MECHANISM OF *CLOSTRIDIUM PERFRINGENS* TYPE A ENTEROPATHY IN RABBIT ILEUM

By

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CERTIFICATE I

This is to certify that this thesis, entitled, "Mechanism of *Clostridium perfringens* type A enteropathy in rabbit ileum" submitted for the degree of M.V.Sc., in the subject of Veterinary Bacteriology and Hygiene, of the Haryana Agricultural University, is a bonafide research work carried out by Shri Raj Singh Khokher 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.

K Gnanaraju

Major Adviser 7.7.78

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Thanks are due to the members of advisory committee: Dr.V.K.Sharma, Professor and Head, Department of Veterinary Bacteriology and Hygiene, Dr.D.S.Kalra, Professor and Head, Department of Veterinary Pathology, Dr.M.U.Kharole, Associate Professor Poultry Pathology for their valuable suggestions from time to time.

Sincere thanks are due to my friends and well wishers, Drs. Jagvinder Singh, Vijay Kuchroo, Yash Pal, Shiv Charan, Azad Kaushik, P.C.Chhabra, Sh.V.P.Rathee and Sh.P.L.Sharma and many others for their timely help and moral support.

Thanks are also due to Sh.Mahabir Singh, Senior Laboratory Assistant and Shri Bhagirath Singh for their sincere and selfless help in day to day work.

The author expresses his thanks to Shri Goodwin Utarid, Senior Photographer, H.A.U. for his keen interest and meticulous care in preparation of photographs used in this manuscript.

Thanks are also due to Indian Council of Agricultural Research, New Delhi for providing financial assistance in the form of Junior research fellowship.

CERTIFICATE II

This is to certify that the thesis entitled, "Mechanism of Clostridium perfringens type A enteropathy in rabbit ileum" submitted by Shri Raj Singh Khokher to the Haryana Agricultural University in partial fulfilment of the requirements for the degree of M.V.Sc., in the subject of Veterinary Bacteriology and Hygiene has been approved by the Student's Advisory Committee after an oral examination on the same, in collaboration with an External Examiner.

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INTRODUCTION

INTRODUCTION

The pathogenic clostridia are of major importance in farm animals and human beings as primary causes of disease. They are all potent producers of exotoxins upon which their pathogenecity depends. Clostridium perfringens type A is an enterotoxin elaborating enteropathogen which produces food poisoning in human beings characterized by diarrhoea and abdominal pain. In animals it is less significant as an enteropathogen although mortality due to enterotoxaemia caused by it has been reported. In ligated ileal loop of rabbit, it causes fluid accumulation.

Pathogenic strains of Cl. perfringens type A are commonly present in soil rich in humus. They are also found in the intestinal contents of normal animals and cause disease only in special circumstances. The ubiquitous character of this organism makes eradication of the disease virtually impossible and necessitates control by prophylactic measures. This can be achieved by identifying the weakest link which can be manipulated in the course of events leading to disease. The study of mechanism of action of this enteric bacterium may reveal such points where manipulation might be possible to thwart its attempt to harm the host. In case of cholera,

for example the possibilities of preventing disease by blocking the toxin receptor sites with choleragenoid are being explored.

In general, three categories of bacterial diarrhoea are recognized:

In the first, the so called toxigenic diarrhoea in which enterotoxin is elaborated by the bacteria in the lumen of small intestine evoking fluid secretion by the intestinal epithelium. This type is best examplified by the disease caused by non-invading Vibrio cholerae and some strains of Escherichia coli. Enterotoxin action on the intestine is believed to be mediated via the stimulation of mucosal adenyl cyclase cyclic adenosine mono-phosphate (AMP) system.

In the second category, invasive organisms such as Shigellae, Salmonellae and some E. coli provoke acute diarrhoea and there is no clear evidence of enterotoxin mediation. These organisms invade and multiply in the intestinal mucosa, causing its damage and fluid loss.

In the third possible category, diarrhoea is caused by an organism which may be both invasive as well as enterotoxin elaborating. Cl. perfringens type A and Salmonella are likely to fall in this category.

While progress in elucidating the mechanism of intestinal fluid loss in toxigenic diarrhoea has been substantial, little is known of the mechanism of excess fluid secretion into bowel caused by Cl. perfringens

type A. The present study was performed to gain some insight into the mechanism of Cl. perfringens type A enteropathy with the following objectives:

1. To study the site of localization of Cl. perfringens and its enterotoxin, in the intestine and their possible association with the physiopathology of intestinal fluid loss.
2. To study the histochemical and histopathological changes taking place in the infected intestine.

REVIEW
OF
LITERATURE

REVIEW OF LITERATURE

Clostridium perfringens as enteropathogen

Cl. perfringens is an enteropathogen which causes food poisoning and possibly enteritis in man, characterized by diarrhoea and abdominal pain (McClung, 1945; Zeissler and Rassfeld-Sternberg, 1949; Hobbs *et al.*, 1953; Egerton and Walker, 1964). Of the six toxigenic types (A-F) of *Cl. perfringens*, type A is the most common human enteropathogen. However, types C (Egerton and Walker, 1964) and type D (Kohn and Warrack, 1965) have also been reported, but rarely. The causative agent of "Enteritis-necroticans", *Cl. perfringens* type F (Zeissler and Rassfeld-Sternberg, 1949) has been merged with type C (Brooks *et al.*, 1957). Skjelkvale and Duncan (1975) reported that certain strains of *Cl. perfringens* type C produced enterotoxin identical with those produced by type A.

Classical strains of *Cl. perfringens* type A causing gas-gangrene produce abundant alpha, theta and kappa toxins and heat sensitive spores. In contrast, the strains causing food poisoning produce heat resistant spores, little quantity of alpha toxin and variable amount of kappa toxin but do not produce theta toxin (Hobbs, 1965). Studies conducted by

Hauschild and Thatcher (1967, 1968), Hauschild et al. (1967) and Chakrabarty (1977) indicated that there was no clear difference between the strains of Cl. perfringens type A causing food poisoning and the ones causing gas gangrene.

Cl. perfringens type A has been frequently incriminated as the cause of human food poisoning in U.K., U.S.A. and other parts of the world (McClung, 1945; Osterling, 1952; Hobbs et al., 1953; Nikodemusz and Felixfne, 1960; Meisel et al., 1961; Dam-Mikkelsen et al., 1962; Sutton and Hobbs, 1968; Woodward et al., 1970; and Hobbs, 1973, 1974). In India, Gupta and Gulati (1974), Chakrabarty et al. (1977) and Prasad et al. (1977) observed Cl. perfringens associated with human diarrhoea. There are evidences that Indian isolates of Cl. perfringens type A are both heat sensitive and heat resistant.

Clostridium perfringens type A enteropathy

Literatures on bacterial enteropathy in general and those of Cl. perfringens type A in particular are reviewed as under:

(i) Site of localization of cells and enterotoxin.

(ii) Histopathological changes.

(iii) Alterations in vascular permeability.

(iv) Histochemical and enzymic alterations.

