 ml/hour. In all, 120 successive fractions each of 4 ml, were collected in tubes using the Ultrarack Fraction Collector. The fractions were then subjected to spectrophotometry at 280 nm and the fractions showing presence of protein were pooled accordingly, and concentrated.

2.4. Studies on Fractions eluted from Sephadex G 75

The fractions collected on Sephadex G 75 column chromatography were subjected to various tests given below -

1. PF activity (2.6.1),
2. Enterotoxicity (2.6.2),
3. Protein estimation (2.2),
4. Molecular weight determination (2.5.2),
5. Absorbance pattern at varying wavelengths in U.V. range (by U.V. Spectrophotometry),
6. LPS estimation (2.5.3),
7. Purity (2.5) and
8. Stability (2.6.3).

2.5. Purity Tests

2.5.1. Polyacrylamide Disc Electrophoresis

Proteins are charged molecules with a specific molecular weight that is characteristic of the species they constitute. The molecular weight and the relative charge of a protein molecule determines its mobility in the polyacrylamide gel, which consists of Acrylamide and Bisacrylamide linked with crossbridges to form a polymer. Ammonium persulphate enhances the polymerization in presence of strong source of light. Spacer gel is used for the primary separation of the proteins and further separation is achieved in the lower gel. Riboflavin polymerizes in the presence of either U.V. light or strong fluorescent light. The larger molecules cannot enter the polymerized gel and are therefore retained at the top of the gel, whereas, the smaller molecules enter the gel with ease and subsequently mobilized towards the electrode depending upon the charge. The minimum concentration of the protein required to develop sharp bands in the gel is 150 to 200 µg while, the maximum amount that can be loaded is 0.4 ml.

As cholera toxin is highly anionic (acidic), Anionic Disc Electrophoresis was employed. The procedure is described below -

In polyacrylamide disc electrophoresis tubes are packed with great care. Gel tubes of uniform diameter and height

were thoroughly cleaned and dried in the oven at 100°C for 30 minutes. These were then fitted tightly in the holes of the gel electrophoresis apparatus and stoppered at the lower edge with clean dry rubber corks. Two ml. of the lower gel was then introduced into the tubes, care being taken not to trap any air bubble in between. Minute air bubbles, which were occasionally trapped at the bottom of the gel, were removed by gently tapping the tubes before polymerization of the gel initiated. A drop of water was slowly introduced on the top of the lower gel so as to get an horizontal upper meniscus. After the gel is completely polymerized, the water drop was removed by a slight jerk and then 0.2 ml. of Spacer gel (upper gel) was introduced. A drop of water was introduced on the top of the spacer gel and the rack was placed under a strong source of U.V. light to achieve polymerization of the spacer gel. On polymerizing the spacer gel, the water drop was removed. The rubber corks were then detached from the bottom of the tubes and the lower trough was filled with the buffer. The samples (in 0.2 ml amounts) were slowly introduced above the gel without trapping any air bubble, this was followed by the introduction of 0.2 ml of 40% sucrose solution and a drop of bromophenol blue indicator. Utmost care was taken to prevent mixing of the sucrose solution and indicator with the sample. The tubes and the upper trough were then filled with the buffer. The apparatus was closed with the lid and electric current (DC) was applied so as to achieve 3 to 4 mA current/tube

from negative to positive. The apparatus was placed at 4°C and the current was switched off when the 'marker indicator' was 1 cm above the lower edge.

The tubes were then removed from the trough and a jet of 5 ml distilled water was introduced from the lower edge of the tubes with a 10 ml syringe, fitted with a 24 gauge needle, so as to separate the gels from the tubes.

The gels were then washed with water and stained with 1% Amido Black B. After 20-30 minutes the gels were destained in several changes of 7% acetic acid and then preserved in 1% acetic acid.

Various reagents used in this technique are listed in Appendix II 7.

2.5.2. Sodium Dodecyl Sulphate (SDS) Gel Electrophoresis

In this method, the charge on the protein molecule is neutralized by the surfactant Sodium Dodecyl Sulphate and as a result, the protein molecules are separated entirely on the basis of their molecular weight.

As the molecular weight of a protein determines its sieving capacity through the gel, the molecular weight of an unknown protein can be determined by comparing the sieving capacity (mobility) with those of known authentic samples that are simultaneously run through the gel.

SDS treatment

To 0.4 ml of the sample, 0.1 ml of SDS (final concentration 1 %) and 0.05 ml mercaptoethanol (final concentration 1 %) were added. The mixture was then heated in boiling water for two minutes. Of the heated mixture, 0.2 ml was loaded for electrophoretic run through the gel.

Packing of the gel was done in the manner described for disc electrophoresis by using SDS reagents (Appendix II 8). The sample was then loaded in the manner described for disc electrophoresis. After running the sample for 6 hours by using 5 mA current/tube, the gels were removed and washed as mentioned in Appendix II 8.

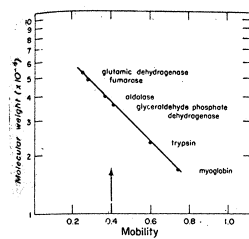
The gels were then stained with 0.25% Coomassie blue for 30 minutes and later destained with 'destaining solution'.

The reagents employed for SDS gel electrophoresis are listed in Appendix II 8.

The mobility of the protein (F II) was calculated by the formula -

$$\text{Mobility} = \frac{\text{distance of protein migration}}{\text{length after destaining}} \times \frac{\text{length before staining}}{\text{distance of dye migration}}$$

From the standard graph (Weber & Osborn, 1969) shown overleaf the corresponding value for molecular weight of F II was determined.



Standard graph for determination of
molecular weight (Weber and Osborn, 1969)

2.5.3. Passive Haemagglutination Inhibition (PHI) Test

For this test, LPS of *V. cholerae* 569 B Inaba (purified by Westphal's method), and LPS-antiserum were obtained from the Department of Bacteriology, Haffkine Institute.

2.5.3.1. Standardization of the Antiserum

Sheep blood was collected in Alsever's solution, and centrifuged to separate out the red blood cells. These cells were then washed 3 - 4 times with normal saline, and suspended in normal saline to obtain 2.5% suspension of RBCs.

Two ml of the 2.5% cell suspension was treated with 0.2 ml of LPS (500 µg/ml) and 1.8 ml of normal saline, and the mixture was incubated at 37°C for 45 minutes, so as to sensitize the sheep RBCs with the LPS. The cells were then washed with saline, and the final volume of the washed cells was adjusted to get 0.5% cell suspension.

Dilutions of the antiserum were obtained in the wells of a microtitre haemagglutination tray as outlined below -

Well No.	1	2	3	4	5	6	7	8	9	10	11	12
Saline	-	.05	.05	.05	.05	.05	.05	.05	.05	.05	.05	.05
Anti-serum (10 ⁻² dilm.)	.10	.05	.05	.05	.05	.05	.05	.05	.05	discard 0.05 ml.		
Fold: 10	20	40	80	160	320	640	1280	2560	Saline Cell	control		
dilm.									cont-1	control		

Sensitized cells in 0.5 ml amount (0.5%) were then added to all the wells, except well No.10.

After incubating the tray at room temperature for 2 hours the results were read and confirmed after overnight incubation at 4°C. The highest dilution showing haemagglutination was taken as 'titre of the antiserum'.

25.3.2. Passive Haemagglutination Inhibition Test

In the above test the titre of the antiserum found was 1:1280, hence, the antiserum was diluted 1:320 so as to obtain a concentration of 4 units of antitoxin, which was employed in the PHI test.

In a microtitre haemagglutination tray, standard LPS was serially diluted as shown below -

Well No.	1	2	3	4	5	6	7	8	9	10	11	12
LPS (500 ug per ml) or test sample	105	105	105	105	105	105	105	105	105	105	105	105
Saline	105	105	105	105	105	105	105	105	105	105	105	105
Mixed and added	105	105	105	105	105	105	105	105	105	105	105	105
												discard

Standard antiserum (1:320 dilution) in 0.05 ml was added to each well and the tray was incubated at 37°C for 30 minutes. To each well, 0.05 ml of 0.5% sensitized RBCs were added and the tray was incubated for 2 hours at room temperature and then

placed overnight at 4°C. The sensitized RBCs and normal saline served as 'cell control', while the serum (1:320 dilution) served as 'serum control' (incubated under the same conditions).

PHI test was interpreted as 'positive' when there was no haemagglutination (button formation), while haemagglutination (no button formation) indicated PHI test 'negative'.

The highest dilution showing button formation was taken as 'titre of the sample'. Results of the test sample (toxin) were compared with the results of standard LPS (that was simultaneously processed), to determine the LPS content of the test sample.

2.6: Toxicity Tests

2.6.1. Skin Test

The dorsal portion of an adult rabbit (weight 1.0 to 1.2 kg) was shaved with an electric clipper and the area was then cleaned by applying a paste containing 1 part of starch and 2 parts of Barium sulphide. After 5 minutes, the paste was removed by gentle rubbing with cotton and the skin was then washed under running tap water. The skin was allowed to dry and with 1 ml tuberculin syringe fitted with 24 gauge 1/2 inch needle, 0.1 ml of appropriate dilution of toxin was injected intradermally into the shaved portion of the rabbit skin. Intradermal injection of 0.1 ml of sterile normal saline served as negative control. Results were read 18 hours after the inoculation. An erythema and oedema of atleast 8 mm diameter

in a non-immunised fasting rabbit. The fluid per centimetre ratios for different concentrations were determined and a graph of Log concentration verses fluid accumulated (ml/cm) was plotted. The stability of the purified cholera toxin (concentrated Fraction II, eluted from Sephadex G 75 column), on storage at 4°C (at 0 day, at 3 days, at 15 days and after 30 days) was determined by the above procedure.

2.7. Toxidation

An ampoule of the freeze dried toxin that was primarily purified by the precipitation method, was opened and the toxin was reconstituted in minimum amount of normal saline. The protein content of the reconstituted toxin was estimated (2.2.) and the toxin was appropriately diluted to obtain a protein concentration of 50 µg/ml. The toxin was then treated with 0.2% final concentration of formaline and incubated at 37°C for 72 hours. Thus, formalinized toxin was obtained.

2.8. Immunological Studies

2.8.1. Immunisation of the Rabbits

Initially, the rabbits (weighing 1.2 to 2.0 kg) to be used for immunisation, were bled by heart puncture or ear vein puncture, the serum was separated, and stored in a closed container at freezing temperature.

The formalinized toxin (2.6.) was used for immunising the rabbits.

Cholera toxoid was mixed with equal volume of Freund's adjuvant (Appendix II 9) and the mixture was homogenized using an 18 gauge needle fitted to a 2 ml syringe. Proper mixing was ensured by placing a drop of the mixture on a column of steady water.

Two ml of the mixture was injected intramuscularly in rabbits. In the four dose schedule, 4 doses spaced one week apart were injected whereas the seven dose schedule consisted of 7 doses spaced a week apart.

The rabbits were bled three weeks after immunization with the last dose, the serum was separated and stored at freezing temperature. This serum was defrosted at room temperature and the following tests were carried out to determine the titre of: 1) antibacterial antibodies and, 2) antitoxic antibodies.

2.3.2. Determination of the Titre of Antibacterial Antibodies;

Bacterial Agglutination Test

The titre of the antibacterial antibodies was determined by serially diluting the serum with normal saline and mixing it with an equal volume of overnight grown culture consisting of 1×10^{10} viable organisms (569 B Ehab and Ogawa, in different sets of tubes). The contents of the tube were properly mixed and incubated at 37°C for 4 hours, and then placed overnight at 4°C in a refrigerator. Before reading the results the tubes were placed at room temperature for one hour. The results were recorded as - 'agglutination positive', when clumps appeared in the tubes and 'agglutination negative', when there were no clumps in the tube.

The highest dilution of the serum showing positive results was recorded as the titre of the antibacterial antibodies.

2.8.3. Determination of the Titre of Antitoxic Antibodies,

Haemagglutination Test

Freshly collected sheep erythrocytes were first sensitized with 'standard' quantity of the toxin and then washed thrice with normal saline. These sensitized cells were employed to determine the antitoxic titre of the antibodies present in the serum. The 'standard' quantity of toxin utilized for sensitization of the sheep erythrocytes was predetermined by the 'standardization of Haemagglutination Test' as described below -

To 4 small test tubes (4" x 3") each containing 1 ml of 2.5% sheep RBCs, 1 ml, 0.5 ml, 0.25 ml and 0.1 ml aliquots of 400 µg/ml of stock cholera toxin (primarily purified by precipitation method) were added respectively. The contents of the tubes were thoroughly mixed and incubated at 37°C for 45 minutes. The sensitized cells were washed thrice with normal saline and the final volume was adjusted to 5 ml with normal saline to obtain 0.5% cell suspension.

The test serum was serially diluted in normal saline from 1:20 to 1:2560 in duplicate sets. To 0.1 ml aliquots of the serum dilutions, 0.05 ml of 0.5% sensitized sheep RBCs were added and incubated at room temperature for 2 hours.

Haemagglutination test was interpreted as 'positive' when there was haemagglutination (no button formation) while no haemagglutination (button formation) indicated Haemagglutination test 'negative'.

Results were compared with the cell control (cells and saline) and serum control (serum and saline) incubated under identical conditions. The results were confirmed by placing the plates overnight at 4°C .

The concentration of the toxin showing highest titre in the haemagglutination 'standardization' test was selected for sensitization of sheep RBCs for the 'test proper'.

The Haemagglutination test was carried out with the cells (RBCs) sensitized with equal volume of $400\text{ }\mu\text{g/ml}$ of cholera toxin. The procedure employed for Haemagglutination test was the same as described above.

2.8.4. Agar Gel Diffusion Test: Microcutcherlow Test

In this method, 5.0 ml of 1% agarose digested in saline was spread evenly on a clean grease free slide and allowed to solidify. Cups of 2.0 mm diameter were bored 4.0 mm apart in solidified agar using a cup borer. The cups at periphery were filled with antigen (the toxin purified by various methods), whereas the central cup was filled with antiserum obtained from immunized rabbits. The slide was left at room temperature in a moist chamber for 3 days to develop the precipitin lines.

* Antigens employed - i) NAG Crude toxin ii) Primarily purified V. cholerae toxin (by Ammonium sulphate precipitation method and by Aluminium phosphate gel adsorption method) and iii) Fraction I and Fraction II eluted from Sephadex G 75 column.

Staining of the slide

Before staining, the slides were washed with repeated changes of saline for 48 to 72 hours; by pouring saline from the side into a petri dish containing the slide, and allowing it to stand for 15 to 20 minutes. The saline was then removed with a capillary pipette without disturbing the gel. The slides were further dried overnight beneath a Whatman filter paper and stained with 0.5% Amido Black B for 15 minutes, and later destained with 2% acetic acid. ☺ ☺ ☺ ☺ ☺ ☺

The reagents used in this test are listed in Appendix II 10.

2.8.5. In vivo Immunoprotection Studies

Three weeks after immunization the rabbits were challenged intraintestinally by ligated ileal loop technique with 569 B Inaba cells (1×10^8 organisms per loop) and different concentrations of 569 B Inaba toxin (10, 50 and 100 µg/loop). In cross-immunoprotection studies 1×10^6 viable cells of NAG vibrios were used for challenge.

The rabbits were said to be successfully protected when

there was no fluid accumulation, and 'susceptible' when
fluid accumulated in the intestinal loops;

.....

CHAPTER III

Results and Discussion

Results and Discussion

3.1. Production of Cholera Exo-enterotoxin

The production of heat labile exo-enterotoxin of cholera tends to be complicated by the strain to strain variation in conditions optimal for toxin production. Various synthetic and semisynthetic media, as well as, simple peptone water have been shown to support high yields of cholera toxin from vibrio strains (Craig 1966, Finkelstein 1966a and Craig 1965).

Datta and Habbu (1955) observed that, highly pathogenic strains such as V.cholerae 569 B Inaba, failed to produce toxin in peptone water media. Their studies on exaltation of 569 B Inaba strain led to the development of 'rabbit passage technique'. 'Serial passages through infant rabbit intestine' is now the widely employed technique for exaltation of not only classical Inaba vibrios but also El-Tor and NAG strains. In the present studies, a rabbit passaged strain of 569 B Inaba was, therefore, employed to obtain high yields of cholera toxin.

As for the medium for toxin production, from his extensive studies with 569 B Inaba strain, Finkelstein (1966a) concluded that Syncase medium provides optimal conditions for maximum toxin yield. Hence, in the present investigations, Syncase medium was employed.

However, in the preliminary studies, the rabbit passaged strain failed to produce sufficiently high titre of cholera toxin

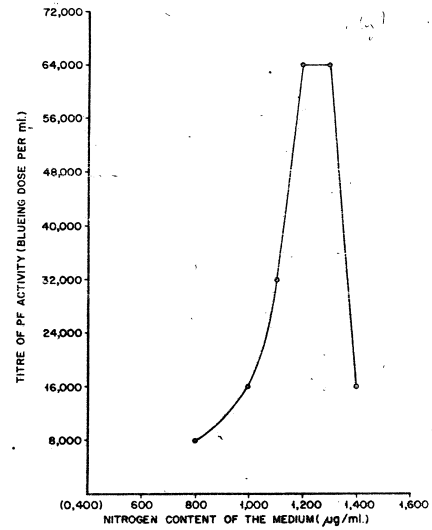
in Syncase medium. It was, therefore, felt necessary to modify the culture medium. Variation in the concentration of nitrogen content i.e. in a concentration of Casamino acids of the medium was tried. Maximum toxin yield was obtained around 1.2 - 1.3 gm % Casamino acids (Figure 1), whereas the original concentration of Casamino acids in Finkelstein's medium was 1.0 gm %. Hence, Syncase basal salt medium supplemented with 1.2 gm % of Casamino acids was used for bulk quantity preparation of crude cholera toxin using 569 B Inaba strain.

Besides nitrogen concentration, various other factors such as inoculum size, aeration rate and incubation period influence toxin production.

The inoculum employed for cholera toxin production (2.1.2.) consists of 0.1 ml of log phase culture i.e. about 10^7 cells per 100 ml. of the medium (Finkelstein 1970). Other investigators have also recommended 1.0 ml inoculum, i.e. about 10^8 cells per 100 ml. of the medium (1.0 ml of the inoculum per 100 ml. of the medium, Richardson *et al.*, 1969). However, the present studies indicated that higher yields of toxin were obtained with 0.1% inoculum (10^7 cells per 100 ml. medium) than with 1.0% inoculum (10^8 cells per 100 ml. medium), and hence, in further studies 0.1% inoculum was used for toxin production.