Site of localization

During the last decade it has been established that the pathogenesis of food poisoning caused by Cl. perfringens type A is dependent on an enterotoxin elaborated by the organism at the time of sporulation (Hauschild et al., 1971). The locus of attachment of the enterotoxin and the cells in the intestinal tissue might provide information relevant to their mode of action. There appears to be no earlier attempt made to trace the localization of Cl. perfringens type A and its enterotoxin in the infected intestine, although similar studies with V. cholerae have been made. Peterson et al. (1972) with the help of fluorescein and horseraddish peroxidase labelled antitoxin antibodies, attempted to follow the cholera toxin into intestinal tissues of adult mice injected intraluminally. The choleragen was specifically and selectively absorbed uniformly to the entire mucosal surface of villi and crypt areas. In a recent study, Gordon and Verway (1976) demonstrated cholera organism in normal and immunized animals by staining the frozen section with specific fluorescent antibody. Study of tissue sections showed that the fluid accumulation was concomittant with the establishment of large masses of organism in the intervillus spaces of the intestine. V. cholera organisms, thus, localized in the mucous zone

of intestine, multiplied there and elaborated enterotoxin (Peterson et al., 1972). In Cl. perfringens infection, enterotoxin was synthesized in the intestine and was released along with mature spores. The organisms have not been reported to be seen in the villus epithelium. The site of localization of Cl. perfringens and its enterotoxin in lumen or in the intestinal tissues is not known.

Histopathological changes (Architectural damage)

Earlier studies revealed cellular alteration in the intestinal tissues of Cl. perfringens infected animals accounting for the massive loss of fluid and electrolytes. Thus the symptoms of food poisoning caused by Cl.perfringens appear to be the result of architectural damage to the intestine. Duncan et al. (1968) first reported damage of ileal epithelium of rabbits exhibiting diarrhoea induced by cells of enteropathogenic strains of Cl. perfringens. Niilo (1973) reported partial loss of epithelium with a cell free extract of sporulating cells given intravenous to calves and sloughing of ileal epithelial cells when administered in the ligated loops. The same was observed in ligated ileal loops of rabbits by Chaturvedi and Narayan (1974). McDonel and Duncan (1975) found that unlike the action of V. cholerae enterotoxin, Cl. perfringens type A enterotoxin caused damage at the tip of ileal epithelium within a concentration

essential to induce fluid accumulation in ligated ileal loop of rabbit. They also observed that the degree of damage was dose dependent. These studies indicated a cytocidal effect of enterotoxin. A direct evidence of the cytotoxic effect of enterotoxin has come from studies in cell line (Kausch and Donta, 1975). Studies by McDonel (1974), McDonel and Asano (1975) and McDonel and Duncan (1975) attempted to associate the functional disturbance brought about by enterotoxin. However, it remained unresolved whether the fluid accumulation in ligated loops of rabbits was due to cytocidal effect of enterotoxin or not.

Alterations of vascular permeability

A method for demonstrating vascular leakage utilising colloidal iron-dextran, was applied by Kausch et al. (1967) in the study of experimental cholera in infant rabbits. An increase of vascular permeability in choleragen infected rabbit ileum was observed. Its onset and duration were consistent with the clinical course of cholera. It is considered to be a major factor in experimental choleraic diarrhoea. Norris and Majno (1968), however, observed no increase in the permeability of the ileal vessels when studied by intravenous markers i.e. Evan's blue and saccharated iron oxide.

Histochemical and enzymic alterations

Recent informations suggested that many enteric pathogens produced protein exotoxins capable of stimulating

secretion of intestinal fluid and electrolytes and these considerably influenced the concepts of pathogenesis of bacterial diarrhoeas (Grady and Keusch, 1971). Much efforts have been directed towards specific investigation on the role of exotoxin of V. cholerae (choleragen) in experimental and human cholera (Pierce et al., 1971; Kimberg et al., 1971; Sharp and Hynie, 1971). These studies have shown that its molecular mechanism of action almost certainly involved the adenyl cyclase- C'AMP pathway. Stimulation of adenyl-cyclase activity by Cl. perfringens type A enterotoxin was also suggested by observing its effect on thyroid hormone production, a cyclic-AMP mediated process (Macchia et al., 1967). However, this could not be substantiated with a study on the effect of Cl. perfringens enterotoxin on Y-1 adrenal cell culture, rather a cytotoxic effect on HeLa cell was observed (Keusch and Donta, 1975).

Studies on the physiopathology of ileum exposed to Cl. perfringens type A enterotoxin showed that it caused fluid and electrolyte secretion and inhibition of glucose uptake in rat ileum (McDonel, 1974; McDonel and Asano, 1975). Experiments done with enterotoxin treated everted rat ileal sacs showed decrease in oxygen consumption with no effect on lactate production (McDonel and Duncan, 1975). Furthermore, McDonel and Duncan (1977) observed 26 to 41 per cent reduction in the rate of oxygen consumption by

rat liver mitochondria infected with Cl. perfringens enterotoxin when various tricarboxylic acid cycle intermediates were used as substrate. However, oxidative phosphorylation ratio were unaltered.

The existence of an energy dependent mechanism for the transport of Na^+ and K^+ in many animal tissues has been established beyond doubt and reviewed by Bouting (1970). Skou (1957) identified a membrane bound enzyme prepared from crab nerve as transport adenosine triphosphatase or Na^+-K^+ pump. This ATPase requires Mg^{++}, Na^+ and K^+ for maximum activity and is inhibited by ouabain. Any process involving this transport, therefore, must be associated with a Mg^{++} dependent (Na^+-K^+) activated and ouabain sensitive ATPase. Giannella et al. (1975) observed that (Na^+-K^+) ATPase activity was unchanged by either salmonella or cholera toxin. They suggested that the net secretory changes observed in these enteropathic infections probably do not arise from reduction of (Na^+-K^+) ATPase mediated absorption of Na^+ and water. The role of this enzyme in Cl. perfringens type A enteropathy has not been reported. However, reversal of net transport of Na^+ from mucosa to serosa in rat ileum treated with Cl. perfringens type A enterotoxin has been observed by McDonel (1974) and McDonel and Asano (1975).

MATERIALS AND METHODS

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Bacterial Strains used

Clostridium perfringens type A, Hobbs serotype III

Clostridium perfringens type A, Hobbs serotype III (FH/8588/66) is a heat sensitive spore forming strain isolated from food poisoning outbreak in England and Wales. It had been earlier obtained from Dr.B.C.Hobbs of the Food Hygiene Laboratory of the Central Public Health Laboratory, Colindale, London and maintained in Public Health Laboratory of the department. This strain was tested by Chakrabarty (1977) and found to be enterotoxic.

Clostridium perfringens, strain PFO

PFO is laboratory identification number of an isolate of Cl. perfringens from poultry feed (Hafed, Rohtak).

Biochemical, biological and serological characters of the above two strains were studied as per the methods described by Chakrabarty (1977).

Vibrio cholerae

Vibrio cholerae, Inaba 569B is a choleragen producing strain obtained from the Central Research Institute, Kasuali.