Although V. cholerae grow more rapidly under partially anaerobic conditions, adequate aeration of the medium is

**FIG-1: THE INFLUENCE OF NITROGEN CONTENT OF
THE MEDIUM ON THE YIELD OF CHOLERA TOXIN**



essential for high titre cholera toxin production. It has also been observed that there is no correlation between growth of the culture and toxin production (Lewis *et al.*, 1977). Maximum enterotoxin is produced when the surface/volume ratio of the medium is 1.25 to 2.5 cm²/ml in stationary culture (De *et al.*, 1962). Roux bottles incubated in horizontal position have been recommended by Craig (1965), while Finkelstein *et al.*, (1965) recommended aerated cultures. Finkelstein and LoSpalluto (1969) reported that aerated fermenter cultures can support higher yields of cholera toxin. Other investigators have used relatively small quantities of culture medium in large Erlenmeyer flasks (50 ml. medium in 250 ml. flasks or 100 ml. medium in 500 ml. flasks) with constant shaking during incubation. In the present studies, 100 ml. medium in 500 ml. flasks on a rotary shaker (180 r.p.m.) produced maximal toxin titre.

As for the release of cholera toxin from the cells, it has been reported that toxin diffuses freely from the cells rather than being released on autolysis (Richardson, 1969). The appearance of the toxin in the medium is parallel to the exponential growth phase of the culture. Whereas Finkelstein *et al.*, (1966a) have reported maximum toxin production during the stationary phase of the culture i.e. 12-16 hours incubation period (inoculum size being 10⁷ cells per 100 ml. medium). On further incubation, the titre of the toxin remains unchanged, however, cell lysis initiates and impurities

such as the cell debris, lipopolysaccharide, (endotoxin, somatic antigen) and other bacterial antigens are added to the crude extract, thus complicating the purification process. It is, therefore, advisable to collect the crude extract before initiation of cell lysis.

The effect of temperature on toxin yield has been studied by Fernandes and Smith (1977). They have reported higher yields of toxin at 30°C than at 37°C. However, in the absence of facilities for growing the cultures at 30°C on a rotary shaker, the present investigation was carried out with cultures grown at 37°C on a rotary shaker for 12-16 hours using 0.1% inoculum (1×10^7 organisms/100 ml. medium).

3.2. Purification of Cholera Etx-enterotoxin

Since past three decades, for the purification of cholera toxin, various methods have been employed. Developments in instrumentation technology have minimized the duration and labour required to achieve pure toxin. With the employment of sophisticated instruments like ultra filters and double gel chromatography, large quantities of highly purified toxin can be obtained, but, the cost increases tremendously and in a developing country like ours, it is not feasible to employ such expensive techniques, especially when good results can be obtained by other simple and economic methods. Hence, in the present studies less expensive methods such as precipitation with Ammonium sulphate and Sodium hexametaphosphate, and adsorption on Aluminium phosphate gel were used.

Whenever possible, the crude extract was processed immediately.

Prior to purification, the crude cholera toxin was subjected to protein estimation by Lowry's method. The protein content of the test sample was calculated from the standard graph of protein estimation presented in Figure 2.

The PF activity (permeability factor activity) of the crude toxin was also determined as described in 2.6.1. This was necessary to obtain a rough idea of the toxin content of the starting material.

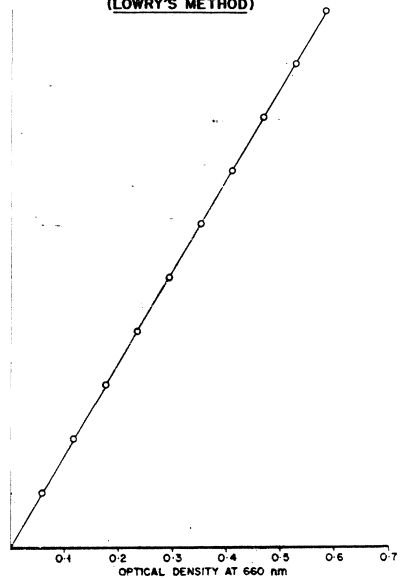
3.2.1. Primary Purification

Various methods were compared for primary purification of cholera toxin. These were -

1. Ammonium sulphate precipitation method (Kaur et al., 1969),
2. Sodium hexametaphosphate precipitation method (Spyrides and Feeley, 1970), and
3. Aluminium phosphate gel adsorption method (Rappaport et al., 1970).

Kaur et al., (1969) used Ammonium sulphate precipitation method for primary purification of cholera toxin, wherein Ammonium sulphate to 90% saturation was employed. In the present studies, 100% saturation with Ammonium sulphate was employed to recover higher yields of the toxin from the supernatant (As per the observations of D.S.Chitnds, Haffkine Institute - personal communication).

**-2: STANDARD CURVE FOR PROTEIN ESTIMATION
(LOWRY'S METHOD)**



However, it was observed that Ammonium sulphate precipitation method though simple, is not specific, for it precipitates large amount of lipopolysaccharide (Table 4) and non-specific proteins (Figure 9). The results of Ammonium sulphate precipitation method are presented in Table 1.

The other methods used in the primary purification of cholera toxin were Sodium hexametaphosphate precipitation method and Aluminium phosphate gel adsorption method. Tables 2 and 3 summarize the results obtained by using these two methods in their original formulae and with the modifications employed in this study. The concentrations of Sodium hexametaphosphate salt and Aluminium phosphate gel used in the original methods appeared to be insufficient as seen from 'percent recovery' and the 'fold increase in specific activity'. As evident from Table 2 and Figure 3, concentration of Sodium hexametaphosphate was increased by four times to precipitate maximum amount of toxin. Similarly, four fold increase in the Aluminium phosphate gel concentration yielded maximum specific activity (Table 3, Figure 4).

Table 1

Primary purification by Ammonium sulphate precipitation method

Stage of purification	Volume (in ml.)	EE activity in bluing dose/ml.	Total skin activity	Protein content (in ug/ml.)	Total protein	Specific activity (bluing dose/ug)	Fold increase in specific activity	% recovery in skin activity	% loss in skin activity
Crude extract	1200	16000	19200000	610	732000	26.23	-	-	-
Supernatant	1200	2000	2400000	N.D.	N.D.	N.D.	N.D.	-	12.5
Toxin dissolved in distilled water (before dialysis)	120	64000	7680000	830	99600	77.1	2.9	40.0	-
Toxin after dialysis	180	32000	5760000	610	91800	52.4	2.0	30.0	-
Toxin lyophilized and reconstituted	50	64000	3200000	450	22500	142.2	5.42	16.66	-

N.D. - Not detectable

Table 2

Primary purification by modifications of Sodium hexametaphosphate precipitation method

Stage of purification	Flask number									
	1	2	3	4	5	6	7	8	9	10
Grams of Sodium hexametaphosphate added per 100 ml. of crude extract	0.255	0.510	0.765	1.02	1.275	1.53	1.785	2.040	2.295	2.55
Supernatant (100 ml.)										
Protein content in mg/ml.	360	355	340	310	280	230	210	205	190	185
Skin activity in bluing dose/ml.	4000	4000	4000	4000	4000	4000	4000	4000	2000	2000
% loss in skin activity	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	6.25	6.25
Toxin suspended in 7 ml. Na ₂ HPO ₄	32000	32000	64000	128000	32000	32000	32000	32000	32000	32000
skin activity in bluing dose/ml.										
Protein content in mg/ml.	215	225	240	250	280	295	320	335	345	360
Specific activity in bluing dose/mg	148.8	142.2	211.7	521.1	144.2	109.4	100	95.5	93.2	88.9
Fold increase in specific activity	3.02	2.89	4.30	10.6	9.23	2.22	2.83	1.94	90.95	0.17
% recovery in skin activity	7.0	7.0	14.0	28.0	7.0	7.0	7.0	7.0	7.0	7.0

Each flask contains 100 ml. of crude extract of following specifications :

Protein content - 650 mg/ml., skin activity - 32000 bluing dose/ml., specific activity - 49.22 bluing dose/mg.

Legend * = 0.255 gm. % addition of Sodium hexametaphosphate (flask No.1) was recommended by Gyrdes and Feeley (1970),
whereas, flask number 2 to 10 are the modifications followed in the present studies.

**FIG-3: TOXIN PURIFICATION: OPTIMAL CONCENTRATION OF SODIUM
HEXAMETAPHOSPHATE**

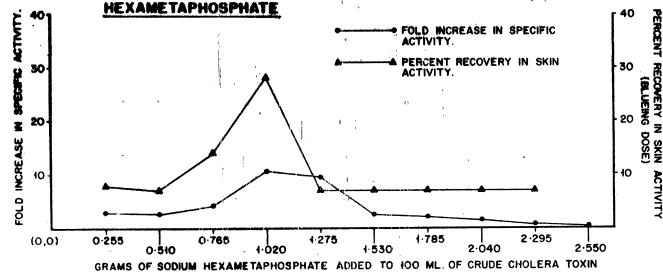


Table 3

Primary purification by modifications of Aluminium phosphate gel adsorption method