Preparation of cell free extract of sporulating cells (CE) and antiserum of Cl. perfringens type A, Hobbs serotype III.

Cell free extract

Clostridium perfringens type A Hobbs serotype III was grown for eight hours in DS medium (Duncan and Strong, 1968). Sporulating cells from 500.0 ml of culture were harvested by centrifugation at 1700 rpm for 45 minutes at 4C. Harvested cell mass was washed thrice with cold saline by centrifugation and finally suspended in 30 ml of 0.025 M tris-HCl buffer (pH 8.0). The suspension was incubated at 37C for one hour before sonication. The cells were sonicated for 40 minutes in the ultrasonic sonicator (Vibrionic Pvt. Ltd., Bombay). The sonicates were centrifuged at 1700 rpm for 45 minutes at 4C. The clear supernatant thus obtained was streaked on lactose egg yolk agar and incubated anaerobically. If growth was observed further sonication was carried out. The cell free supernatant was dialysed against 20.0 per cent solution of polyethylene glycol (MW 6000) at 4C until 80 to 90 per cent concentration was obtained. The concentrate (CE) thus obtained was kept in sterile vials at 4C for future use. Freedom of CE from lecithinase and any viable cell or spore was tested by spotting CE in one half and the respective culture on the

other half (positive control) of egg yolk agar media and incubating the plate anaerobically at 37C for 24 to 48 hours. Sufficient quantity of CE so as to last for the entire study was raised in a single batch eliminating chance of any variability on this account. Protein content of CE was determined by the method of Lowry et al. (1951) with crystaline bovine serum albumin as standard.

Antiserum

It was raised as per the method described by Chakrabarty (1977). It consisted of injecting equal volume of the CE and Freund's complete adjuvant (Difco) intramuscularly to each of the two adult rabbits at three days interval. The amount of CE protein injected was 4.0, 16.0, 32.0 and 32.0 microgramme on each occasion. After 30 days of the last injection, the animals were challenged with a massive dose of 160 microgramme of CE protein. Bleeding was done seven days post challenge. Serum was harvested, heated at 56C for 30 minutes and then tested for the presence of antibody by immuno-diffusion test using homologous antigen. The antiserum was absorbed with the sonicated extract of the vegetative cells of the Hobbs serotype III to eliminate non-enterotoxin antibodies. Thus enterotoxin specific serum was prepared which gave single precipitin band

with CE of homologous strain and no precipitin band with vegetative cell extract. Thiomersalate in 1:10,000 was added to the absorbed serum which was stored at 4C.

Preparation of spore antigen and antiserum of Cl. perfringens type A Hobbs serotype III.

Spore antigen

Spore antigen of Hobbs serotype III was raised by the method described by Chakrabarty (1977). The stock spore suspension in 0.85 per cent saline was matched with Brown's opacity tube No.8. The spore suspension was then autoclaved at 121C for 15 minutes and stored at 4C in tightly sealed vials. Autoclaving was done to prevent possible germination of spores *in vivo* with consequent production of antibody against vegetative cells (Walker, 1963).

Antiserum

Method described by Walker (1963) was followed for raising antisera against Hobbs serotype III. Two adult rabbits weighing 1.5 to 2.0 kg were used. Preimmunization antispore antibody titre of the sera of these animals were determined. Sera from these animals did not show any antibody against spore. Such sera were preserved at 4C for use as controls in subsequent tests. Suspension of killed spore antigen

were injected intravenously to each animal at three days intervals. The first three injections were 0.5, 1.0 and 1.5 ml, respectively while the subsequent five injections were of 2.0 ml each. Test bleeding was done on the 35th day. When the antibody titre was satisfactory then large quantity of blood was drawn through cardiopuncture. The serum was separated, heated at 56C for 30 minutes and then preserved with 1:10,000 thiomersalate and stored at 4C. Spore agglutination test was done as described by Lamanna and Eisler (1960) with slight modification suggested by Lamanna and Jones, (1961).

Preparation of somatic antigen and antiserum of Cl. perfringens type A Hobbs serotype III.

Somatic antigen

Somatic antigens were prepared by using Hobbs serotype III as per the method of Henderson (1940). Vegetative cells were raised in VF broth (Fredette, 1956) as described by Chakrabarty (1977). The vegetative cell stock was suspended in saline and matched with opacity tube No.8. It was steamed for one hour on three successive days and stored at 4C.

Antiserum

A pair of rabbits weighing 1.5 to 2.0 kg was used for raising antisera against Hobbs serotype III.

Preimmunization somatic antibody titre of sera of rabbits was determined. Each rabbit received six successive increasing doses of cell suspension at an interval of three days. The doses were administered intravenously as per the following schedule:

0 th day	0.5 ml	24th day	3.0 ml
4th day	1.0 ml	28th day	3.0 ml
8th day	1.5 ml	32nd day	3.0 ml
12th day	2.0 ml	36th day	3.0 ml
16th day	2.5 ml	44th day	Test bleeding by venipuncture.
20th day	3.0 ml		

Serum was separated, inactivated, preserved and stored at 4C. The antibody titre (agglutinins) was determined against homologous somatic antigen. Somatic cell agglutination test was carried out according to the method described by Hobbs et al. (1953). When the desired titre was achieved the animals were finally bled to collect large quantity of blood by cardiopuncture.

Preparation of rabbit antiglobulin serum for indirect fluorescent antibody technique

The procedure recommended by Proom (1943) was followed. 25 ml of the normal rabbit serum was diluted by adding 80 ml of distilled water. To this 90 ml of a 10 per cent solution of ptassium alum in water was added (Aluminium Potassium sulfate, $Al_2 (SO_4)_3 2H_2O$ M.W.

948.7). The mixture was centrifuged after adjusting the pH to 6.5 with 5 N NaOH. The precipitate was washed twice using 200 ml of saline containing 1:10,000 thiomersalate for each washing. The final precipitate, thus obtained was dissolved in 1:10,000 thiomersalate saline that gave a volume of 100 ml. Ten ml of this suspension was equivalent to 2.5 ml of original serum. A healthy goat of about 20 kg body weight was selected. The above antigen (20 ml) was injected intramuscularly into each hind leg of the goat. Subsequently, two more injections were given after 15 days of interval. The trial bleeding was made on the 15th day of the last injection. When the titre was found to be satisfactory, food was withheld for 24 hours and the goat was bled for the required quantity.

The clear serum from the goat was inactivated at 56C for 30 minutes and preserved by additionof 1:10,000 thiomersalate and stored at 4C.

Later, globulin was obtained with ammonium sulphate precipitation, and conjugated with fluorescein isothiocyanate (Appendix A) for the indirect fluorescent antibody techniques.

Localization of Cl.perfringens enterotoxin, cells and spores in rabbit ileum

Staining with indirect fluorescent antibody technique(IFAT)

Frozen sections of the infected and control loops were cut at 5 /u. The sections were transferred on to

the slides fixed with methanol for 5 minutes and washed in phosphate buffer saline (PBS), pH 7.5. Few drops of the immune serum diluted to 1:80 (anti-somatic and antispore) and undiluted anti enterotoxin were applied, and the slides were incubated at 37C for 30 minutes in a humid chamber. For control, the frozen sections were treated in the same manner except that 0.85 per cent saline (NSS) was applied in place of antiserum. After incubation the slides were washed twice for 15 minutes each in PBS, pH 7.5. Two to three drops of FITC labelled globulin diluted to 1:16 or 1:32 with PBS, pH 7.5 were applied, incubated for the same period. Slides were washed with PBS twice, mounted in buffered glycerol, pH 9.5 and examined under fluorescent microscope (Fluoroluma, American Optical Co., exciter filter 702).

Acridine orange staining

The method of Kronvall and Myhre (1977) was followed.

Fifty mg of acridine organe (BDH) was dissolved in one liter of 0.15 M acetate buffer, pH 4.0. This was used for staining.

The sections or smears were fixed in methanol for two minutes and washed in tap water thoroughly. These were stained in troughs containing about 100 ml of the

staining solution, washed in tap water, dried, and examined under fluorescent microscope. The bacteria gave a strong orange fluorescence and the epithelial cells, green to yellow fluorescence.

Gram's technique for bacteria in sections

The procedure described by Lillie (1965) was followed. The paraffin sections were taken to water as usual. Crystal violet was applied for two to three minutes. After washing the slides were flooded with Grams iodine and which was left for two to three minutes on slides. The sections were then decolourized with acetone and stained with one per cent aquous solution of neutral red, rinsed rapidly in distilled water, blot dried and rapidly cleared in aniline-xylene and xylene and mounted with DPX.

Haematoxyline-eosin staining

The control and test loops of ileum were processed after fixation in buffered formaline, embedded in paraffin, and stained with haematoxylin and eosin.

The same slides were also used for histopathological studies.

Vascular Permeability Studies

In order to gather evidences whether Clostridium perfringens type A causes an alteration of vascular permeability in the intestinal villi of rabbits, this

test was conducted with vegetative cells suspended in sporulation medium and enterotoxin of this organism. For this, the method of Keusch et al. (1967) was followed.

The rabbits were prepared as usual, and a single dose of imferon (Rallies India Limited) at the rate of 1.25 mg iron per 100 gm body weight and Evan's blue or trypan blue at the rate of 30 mg per kg body weight were injected intravenously through ear vein one hour before the animals were sacrificed. The abdominal wall was opened and 2 to 3 cms length of intestine was removed, opened lengthwise and pinned out on paraffin under formaline (10 per cent). After 24 hours of fixation, villi were scrapped off with a scalpel blade and fixed to glass slides with Mayer's egg albumin. The intestinal loop content was also visually examined for colouration. The remaining 2 to 3 cm length of the loop was processed for paraffin section. The scrapings and paraffin sections were stained for iron by Gomori technique (Appendix B) and examined under transmitted light.

Histochemical Studies

Nitro Blue Tetrazolium Method for succinic dehydrogenase (Nachlas et al., 1957).

Method for demonstration of succinic dehydrogenase is based on the principle that colourless, soluble tetrazolium compounds (Nitro BT) are converted by the

addition of hydrogen into water insoluble, deeply coloured pigments, called formazans. In tissues, this is accomplished by enzyme system.

Frozen sections of fresh tissue were incubated in the substrate (containing 0.2 M sodium succinate, Nitro BT in 0.2 M phosphate buffer, pH 7.6) at 37C for 15 minutes. These were washed in saline for 10 minutes and then fixed in 10 per cent formal saline for the same period. sections were rinsed in distilled water after washing for 10 minutes in 15 per cent alcohol. Finally these were mounted in glycerine jelly and examined under light microscope. Sites of enzymic activity were located by observing blue deposits.

Cytochrome oxidase (Person and Fine, 1961)

The demonstration of this enzyme is based on the fact that cytochrome oxidase acts as a catalyst for oxidation reaction between alpha nephthol and dimethyl-p-phenylaminediamine hydrochloride to form indophenol blue (Nadi-reaction).

Frozen sections of the fresh and unfixed tissues were cut and incubated in Nadi reagent (0.1 per cent alpha naphthol, 0.12 per cent dimethyl-p-phenylamine-diamine hydrochloride in phosphate buffer, pH 7.5) for one hour at 37C. The sections were transferred to normal saline, floated on to slides drained and mounted in 20 per cent potassium acetate and then looked for

blue violet colour under light microscope.

Enzymic Assay

(Na^+-K^+) Adenosine triphosphatase

(Na^+-K^+) ATPase activities were measured by the method of Charney et al. (1974).

1. Mucosal scrapings from test and control ileal loops were taken in solution A containing 130 mM NaCl, 5 mM disodium, EDTA, 30 mM imidazole, and 2.4 mM sodium deoxycholate.
2. Centrifuged at 770 g for 10 minutes.
3. Supernatant was taken and centrifuged at 10,000 g for 10 minutes.
4. Pellets were taken in imidazole buffer. This constituted the enzyme homogenate. Protein estimation of this was done by method of Lowry et al. (1951).

The enzyme homogenate was further processed as follows:

Solutions added in millilitres

Solutions	Added in millilitres					
	Test loop		Control loop		Blank	
	1	2	1	2	1	2
Buffer imidazole (20 mM)	0.3	0.3	0.3	0.3	0.3	0.3
Solution B *	0.1	-	0.1	-	0.1	-
Solution C **	-	0.1	-	0.1	-	0.1
ATP (33.6 mM)	0.1	0.1	0.1	0.1	0.1	0.1
Enzyme homogenate	0.1	0.1	0.1	0.1	-	-
	Incubated at 37 C for 15 minutes					
35 per cent Tricholoroacetic acid	0.2	0.2	0.2	0.2	0.2	0.2

*600mM NaCl, 120mM KCl, 20mM imidazole, 33.6 mM $MgCl_2$
**600mM NaCl, 20mM imidazole, 33.6 mM $MgCl_2$

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After terminating the reaction with 35 per cent trichloroacetic acid, the inorganic phosphate liberated was measured by the method of Chen et al. (1956). Procedure to obtain a standard curve of inorganic phosphorus is appended (Appendix C).

To 0.1 ml of the reaction mixture, 2.8 ml of the working solution (1 part of 10 per cent ascorbic acid and 6 parts of 0.42 per cent ammonium molybdate in 1.0 N sulphuric acid) was added and incubated for 20 minutes at 45C. This was read at 820 mu in Spectronic-20. The values were extrapolated from standard curve (Fig.1).

(Na^+-K^+) ATPase activity was defined as the difference between the inorganic phosphate released in presence and absence of potassium. Results were expressed as micromoles of inorganic phosphate liberated per mg of protein per fifteen minutes.