Stage of purification	Flask number									
	1	2	3	4	5	6	7	8	9	10
Alum potash added (20% w/v in ml.)	1	2	3	4	5	6	7	8	9	10
Aluminium phosphate gel conc.	0.2%	0.4%	0.6%	0.8%	1.0%	1.2%	1.4%	1.6%	1.8%	2.0%
Supernatant (100 ml.)										
Protein in $\mu\text{g/ml.}$	605	580	560	530	520	535	555	560	575	585
Skin activity (B.D./ml.)	8000	4000	4000	2000	2000	2000	4000	4000	4000	4000
% loss in skin activity	25.0	12.5	12.5	6.25	6.25	6.25	12.5	12.5	12.5	12.5
Toxin in 10 ml. Sodium citrate before dialysis										
Protein in $\mu\text{g/ml.}$	135	150	175	210	225	200	185	170	165	150
Skin activity (B.D./ml.)	64000	64000	80000	256000	256000	128000	64000	64000	32000	32000
% recovery in skin activity	20	20	25	80	80	40	20	20	10	10
Specific activity	474.1	426.7	457.0	1219.1	1137.8	640.0	346.0	376.5	193.9	213.3
Fold increase in specific activity	9.6	8.7	9.3	24.8	23.1	13.0	7.0	7.7	3.9	4.3
Toxin after dialysis (in ml.)	14.5	14.5	14.5	15.0	15.0	20.0	25.0	27.0	30.0	32.0
Protein in $\mu\text{g/ml.}$	85	100	115	130	140	100	70	55	50	45
Skin activity (B.D./ml.)	40000	40000	64000	128000	128000	32000	16000	16000	8000	8000
% recovery in skin activity	18.0	18.0	29.0	60.0	60.0	20.0	12.5	13.5	7.5	8.0
Specific activity	470.6	400.0	278.3	984.6	914.3	320.0	228.5	250.0	160.0	177.8
Fold increase in sp.act.	9.6	8.2	5.7	20.0	18.6	6.5	4.6	5.9	3.3	3.6
Amount of conc. toxin in ml.	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Protein in $\mu\text{g/ml.}$	230	320	360	380	450	485	430	365	365	360
Skin activity (B.D./ml.)	128000	128000	256000	512000	512000	128000	64000	64000	32000	32000
% recovery in skin activity	16.0	16.0	32.0	64.0	64.0	16.0	8.0	8.0	4.0	4.0
Specific activity	556.5	400.0	711.1	1347.4	1137.8	263.9	148.8	175.2	87.7	88.9
Fold increase in sp.act.	11.3	8.13	14.4	27.4	23.1	5.4	3.0	3.6	1.8	1.8

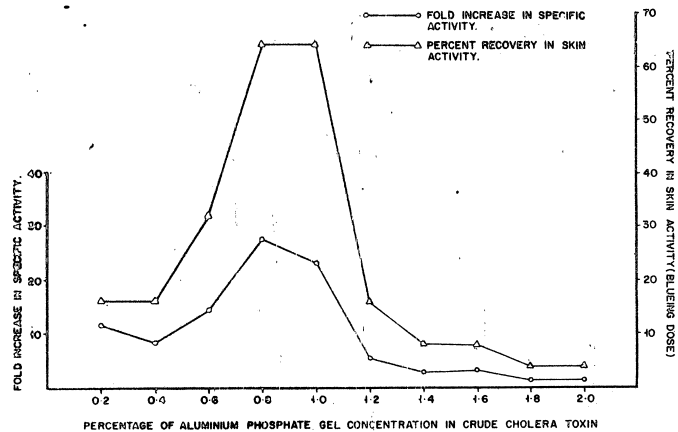
Each flask contained 100 ml. of crude extract of following specifications : Protein content - 650 $\mu\text{g/ml.}$, Skin activity - 32000 B.D./ml.
Specific activity - 49.22.

Legend * = Bluing dose

** = Specific activity

@ = 0.2% concentration of Aluminium phosphate gel (flask No.1) for adsorption of cholera toxin was recommended by Rappaport et al., (1970) whereas flask No.2 to 10 are modifications employed in the present studies.

FIG-4: TOXIN PURIFICATION: OPTIMAL CONCENTRATION OF $AlPO_4$ GEL



The toxin purified by using five primary purification methods -

1. Ammonium sulphate precipitation method,
2. Unmodified Sodium hexametaphosphate (0.255 gm.%) precipitation method,
3. Modified Sodium hexametaphosphate (1.02 gm.%) precipitation method,
4. Unmodified Aluminium phosphate gel (0.2 %) adsorption method and
5. Modified Aluminium phosphate gel (0.8 %) adsorption method

was subjected to purity (2.5.) and toxicity (2.6.) tests.

The purity of the toxin was tested in two different ways -

1. The presence of a single protein species was determined by polyacrylamide (2.5.1.) and SDS (2.5.2.) gel electrophoresis and

2. Total absence of somatic antigen (LPS) by Passive Haemagglutination Inhibition (PHI) test (2.5.3., Figure 5).

The toxicity was attributed to two factors -

1. PF activity as detected by skin test (2.6.1., Figure 6) and
2. Enterotoxin activity by adult rabbit ligated ileal loop technique (2.6.2., Figure 7).

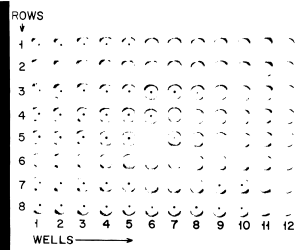


Figure - 5 PH1 Test

Button formation - PH1 test positive
i.e. LPS present

No button formation - PH1 test negative
i.e. LPS absent

First row - LPS standard

Sixth row - Fraction II (F II)
eluted from Sephadex G 75

Seventh row - Wells 9 & 10 - Cell control
Wells 11 & 12 - Serum control

Remaining rows elucidate results for LPS
content of different ' toxin ' samples

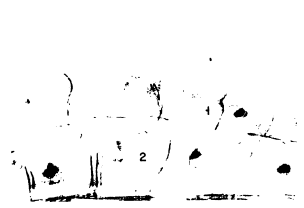


Figure - 6 Rabbit Skin Test

- Legend
1. Skin test negative
(saline control)
 2. Skin test positive

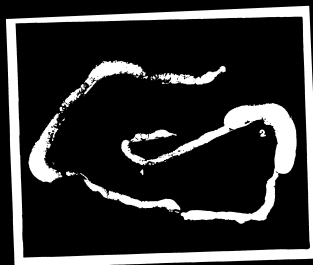


Figure - 7 Adult Rabbit Ileal Loop Test

Legend 1. No fluid accumulation
(saline control)
2. Fluid accumulation;
enterotoxin present in the
sample

Comparison of the original and modified methods on the basis of 'percent recovery of skin activity', 'fold increase in specific activity', number of bands as detected by polyacrylamide disc electrophoresis (Figures 9,10 and 11) and efficiency to eliminate the somatic antigen impurities (LPS, Table 4 and Figure 8) indicates that when modified methods are employed, along with the increase in specific activity and percent recovery, the LPS content of a purified product is also increased considerably. However, when gel filtration is to follow a primary precipitation method, the criterion is to obtain maximum percent recovery and specific activity of the enterotoxin ignoring the increased LPS contamination as the LPS is easily separated on gel-filtration.

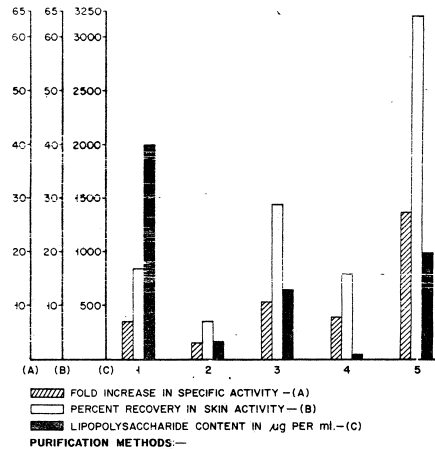
Therefore, the modified Aluminum phosphate gel adsorption method using 0.8 % gel, which yields the maximum percent recovery and maximum increase in specific activity of enterotoxin was selected inspite of the high LPS contamination for the primary purification of cholera toxin. Moreover, this method was found relatively selective for cholera toxin precipitation as the number of contaminating proteins are significantly reduced (2 bands were observed in polyacrylamide gel, Figure 9). The toxin obtained by this method was then subjected to further purification.

Table 4

Comparison of the methods used for primary purification

Method	Fold increase in Specific activity	Percent recovery in skin activity	Percent loss of toxin in supernatant	LF8 content (in $\mu\text{g/ml.}$)	Number of bands in poly- acrylamide gel
Ammonium sulphate precipitation	5.421	16.66	12.58	2000	15
Unmodified Sodium hexametaphosphate (0.25%) precipitation	3.02	7.00	12.5	160	9
Modified Sodium hexametaphosphate (1.02%) precipitation	10.59	28.00	12.5	640	9
Unmodified Aluminium phosphate gel (0.2%) Adsorption	7.96	16.00	25.0	50	2
Modified Aluminium phosphate gel (0.8%) Adsorption	27.37	64.00	6.25	1000	2

FIG-8: TOXIN PURIFICATION : COMPARITIVE STUDY.



1. AMMONIUM SULPHATE PRECIPITATION.
2. SODIUM HEXAMETAPHOSPHATE PRECIPITATION (UNMODIFIED).
3. MODIFIED SODIUM HEXAMETAPHOSPHATE (4.02 %) PRECIPITATION.
4. ALUMINIUM PHOSPHATE GEL ADSORPTION (UNMODIFIED).
5. MODIFIED ALUMINIUM PHOSPHATE GEL (0.8%) ADSORPTION.