Rabbit ileal loop test

Rabbits of 900 to 1500 g body weight were selected. Under ether anaesthesia, laprotomy was performed and constricting ligatures were applied to the ileum so as to segment it into a series of closed loops, 5 cm in length. Care was taken not to compromise any of the vascular arcades. Between adjacent test loops a 4 cm segment of blank ileum was interposed. Control loops received 0.8 ml NSS. Each test loop was inoculated with a total

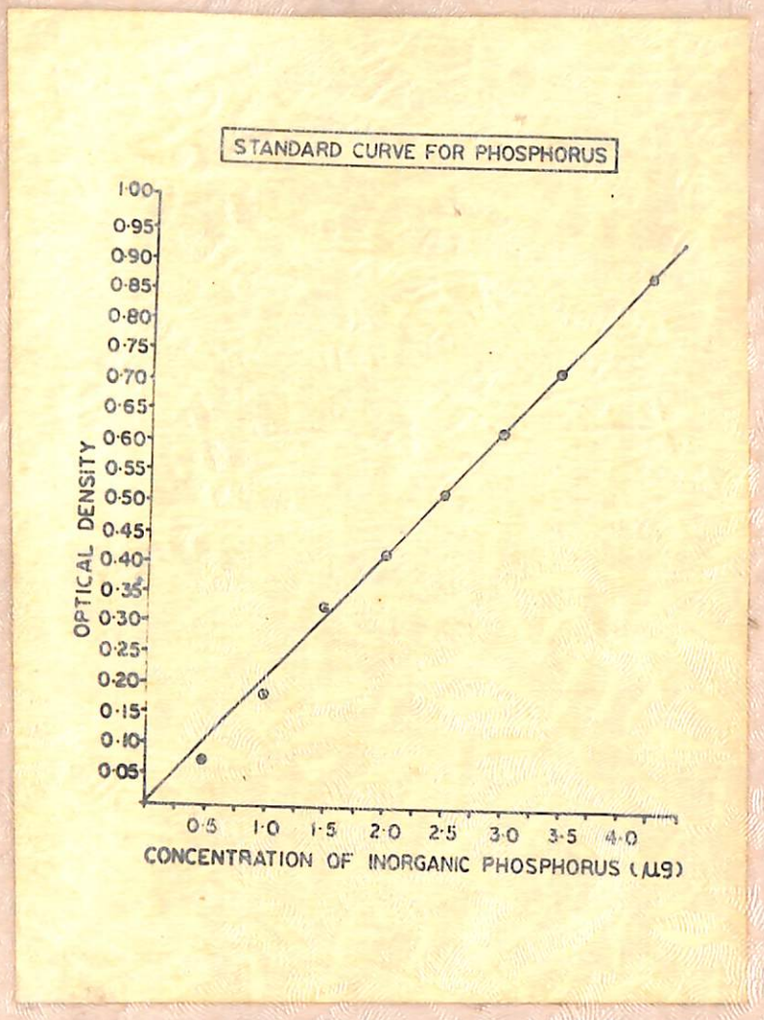

STANDARD CURVE FOR PHOSPHORUS
OPTICAL DENSITY
1·00
0·95
0·90
0·85
0·80
0·75
0·70
0·65
0·60
0·55
0·50
0·45
0·40
0·35
0·30
0·25
0·20
0·15
0·10
0·05
0·5
1·0
1·5
2·0
2·5
3·0
3·5
4·0
CONCENTRATION OF INORGANIC PHOSPHORUS (µg)

Fig.1.

volume of 0.8 ml containing 3.2 mg of crude enterotoxin of HSIII or 0.8 ml containing 1 x 10^6 cells of both the strains of Cl. perfringens, 3 x 10^{11} cells of V. cholerae or 1.68 mg of crude cholera toxin (Appendix D and E). After eight hours of inoculation, the animals were sacrificed, fluid secretion measured and mucosal homogenates assayed for (Na^+-K^+) ATPase and protein concentrations. Tissues of all the experimental and control loops were processed and the microsections used for different histopathological, histochemical and immunohistochemical studies. For histopathological studies, the tissues were fixed in 10 per cent buffered formaline and stained by HE, acridine orange and Grams tissue stains. For immunohistochemical studies liquid nitrogen fixed frozen sections were cut (Appendix E) and stained by IFAT and acridine orange to trace out the localization of bacterial cells, spores and enterotoxin. For histochemical studies similar sections (frozen) were used to demonstrate succinic dehydrogenase and cytochrome oxidase activities.

RESULTS

RESULTS

Localization of enterotoxin of *Cl. perfringens* type A, Hobbs serotype III in rabbit ileum

The site of localization of enterotoxin in ligated ileal loop exposed to crude enterotoxin (CE) was traced by employing indirect fluorescent antibody technique (IFAT). In all, five different rabbits were used. In each, two ileal loops were inoculated with CE. Frozen sections of the loop showing fluid accumulation (Fig.2) were examined for enterotoxin specific fluorescence. Figure 3 a, b illustrate the brilliant specific fluorescence of toxin antigen at the surface of intestinal epithelium. Section from the same ileal segment treated with normal saline solution (NSS) instead of antitoxin showed no specific fluorescence. The results were also negative when section of ileal loop inoculated with NSS was stained for enterotoxin specific fluorescence (Fig.4). The enterotoxin appears to have absorbed to the surface of villi. No specific fluorescence for presence of enterotoxin could be detectable in any other layer of intestine.

Localization of cells and spores of *Cl. perfringens* type A, Hobbs serotype III in rabbit ileum

To locate the cells and spores in the ileal loop exposed to HSIII cells suspended in sporulation medium,

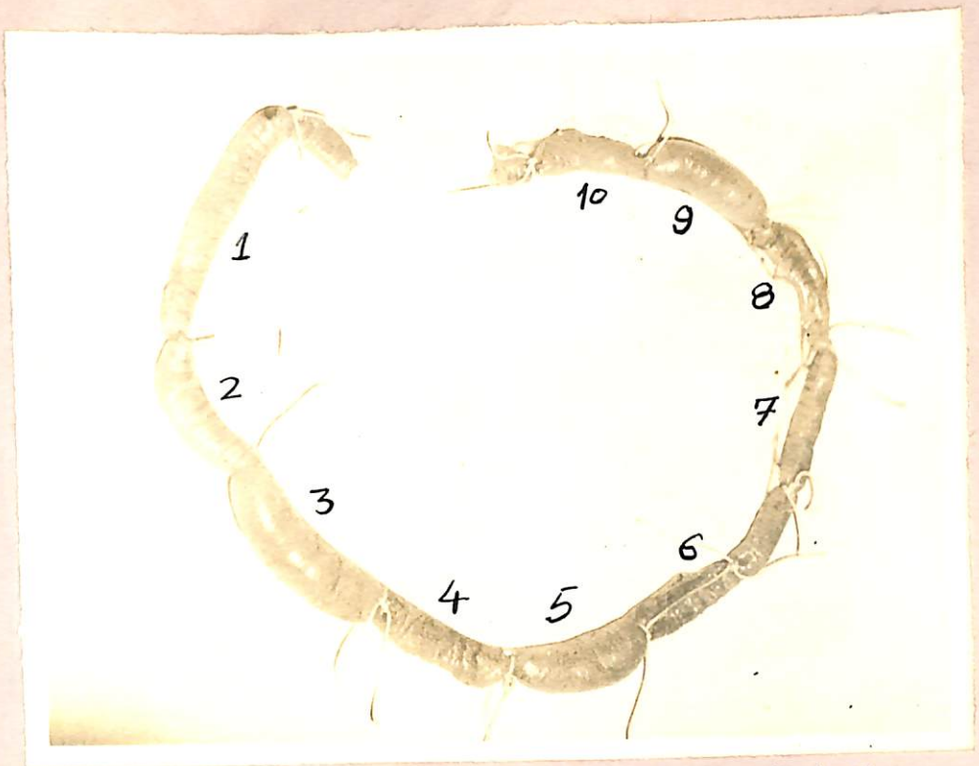


Fig.2. Cl.perfringens type A HSIII CE inoculated segments 3,5 and cells inoculated segment 9 showing fluid accumulation. Segments inoculated with DSM(6,10), NSS(2,4,8) and CE of a non-enterotoxigenic strain(7) showingno fluid accumulation, Segment 1 uninoculated.

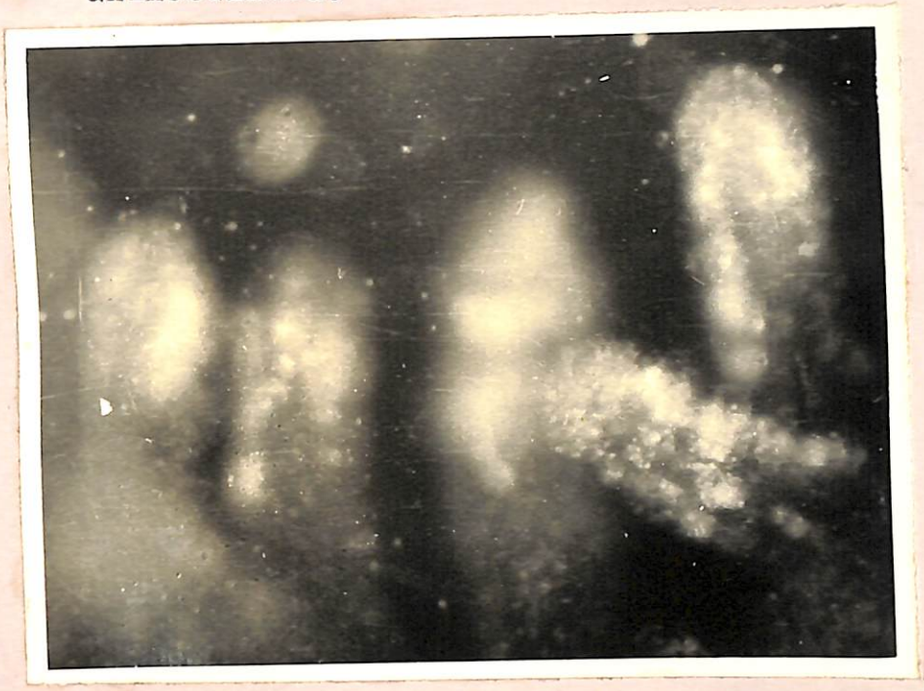
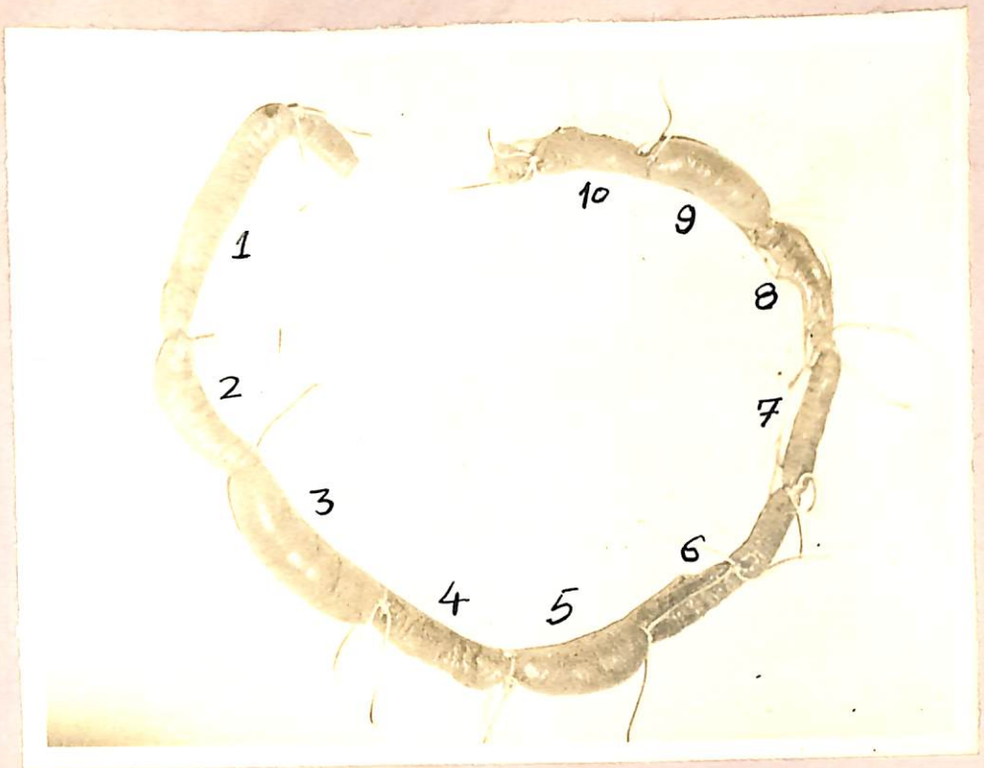


Fig.3a. A number of ileal villi showing localization of Cl.perfringens type A HSIII enterotoxin detected by IFAT in frozen section. 15x40.



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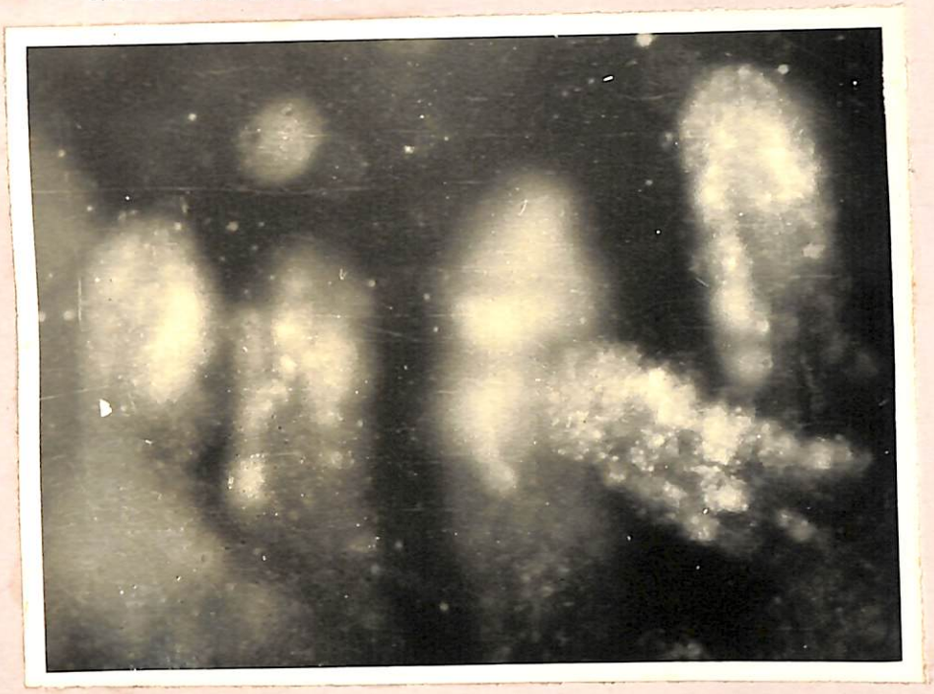

Fig.3a. A number of ileal villi showing localization of Cl.perfringens type A HSIII enterotoxin detected by IFAT in frozen section. 15x40.