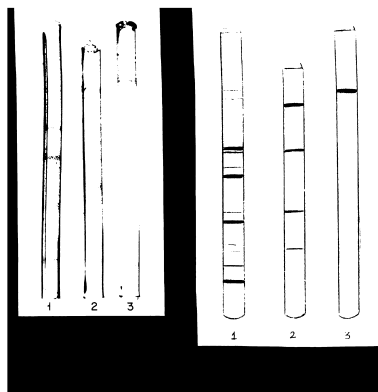
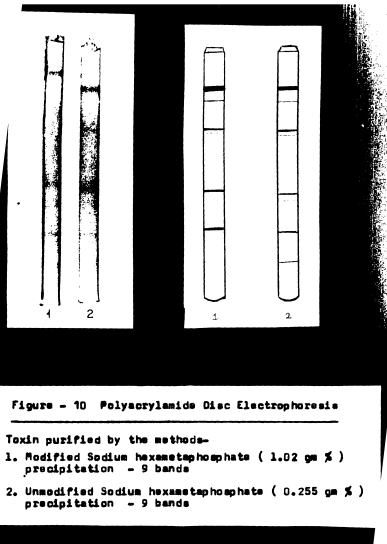


Figure - 9 Polyacrylamide Disc Electrophoresis :

Comparison of the toxin primarily purified by the methods -

1. Ammonium sulphate precipitation - 15 bands
2. Sodium hexametaphosphate (0.255 gm %) precipitation - 9 bands
3. Aluminium phosphate gel (0.2 %) adsorption - 2 bands



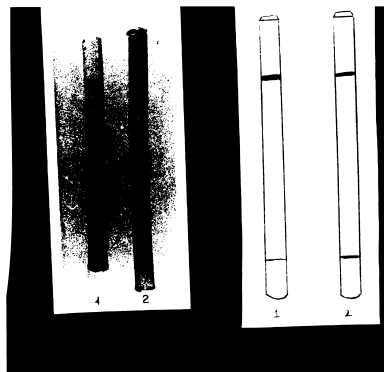


Figure - 11 Polyacrylamide Disc Electrophoresis

Toxin purified by the methods-

1. Modified Aluminium phosphate gel (0.8 %)
adsorption - 2 bands
2. Unmodified Aluminium phosphate gel (0.2 %)
adsorption - 2 bands

3:2:2. Secondary purification

The partially purified toxin obtained by adsorption on Aluminium phosphate gel (0.8%) was subjected to gel filtration using Sephadex G 75 column chromatography (2:3.2.).

The elution pattern of G 75 filtration (Figure 12) was similar to that obtained by Finkelstein *et al.*, (1970). The high molecular weight somatic impurities formed the first peak (tubes No. 18 to 20) designated as F I, while the second peak; F II (tubes No. 33 to 57) contained the toxin exhibiting both the PF activity and the enterotoxin activity.

Complete characterization of the two fractions eluted from Sephadex G 75 is summarized in Table 5.

The absorbance pattern of these fractions in the U.V. range showing maximum absorbance between 260 and 280 nm (characteristic of a protein, Figure 13) is similar to that obtained by Rappaport *et al.*, (1974b).

The dose response curve of F II is shown in Figure 14. The effective toxicity dose 50, i.e. the concentration of toxin giving half the maximum fluid accumulation can be determined from this curve. It is also seen from this figure that the maximum amount of fluid accumulated was 310 ml. with a dose of 0.4 µg. The maximum amount reported in the literature is 275 ml. with a dose of 1.3 µg (Kasai and Burrows, 1966; Burrows and Mustekis, 1966).

FIG.12: FRACTIONATION ON SEPHADEX G-75

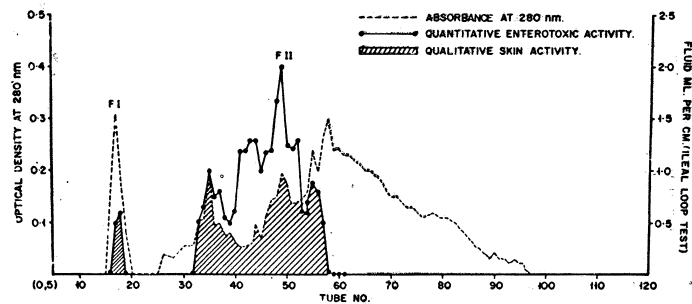


Table 5

Characterization of the two fractions eluted from Sephadex G 75

Parameters	F I	F II
Volume (in ml.)	1.6	3.6
Protein content (in $\mu\text{g/ml.}$)	250	100
Approximate molecular weight in daltons	1,00,000	45,000-50,000
LPS content (per ml.)	400 μg	< 80 ng
Enterotoxin units (1.0 ml. of the highest dilution giving positive reaction)	2	4800
PF activity (bluing dose per ml.)	100	2,56,000
Specific activity (PF units per $\mu\text{g.}$)	0.4	2,560
Fold increase in specific activity (with respect to the crude toxin)	8.13×10^{-3}	6446
Percent recovery of the fraction (Total 1920 μg of the protein were loaded in the column)	26.32	23.7
Nature of the precipitin lines formed in agar gel (Microcutcherry test)	Single, brownish precipitin line, hence, homogenous	Double, white opalescent precipitin lines, hence, heterogenous

**FIG.13: ABSORBANCE PATTERN OF
F I AND F II FRACTIONS IN THE U.V. RANGE.**

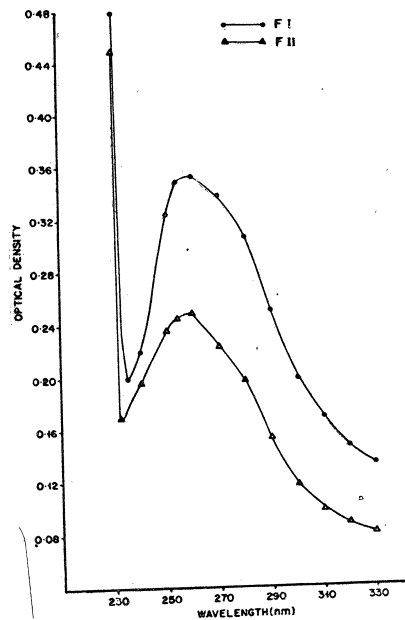
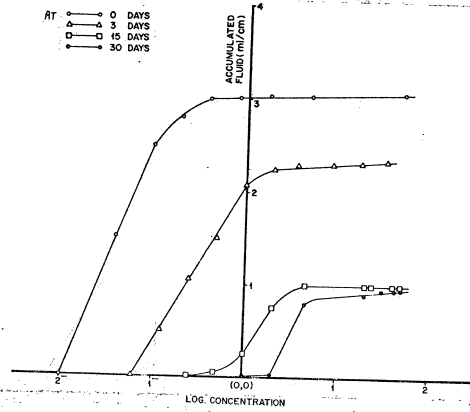


FIG-14: DOSE RESPONSE CURVE



The molecular weight of F I was estimated to be approximately 1,00,000 from the elution pattern, which is characteristic of bacterial endotoxin. Passive haemagglutination inhibition test (Figure 5) confirmed the endotoxic nature of F I (Table 5).

The molecular weight of cholera toxin has been worked out by different investigators and these estimates fall in a very wide range, 3397 daltons being the least and 180,000 the highest as given below -

<u>Method employed</u>	<u>Molecular weight in daltons</u>
Elution profile of standard dextran (Coleman <i>et al.</i> , 1967)	50,000 to 1,00,000
Dialysis retentate level (Finkelstein and LoSpalluto, 1970)	1,00,000
Thin layer gel filtration (Coleman <i>et al.</i> , 1968)	12,000 to 14,000
Agarose gel filtration (Finkelstein and LoSpalluto, 1969)	20,000
Gel filtration (Holmgren <i>et al.</i> , 1971)	25,000 to 38,000
Sephadex G 75 filtration (Finkelstein and LoSpalluto, 1969)	61,000
Sephadex G 150 filtration (Duhamel <i>et al.</i> , 1970)	60,000
Sephadex G 75 filtration (Holmgren <i>et al.</i> , 1971)	55,000 to 60,000
Sedimentation-velocity diffusion method (Finkelstein and LoSpalluto, 1972)	82,000

<u>Method employed</u>	<u>Molecular weight in daltons</u>
Membrane depletion method (Finkelstein and LoSpalluto, 1972)	84,000
Amino acid analysis - (Coleman <i>et al.</i> , 1968)	7,740
(Inwood and Tyrell, 1970)	3,397
(Finkelstein and LoSpalluto, 1972)	7,664

From the elution pattern obtained in the present studies, the molecular weight of the enterotoxin can be estimated as approximately 45,000 (Figure 12). On the basis of SDS gel electrophoretic mobility (Figure 15), the molecular weight of F II falls around 48,000 - 50,000 daltons (calculated as per Weber and Osborn, 1969; 2.5.2.).

Further, F II was subjected to stability studies using ligated ileal loop technique (2.6.3.). The results elucidated in Table 6 confirms the findings of Grady and Chang (1970) that, cholera toxin is unstable in suspended state.

On final purification on Sephadex G 75, the total increase in the specific activity of the toxin over the crude toxin was 65.46 (Table 5). This toxin was free from other protein impurities as well as from LPS, as seen from the polyacrylamide disc electrophoretic pattern and PHI test (Figures 16 and 5), which is in agreement with the recent reports of Finkelstein *et al.*, (1976) that Sephadex G 75 column chromatography renders cholera toxin free from all the impurities.