Fig.3b. Single villus of ileum showing localization of Cl.perfringens type A HSIII enterotoxin detected by IFAT in frozen section. 15x40.

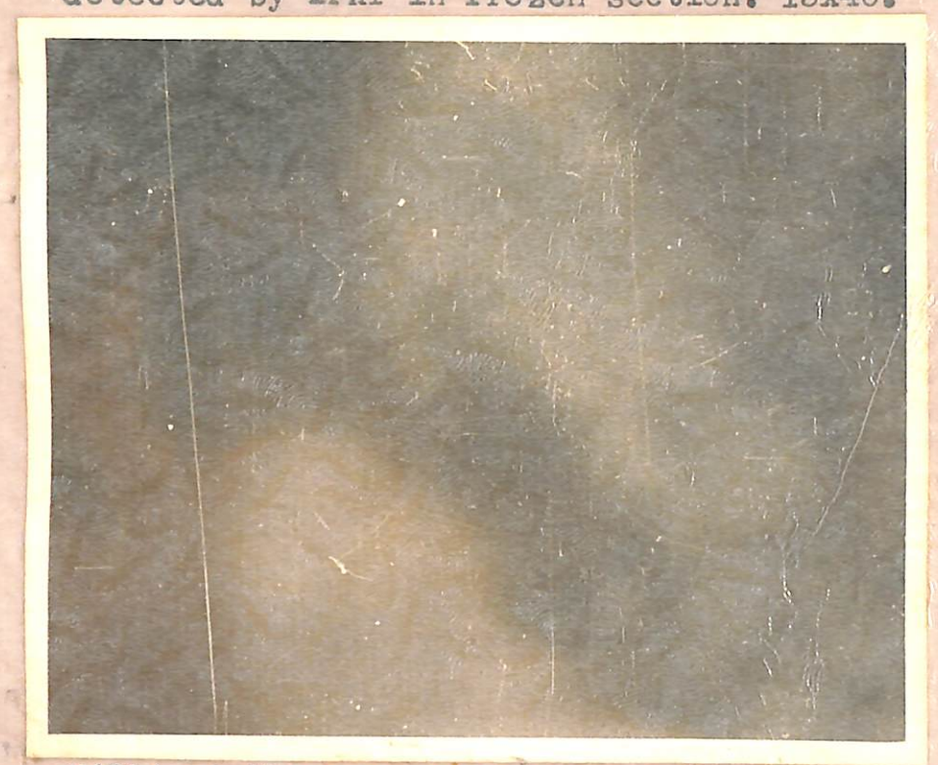

Fig.4. Villi of ileum exposed to normal saline stained with anti-enterotoxin serum showing no fluorescence by IFAT in frozen section. 15x40.

Fig.5. Smear of content of loop exposed to Cl. perfringens type A HSIII cells showing fluorescing bacilli. (Acridine orange stain 15x20).

the same number of loops and rabbits were employed as in the case of enterotoxin. To locate these, IFAT, Grams, haemotoxyline eosin and acridine orange staining procedures were employed in both the frozen and paraffin sections. In paraffin sections no organisms were detectable by any of the staining procedures, although smears of the loop content when stained by acridine orange (Fig.5) showed brilliant orange fluorescence. For comparison, a smear of the culture of the same strain was also examined. Besides, the smear of the contents of ileal loop inoculated with NSS was stained with acridine orange and it was found to be negative. In frozen sections, the organisms could be demonstrated by IFAT and acridine orange staining procedures. The sections were stained for all the three antigens i.e. cells, spores and enterotoxin by IFAT. It was found that the enterotoxin localized at the surface of the villus epithelium, as had also been observed when loops were exposed to enterotoxin and stained for it. When stained with anti-somatic and anti-spore sera, the respective antigens were found to be located in the lumen and the adjoining villi (Table 1). With acridine orange staining also, a brilliant orange fluorescence of the cells in the lumen of the bowel and the adjoining villi was observed (Fig.6). No organism was detectable in any other layer of the intestine with either of the staining procedures. Similarly, in frozen sections of

Table 1

Localization of vegetative cells, spores and enterotoxin of Cl. perfringens type A Hobbs serotype III in ligated ileum of rabbit by IFAT

Specific staining for	Localization of cells, spores, enterotoxin in ligated loops exposed to		
	Cells in DSM	CE (enterotoxin)	NSS
Enterotoxin	Villus epith.	Villus epith.	-
Vegetative cells	Lumen and adjoining villi	-	-
Spores	-do-	-	-

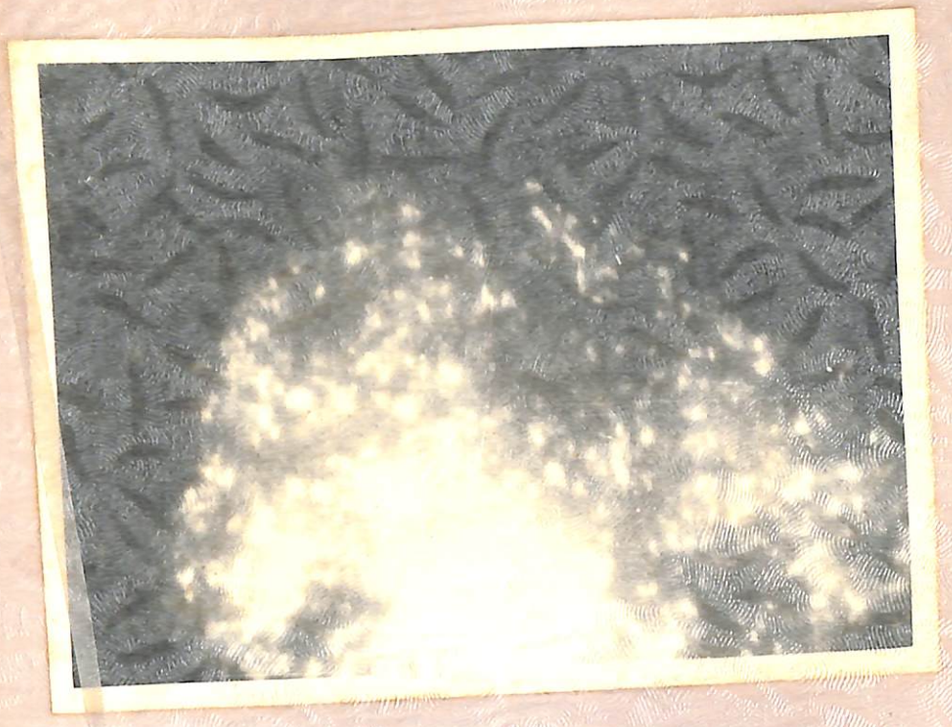

Fig.6. Ileum exposed to Cl.perfringens type A HS II cells: Frozen section showing the brilliant fluorescence of cells in the lumen of bowel and adjoining villus. (Acridine orange stain 15x20).