Figure - 15 SDS Gel Electrophoresis

Fraction II (F II) eluted from Sephadex G 75
exhibiting single band

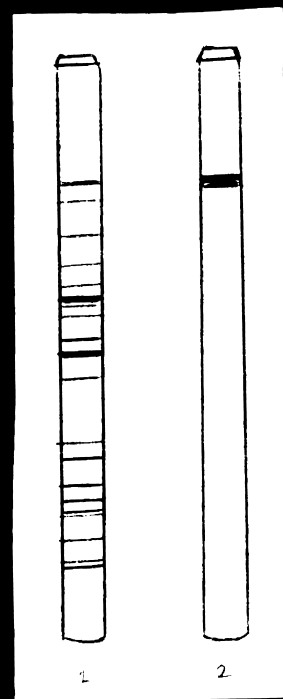
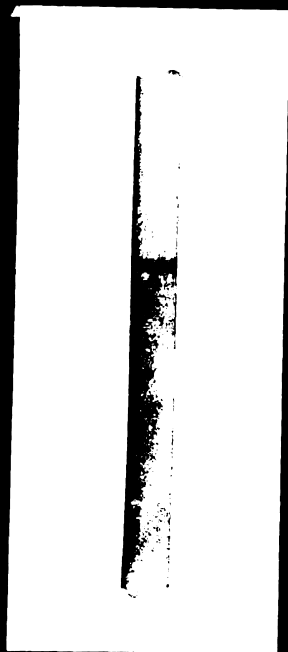


Figure 16 - Polyacrylamide Disc Electrophoresis

- Legend -
1. Crude cholera toxin - 20 bands
 2. F II - highly purified cholera toxin - single band

Table 6

Stability of the purified toxin (F II)

Toxin (ug/ml.)	Storage : Number of days			
	0	3	15	30
Control (saline)	0	0	0	0
0.125	2.6 *	0.5	0	0
0.25	2.88	1.08	0	0
0.5	3.0	1.5	0.05	0
1.0	3.03	2.1	0.25	0
2.0	3.05	2.25	0.75	0.07
4.0	3.05	2.3	0.95	0.6
8.0	3.05	2.3	1.0	0.87
16.0	3.05	2.33	1.0	0.9
32.0	3.05	2.35	1.0	0.94

Legend * = ml/cm. of fluid accumulated on injecting in Adult rabbit ileal loop.

3.3. Immunoprotection studies

Convalescent cholera patients exhibit rise in both antibacterial and antitoxic antibody titres (Pierce et al., 1970), however, the conventional cholera vaccines that contain killed cholera vibrios stimulate high titre of antibacterial antibodies but negligible titre of antitoxic antibodies (Mosley et al., 1970). This probably accounts for the fact that, the conventional cholera vaccine prove inefficient immunising agents (Basu et al., 1970). Field trials with vaccine that contained Vibrio cholerae somatic antigens (Killed cells) as well as purified toxoid have brought out encouraging results (Basu et al., 1970).

In the present investigation, in order to determine the efficiency of the cholera toxoid prepared by formalinization of the primarily purified toxin (using Ammonium sulphate precipitation method), rabbits were immunised with the toxoid and challenged intrainstestinally with different concentrations of cholera toxin as well as with viable classical (569 B Inaba) and NAG vibrios (2.8.5.).

As described in 2.8.3. the standardization of haemagglutination test was carried out. Table 7 depicts the results for this test, while Table 8 shows that the immunised rabbits developed both the antitoxic and antibacterial antibodies, as the partially purified toxin containing a large amount of

Table 7
Standardization of Haemagglutination test

Sheep RBCs sensitized with	Titre for Haemagglutination test
400 µg toxin	1 : 160
200 µg toxin	1 : 80
100 µg toxin	1 : 40
40 µg toxin	1 : 40

Table 8
Results for 'Haemagglutination' and 'Bacterial Agglutination' test

Test employed	Mean titre		
	Antibodies	Before immunization	After immunization
Haemagglutination test	Antitoxic	Absent	1:2560
Bacterial agglutination test	Antibacterial (against somatic antigen)	1 : 10	1:1280

Table 9

In vivo Immunoprotection Studies

Challenged with	Fluid accumulation*		
	Control rabbit	Rabbit immunized with 569 B Inaba toxoid 4 dose schedule [@]	7 dose schedule [@]
Saline (negative control)	-	-	-
10 µg toxin	+	-	-
50 µg toxin	+	-	-
100 µg toxin	+	+	-
569 B Inaba (10^6 cells)	+	-	-
NAG C 128 (10^6 cells)	+	+	+
JPW 8 NAG (10^6 cells)	+	+	+
NAG Vibrio 2 (10^6 cells)	+	+	+
Ram NAG (10^6 cells)	+	+	+
NAG 280 (10^6 cells)	+	+	+

Legend * = Tested by adult rabbit ligated ileal loop technique (2.6.2.)

@ = Average results obtained from two rabbits

- = No fluid accumulation (ratio of fluid in ml. per centimetre less than 1.0)

+ = Fluid accumulation (ratio of fluid in ml. per centimetre more than 1.0)

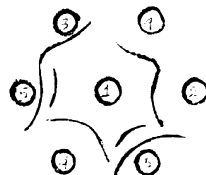
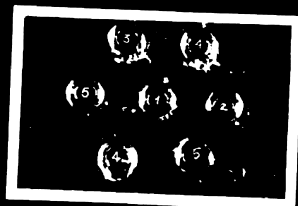


Figure - 17 Microoutchertony Test

- Legend -
1. Cholera antiserum
(against V. cholerae 569 B Inaba)
 2. NAG crude toxin
 3. 569 B Inaba toxin
primarily purified by Aluminium
phosphate gel adsorption method
 4. F I
 5. F II

CHAPTER IV

5

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Summary and Conclusions

Summary and Conclusions

The aim of this study was to obtain purified high titre cholera toxin using simple, economic procedures.

Efforts were also directed towards the immunoprotection studies using a primarily purified and formalinized toxin as an immunogen and challenging the rabbits intraintestinally with classical as well as NAG strains and 569 B Inaba toxin.

Modified Syncase medium i.e., basal salt medium supplemented with 1.2 gm.% Casamino acids was used to obtain high titre crude toxin. The NAG strains which have been reported as nontoxigenic *in vitro*, also yielded high titre of toxin in the same medium. It was also observed that for toxin production by NAG vibrios, enrichment with extra proteinic source such as rabbit intestinal extract was not required.

The efficiency of various methods used for cholera toxin purification was assessed. Modified Aluminium phosphate gel (0.8%) adsorption method proved the best for primary purification while gel filtration on Sephadex G 75 effectively eliminated the endotoxin (LPS). In fact, the final purified toxin obtained was totally free from endotoxin impurities as detected by an extremely sensitive method such as Passive Haemagglutination Inhibition (PHI) test. Moreover, absence of protein impurities was confirmed by polyacrylamide disc electrophoresis and SDS

gel electrophoresis.

The studies on purified cholera toxin revealed that, the absorbance pattern (in the U.V. range) of cholera toxin was characteristic of a protein with maximum absorbance between 260 and 280 nm. The studies on stability of cholera toxin showed cholera toxin to be highly unstable in liquid form. A significant increase in specific activity (64.46) was obtained by the combination of two methods employed in the present investigation viz. modified Aluminium phosphate gel adsorption method as a primary purification, followed by Sephadex G 75 column chromatography as a secondary purification method.

Primarily purified toxin was formalinized and used as an immunogen. Rabbits thus immunized were completely protected against 569 B Inaba infection, but, not against the NAG vibrios.

The *in vitro* immunological studies (agar gel diffusion using microcutcherry test) presented some interesting results. It was observed that, the endotoxin of classical and NAG vibrios were similar if not identical but, the enterotoxin of both these vibrios were antigenically different.

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Appendix

Appendix

Media and Reagents

I. Media

1. Syncase medium (Finkelstein et al., 1966)

Na_2HPO_4	...	5.0 g.	
KH_2PO_4	...	5.0 g.	
Sucrose	...	5.0 g.	
NH_4Cl	...	1.18 g.	
Na_2SO_4	...	0.089 g.	1% Solution
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	...	0.042 g.	8.9 ml.
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	...	0.004 g.	0.4 ml.
$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	...	0.005 g.	0.5 ml.
Casamino acids	...	10.0 g.	
Distilled water	...	1000.0 ml.	
pH	...	7.5	

The medium was dispensed in 100 ml. amount in 500 ml. capacity Erlenmeyer flasks and sterilized by autoclaving at 116°C for 20 minutes.

2. Modified Syncase medium

Syncase medium less Casamino acids was prepared and to 100 ml. of the medium required quantities of casamino acids were added -

Casamino acids (in g/l)	Nitrogen concentration (in ug per ml.)
0.8	800
1.0	1000
1.1	1100
1.2	1200
1.3	1300
1.4	1400

(1 g. Casamino acids contains 100 mg. of Nitrogen)

The pH of the medium was adjusted to 7.5.

The media were dispensed in 100 ml. amount in 500 ml. capacity Erlenmeyer flasks and sterilized by autoclaving at 116°C for 20 minutes.

III. Reagents

1. Merthiolate solution

One gram percent merthiolate solution was prepared in distilled water and stored at 4°C. One ml. of the solution was added to 100 ml. culture so as to achieve a final concentration of 0.0001 g/ml.

2. Levy's method

1) Standard protein solution

Stock solution (1 mg/ml) : Gamma globulin ampoule from Processing Unit of H.B.P.C.L. labelled 10743 60% was

carefully opened and mixed thoroughly with 103.3 ml² of 0.1 N NaOH, stored at 4°C.

'Working protein standard' (100 µg/ml): This was freshly prepared by 1:10 dilution of stock solution in saline.

ii) 0.1 N NaOH

0.4 grams of NaOH were dissolved in 100 ml. of distilled water. Stored at room temperature.

iii) Alkaline CuSO₄ or Solution 'C'

Solution 'A': To the solution of 100 mg. Na.K.Tartarate in 10 ml. distilled water add 50 mg. of CuSO₄·3H₂O. Stored at room temperature.

Solution 'B': 2% Na₂CO₃ anhydrous in 0.1 N NaOH. Stored at room temperature.

Solution 'C': 1 ml. of Solution 'A' was mixed with 50 ml. of Solution 'B' to get Solution 'C'. This solution was prepared freshly before use. (The solution should not be turbid)

iv) Folin-Phenol Catecholic Reagent

Stock solution was obtained from M/s Beland Fine Chemicals Private Limited, Bombay. Stored at 4°C.

Stock solution was freshly diluted 1:1 with 0.1 N NaOH and used.

3. Ammonium sulphate method2% BaCl₂ solution

2 grams of BaCl₂ were dissolved in 100 ml. of distilled water. Stored at 4°C.

4. Sodium Hexametaphosphate precipitation method1) 0.2 M NaCl, pH 4.5

11.69 grams of NaCl were dissolved in 1000 ml. of distilled water. The pH was adjusted to 4.5 using concentrated HCl. Stored at room temperature.

11) 0.15 M Na₂HPO₄, pH 8.0

21.30 grams of Na₂HPO₄ were dissolved in 1000 ml. of distilled water. The pH was adjusted to 8.0 using 10 N NaOH. Stored at room temperature.

5. Ammonium phosphate Gel adsorption method1) 20% w/v alum potash

5 grams of alum potash were added to 25 ml. of distilled water and heated gently to get uniform solution. Prepared freshly before use.

11) 0.1 M Phosphate buffer, pH 6.4

Solution A : 0.1 M NaH₂PO₄·2H₂O was prepared by adding 3.9 grams of NaH₂PO₄·2H₂O to 400 ml. of distilled water. Stored at room temperature.

Solution B : 0.1 M $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ was prepared by adding 5237 grams of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ to 150 ml of distilled water. Stored at room temperature.

367.5 ml of Solution A was mixed with 132.5 ml of Solution B to get 0.1 M Phosphate buffer, pH 6.4. Stored at room temperature.

iii) 0.1 M Sodium citrate, pH 8.5

14.706 grams of Sodium citrate were dissolved in 500 ml. of distilled water. The pH was adjusted to 8.5 using 10 N NaOH. Stored at room temperature.

6. Gel chromatography

0.4 M Ammonium formate buffer

23 grams of Ammonium formate were dissolved in 1000 ml. of distilled water. The pH was adjusted to 8.2. To this, 0.2 grams of Sodium azide were added as a preservative. Stored at 4°C.

7. Anionic Polyacrylamide Disc electrophoresis

1) Lower gel

<u>Solution 'A'</u> :	1 N HCl	2470 ml.
	Tris (EDTA)	18.2 g.
	TEMED	0.23 ml.
	Distilled water to	100 ml.
	pH	8.9

Solution 'C' : Acrylamide 30%0 g.
 Bis-acrylamide 0.8 g.
 Water to 100 ml.

Solution 'F' : Ammonium persulphate 0.24 grams of
 Ammonium persulphate were dissolved in 100 ml² distilled water.
 (freshly prepared).

One part of Solution 'A' (6.0 ml.), one part of
 Solution 'C' (6.0 ml.), two parts of Solution 'F' (12.0 ml.)
 were mixed and used for 10 tubes (2.0 ml./tube).

11) Upper gel (Spacer gel)

Solution 'B' : 1 M Phosphoric acid 25.6 ml.
 Tris 5.7 g.
 TEMED 0.46ml.
 Distilled water to 100 ml.
 pH 6.9

Solution 'D' : Acrylamide 10.0 g.
 Bis-acrylamide 2.5 g.
 Distilled water to make 100 ml.

Solution 'E' : Riboflavin 4.00 mg.
 Distilled water to make 100 ml.
 (Stored at 4 °C)

One part of Solution 'B' (0.3 ml.), two parts of
 Solution 'D' (0.6 ml.), one part of Solution 'E' (0.3 ml.),
 four parts of distilled water (1.2 ml.) were mixed and used
 for 10 tubes (0.2 ml./tube).

iii) Staining solution

1% Amido Black B in 7% Acetic acid, stored in amber coloured bottle for 3 days, filtered and used.

iv) Destaining solution

7% Acetic acid

v) Solution for preservation

1% Acetic acid

vi) Tris Glycine buffer

Tris 6.0 g.

Glycine 28.8 g.

Distilled water to 1000 ml.

pH 8.3

Reagents were stored at room temperature.

8. SDS Gel electrophoresisi) Gel buffer

$\text{Na}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ 7.38 g.

$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ 38.6 g.

SDS 2.78 g.

Distilled water to 1000 ml.

ii) 22% Acrylamide solution

Acrylamide 22.0 g.

Methylene Bisacryl 0.3 g.

Distilled water to 100 ml.

Both the reagents were stored at room temperature.

iii) Ammonium persulphate

The solution was freshly prepared in distilled water (4 mg./ml.).

For Gel solution mix per gel	1.25 ml.	gel buffer
	1.13 ml.	Acrylamide solution
	0.13 ml.	Ammonium-persulphate
	4 >	TEMPED
	(1 > TEMPED = 0.01 ml.)	

iv) Buffer

For upper and lower buffer dilute the gel buffer with an equal volume of water.

v) Washing solutions

Solution 'A' : 25% aqueous isopropanol in 10%

Acetic acid for overnight washing.

Solution 'B' : 10% aqueous isopropanol in 10%

Acetic acid for 7 to 8 hours washing.

Solution 'C' : 10% aqueous acetic acid for overnight washing.

vi) Staining solution

0.25% Coomassie blue

1 gram of Coomassie blue in 36 ml. of glacial acetic acid was mixed with Methanol to make final volume of 400 ml.

vii) Destaining solution

Methanol 100 ml.
Glacial Acetic acid 140 ml.
Distilled water to 2000 ml.

9. Freund's Adjuvant

Arcenol 1.5 ml.
Biol 8.5 ml.
Mycobacterium powder 50.0 mg.

(Mycobacterium phlei were grown on Löwenstein Jensen slants, 6 - 8 days growth was autoclaved and preserved in the desiccator).

10. Gel Diffusion test (Microcutcherylow test)

1% Difco agar was prepared in normal saline (0.85% saline)

Staining solution : 0.5% Amido Black B in methanol

Amido Black B 0.5 g
Methanol 400 ml.
Glacial Acetic acid 100 ml.
Distilled water 500 ml.

Stored overnight at room temperature in amber coloured bottle for maturation, filtered and used.

Destaining solution : 2% Acetic acid.

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Bibliography

Bibliography

1. Aziz K.M.S., K.M. Mohsin, 1968 Nature (London)
W.K. Hare and R.A. Phillips 220 : 814-815
2. Banwell J.G., N.F. Pierce, 1970 J. Clin. Invest.
R.C. Mitra and 49 : 183-192
G.J. Caranasos
3. Barua D.; and W. Burrows 1974 Cholera : W.B. Saunder's
Company, Philadelphia
4. Basu S., and M.J. Pickett 1969 J. Bacteriol.
100 : 1142-1143
5. Basu S., R.L. Robinson and 1970 J. Infect. Dis.
M.J. Pickett 121 (Suppl.) : 8 56-5862
6. Buchanan R.E., E.E. Gibbons, 1974 Bergey's Manual of
S.T. Cowan, J.G. Holt, Determinative Bacteriology,
J. Kistner, R.G.E. Murray, Eighth Ed., William and
C.F. Miven, A.W. Rayn and Wilkins, Baltimore
R.Y. Stanier
7. Burrows Williams 1965 Proc. Cholera Res. Symp.
Honolulu : pp 120-124
(Public Health Service
Publication)
8. Burrows W., J.M. Mustekis, 1965 J. Infect. Dis.
N.B. Oza and M.K. Datta 115 : 1-8
9. Burrows W., and 1966 J. Infect. Dis.
G.M. Mustekis 116 : 183-190
10. Burrows W., J. Kaul and 1971 Ann. N.Y. Acad. Sci.
L. Cercavski 276 : 323-329
11. Burrows W., and Sack 1974 Cholera : W.B. Saunder's
Company, Philadelphia
12. Callanhan L.T., R.C. Ryder 1971 Infect. Imm.
and S.H. Richardson 4 : 611-618
13. Carpenter C.C.J., 1974 Cholera : Barua and Burrows,
W.B. Greenough III and W.B. Saunder's Company,
R.S. Gorden Philadelphia
14. Chaiampa W., and D. Rowley 1972 J. Infect. Dis.
121 (Suppl.) : 5132-5136

15. Coleman W.H., G.J.Kasai and W.Burrows 1967 Bacteriol. Proc. M 155
16. Coleman W.H., J. Kaur, M.E.Iwert, G.J.Kasai and W. Burrows 1968 J. Bacteriol. 96 : 1137-1143
17. Craig J.P. 1965 Proc. Cholera Res. Symp. Honolulu : pp. 153-158 (Public Health Service Publication)
18. Craig J.P. 1966 J. Bacteriol. 92 : 793-795
19. Craig J.P. 1970 J. Infect. Dis. 121 (Suppl.) : S100-S110
20. Craig J.P. 1971 Microbial Toxins, Vol.IIA (pp. 189-274 (Academic Press, New York)
21. Curlin G.T., J.P.Craig C.C.J. Carpenter and A. Subong 1970 J. Infect. Dis. 121 : 463-470
22. Datta N.K., and M.K.Habbu 1955 Brit. J. Pharmacol. Chemother. 10 : 153-159
23. De S.N., and D.N.Chatterjee 1953 J. Pathol. Bacteriol. 66 : 559-562
24. De S.N., and D.N.Chatterjee 1959 Nature (London) 183 : 1533-1534
25. De S.N., and M.L.Ghose 1960 J. Pathol. Bacteriol. 79 : 373-380
26. De S.N., M.L.Ghose and J. Chandra 1962 Trans. Roy. Soc. Trop. Med. Hyg. 56 : 241-245
27. Determann E. 1969 Gel Chromatography : Springer - Verlag - New York
28. Duhamel R.C., P. Talbot and G.F. Grady 1970 J. Infect. Dis. 121 (Suppl.) : S85-S91
29. Finkelstein R.A., and C.E.Lankford 1955 Bacteriol. Proc. G 60
30. Finkelstein R.A., H.T.Norris and N.K.Datta 1964 J. Infect. Dis. 114 : 203-216

31. Finkelstein R.A. 1965 Proc. Cholera Res. Symp.
Honolulu (Public Health
Service Publication)
32. Finkelstein R.A.,
A. Athasampunna,
M. Chulasamaya and
P. Charunamethee 1966a J. Immunol.
96 : 440-449
33. Finkelstein R.A.,
P.Z. Sobosinski,
A. Athasampunna, and
P. Charunamethee 1966b J. Immunol.
97 : 25-33
34. Finkelstein R.A. and
J.J. LoSpalluto 1969 J. Exptl. Med.
130 : 185-202
35. Finkelstein R.A. and
J.J. LoSpalluto 1970a J. Infect. Dis.
121 (Suppl.) : 863-872
36. Finkelstein R.A. and
J.W. Peterson 1970b Infect. Imm.
1 : 21-29
37. Finkelstein R.A. and
J.J. LoSpalluto 1972 Science
175 : 529-530
38. Fernandes and Smith 1977 J. Gen. Microbiol.
98 : 77-86
39. Fuhrman G.V. and
F.A. Fuhrman 1960 Nature (London)
188 : 71-72
40. Gallut E. 1971 Cholera : Barua and Burro
W.B. Saunders's Company,
Philadelphia
41. Gardner E.W., S.T. Lyles,
C.E. Lankford and S.V. Hagens 1963 J. Infect. Dis.
112 : 269-272
42. Gardner E.W., S.T. Lyles,
and C.E. Lankford 1964 J. Infect. Dis.
114 : 412-416
43. Germanier R., E. Furer,
S. Varallyay and
T. Anderjaska 1977 J. Infect. Dis.
135 : 512-516
44. Ghosh A.K., G.J. Kasai and
W. Burrows 1968 Bull. W.H.O.
39 : 964-967
45. Grady G.F., M.A. Madoff,
R.C. Duhamel, E.W. Moore
and T.C. Chalmers 1968 J. Infect. Dis.
118 : 263-270

46. Grady G.F., and M.C.Chang 1970 J. Infect. Dis.
121 (Suppl.) : 892-895
47. Greer W.E., Z.Jiricka and
O. Felsenfeld 1968 Proc. Soc. Exptl. Biol.
Med. 127 : 551-555
48. Hansen S.T. 1965 Proc. Cholera Res. Symp.
Honolulu : pp. 276-278
(Public Health Service
Publication)
49. Heckly R.J. and
H. Wolochow 1970 J. Infect. Dis.
121 (Suppl.) : S80-S84
50. Heyningen W.E.,
C.C.J. Carpenter,
N.F.Pierce and
W.B.Greenough III 1971 J. Infect. Dis.
124 : 415-418
51. Hochstein H.D., J.C.Feely
and S.H.Richardson 1970a Proc. Soc. Exptl. Biol.
Med. 133 : 120-124
52. Hochstein H.D., J.C.Feely
and W.E.Dewitt 1970b Appl. Microbiol. Biol.
19 : 742-745
53. Holmgren J., I.Lonnroth
and O. Outcherlony 1971 Infect. Imm.
3 : 747-755
54. Holmgren J.,
Ann Svennerholm and
I. Lonnroth 1973 Infect. Imm.
8 : 208-214
55. Holmgren J.,
Ann Svennerholm and
I. Lonnroth 1977 Nature (London)
269 : 602-604
56. Hugh A. 1965 Quoted from Cholera :
Barua and Burrows, 1974,
W.B.Saunders 's Company,
Philadelphia
57. Iber F.L., McGangle, Carol
Serebro, Luebbers and
Baylors 1969 Amer. J. Med. Sci.
258 : 340-350
58. Inwood J. and
D.A.J. Syrell 1970 Brit. J. Exptl. Pathol.
51 : 577-603
59. Jenkin C.R. and D.Rowley 1959 Brit. J. Exptl. Pathol.
40 : 474-481
60. Kasai G.J. and W.Burrows 1966 J. Infect. Dis.
116 : 606-614

61. Kaur J., W.Burrows and 1969 J. Bacteriol.
L. Cercovski 100 : 985-993
62. Lapage S.P., J.E.Shelton, 1970 Norris and Ribbon,
T.G.Fitchell and Vol.III A, Academic Press,
A.H.Mackenzie New York
63. Lavrovskaia V.M., and 1964 Cited from Cholera :
M.E.Elant Barua and Burrows,
W.B.Saunders's Company,
Philadelphia
64. Lewis A.C., and B.A.Freeman 1969 Science
165 : 808-809
65. Lewis A.C., S.H.Richardson 1976 J. Appl. Environ.Microbiol.
and Barbara Sheridan 32 : 288-293
66. Linggood F.V., M.F.Stevens, 1963 Brit. J. Exptl. Pathol.
A.J.Pulthorpe, A.J.Wainod 44 : 177-183
and C.G.Rope
67. Love A.H. 1965 Proci. Cholera Res. Symp.
Hemolulu (Public Health
Service Publication)
68. Lowry, H.Oliver, Rosebrough, 1951 J. Biol. Chem.
T. Nira, Parr, A.Lewis, 193 : 265-276
Randall and J. Rose
69. Mosley W.H., W.E.Woodward, 1970 J. Infect. Dis.
K.M.A.Azis, A.S.M.Mizanur 121 (Suppl.) : 81-89
Rahman, A.K.M.Alauddin
Chowdhury, Ansaruddin Ahmed
and J.G.Fockey
70. Nikonov A.G., V.I.Verseyeva, 1958 Zh. Mikrobiol.
P.D.Vibikova and K.G.Bichul 29 : 1925-1927 (translated)
71. Northrup R.S., and 1972 J. Infect. Dis.
F.V.Chisari 125 : 471-479
72. Pierce N.F., R.B.Sack, 1970 J. Infect. Dis.
J.G.Bennell, R.C.Mitra and 121 (Suppl.) : 831-835
Arabindo Mondal
73. Pierce N.F., W.B.Greenough 1971 Bacteriol. Rev.
III and C.C.J.Carpenter 35 : 1-12
74. Pierce N.F., E.A.Kanlecki 1972 J. Infect. Dis.
and R.S.Northrup 126 : 606-616

75. Pollitzer R. 1959 Cholera : Monograph series No. 43, W.H.O. Publication, Geneva
76. Fribram 1933 Quoted from Bergy's Manual of Determinative Bacteriology, Eighth Ed., William and Wilkins, Baltimore
77. Rappaport R.S., G.Bonde, T. McLam, B.A.Rubin and H. Tint 1974a Infect. Imm. 9 : 304-317
78. Rappaport R.S., B.A.Rubin and H. Tint 1974b Infect. Imm. 9 : 294-303
79. Rappaport R.S. 1976 Infect. Imm. 14 : 687-693
80. Relyveld E.H. 1969 Prog. Immunobiol. Standard 3 : 253-263
Quoted from Microbial Toxins, Vol. 1, Academic Press, New York
81. Richardson S.H. and D.J.Evans 1968 J. Bacteriol. 96 : 1443-1445
82. Richardson S.H. 1969 J. Bacteriol. 100 : 27-34
83. Richardson S.H., and K.A.Nortie 1970 J. Infect. Dis. 121 (Suppl.) : 873-879
84. Sack R.B., and C.C.W. Carpenter 1969 J. Infect. Dis. 119 : 138-149
85. Scheibel I., and P.E.Christensen 1965 Acta. Pathol. Microbiol. Scand. 65 : 117-128
86. Spyrides G.J., and J.C.Fealey 1970 J. Infect. Dis. 121 (Suppl.) : 896-899
87. Stainer D.W. 1968 Can. J. Microbiol. 14 : 327-330
88. Swallow J.H., C.F.Code and R.Freter 1965 Proc. Cholera Res. Symp. Honolulu : pp. 283-289 (Public Health Service Publication)
89. Ujiiye A.M., Makimoto, A.Utsunomiya, K.Mitsui, S.Sogami, M.Iwanaga and K.Kobori 1968 Trop. Med. 10 : 65-71

90. Vaughan M., H.F. Pierce and 1970 Nature (London)
W.B. Greenough III 225 : 658-659
91. Vitakar R.P. 1973 M.Sc. Thesis,
Bombay University
92. Weber K., and M. Osborn 1969 J. Biol. Chem.
244 : 4406-4411
93. Wilson A. T. 1946 J. Exptl. Med.
84 : 305-322

Erratum

Page	Line	Misprint	Correction
2	19 th	Group O	Group O:1
6	14 th	Fuhrman and Fuhrman	Fuhrman and Fuhrman
	11 th	Burrows, 1968	Burrows, 1974
7	7 th and 11 th	Picket	Pickett
	10 th	Larvovskaya	Lavrovskaia
11	9 th	1970	1970 a
13	11 th	1970	1973
19	12 th	Room tempera- ture	4 °C
24	20 th	Acidic	-
26	16 th	Neutralised	Rendered equally negative
29	1 st	0.5 ml	0.05 ml
32	23 rd	2.6.	2.7.
Table 2	Flask no. 9	90.95	1.93
	8 th line		
	Flask no. 5	9.23	2.32
	8 th line		

Figure 3