Fig.7. Ileum: Frozen section of NSS inoculated loop showing no organism in the lumen of villi. (Acridine orange stain, 15x20).

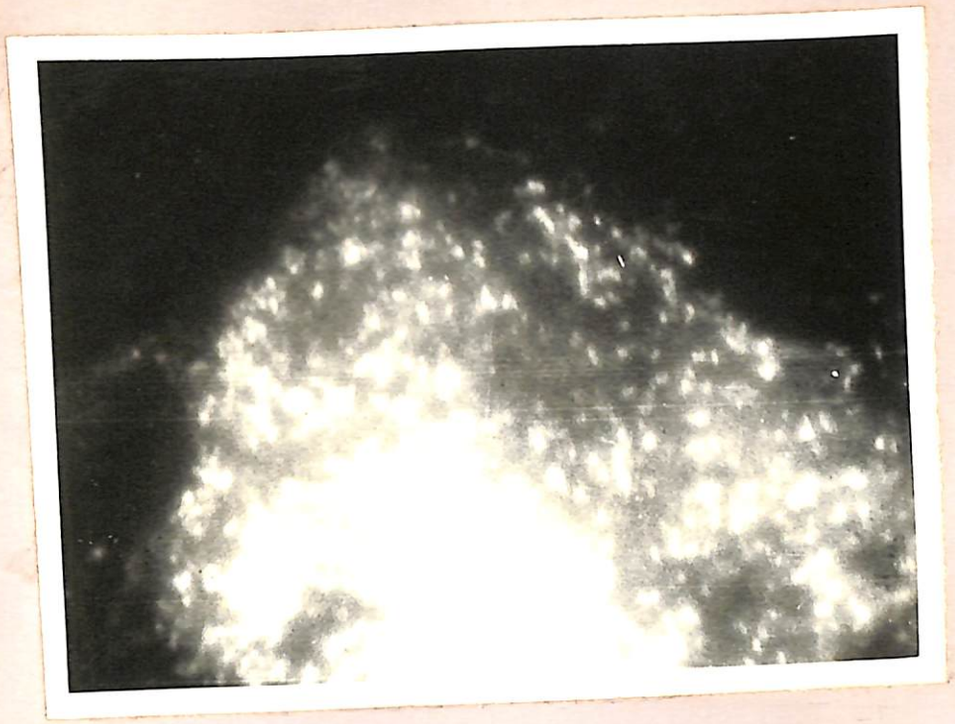

Fig.6. Ileum exposed to Cl.perfringens type A HSIII cells: Frozen section showing the brilliant fluorescence of cells in the lumen of bowel and adjoining villus. (Acridine orange stain 15x20).

Fig.7. Ileum: Frozen section of NSS inoculated loop showing no organism in the lumen of villi. (Acridine orange stain, 15x20).

NSS inoculated loops, organisms could not be demonstrated (Fig.7).

A summary of the observations on localization of Cl. perfringens type A Hobbs serotype III cells and spores in different layers of intestine is presented in table 2. This table reflects that no organisms were detectable in any layer of intestine starting from lumen upto muscularis and serosal layer in paraffin sections stained by acridine orange, haematoxyline-eosin and Gram's staining methods. In the case of frozen section, however, it was possible to locate the cells and spores of Cl. perfringens in the lumen of ileum and adjacent to villus epithelium when stained by IFAT and acridine orange.

Data shown in tables 1 and 2 clearly indicate that Cl. perfringens type A Hobbs serotype III cells are non-invasive and fluid accumulation may be ascribed to the enterotoxin alone which gets absorbed to the villus epithelium.

Localization of cells of Cl.perfringens, poultry feed isolate (PFO) in rabbit ileum

As shown in table 3, PFO, is a non-enterotoxin producing poultry feed isolate conforming to the characters of Cl. perfringens.

The site of localization of this organism was determined by employing acridine orange, Grams and HE stains. Both the paraffin and frozen sections of ileum

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Table 2

Localization of Cl. perfringens type A Hobbs serotype III-cells and spores in rabbit ileum

Sites of localization	Paraffin section Staining			Frozen section Staining	
	AO	HE	Gram's	AO	IFAT
Lumen	-	-	-	+	+
Adjacent to villus epithelium	-	-	-	+	+
Villus core	-	-	-	-	-
Lamina propria	-	-	-	-	-
Crypts	-	-	-	-	-
Submucosa	-	-	-	-	-
Muscularis and serosa	-	-	-	-	-

AO = Acridine orange; HE = Haematoxylene and eosin;
IFAT = Indirect fluorescence antibody technique.

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Crypts	-	-	-	-	-
Submucosa	-	-	-	-	-
Muscularis and serosa	-	-	-	-	-

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IFAT = Indirect fluorescence antibody technique.

Table 3

Biochemical, biological and serological characters of Cl. perfringens strains studied

Characters studied	Hobbs serotype (HS III)	Poultry feed isolate (PFO)
Source	Food poisoning	Pouktry feed
Morphology	Rod	Rod
Gram's staining	+	+
Lecithinase production on egg yolk agar	+	+
Specific inhibition of lecithinase by antialpha toxin	+	+
Motility	-	-
Nitrate reduction	+	+
Fermentation of:		
Glucose	+	+
Mannitol	-	+
Starch	+	-
Sucrose	+	+
Lactose	+	+
Maltose	+	+
Raffinose	+	+
Salicin	+	-
Inositol	+	+
Melibiose	+	+
Cellobiose	+	-

contd...

Table 3 (contd.)

Agglutination with antiserum to HSIII cells	+	-
Agglutination with antiserum to HSIII spores	+	-
Gel-diffusion with antiserum to enterotoxin of HSIII	+	-
Biological test in ligated ileum of rabbits with cells	+	+
With cell extract	+	-

Table 3 (contd.)

Agglutination with antiserum to HSIII cells	+	-
Agglutination with antiserum to HSIII spores	+	-
Gel-diffusion with antiserum to enterotoxin of HSIII	+	-
Biological test in ligated ileum of rabbits with cells	+	+
With cell extract	+	-

were used. These were harvested from two different rabbits, in each of which two ileal loops were inoculated with PFO cells. Innumerable bacilli along with large number of erythrocytes and desquamated cells in the lumen of intestine were seen in sections stained with HE (Fig.8). In paraffin sections stained with acridine orange, organisms were found invading the intervillus space (Fig.9). With HE staining these were also located in the close vicinity of villus tip (Fig.10), approaching towards the blood vessel in the villus core (Fig.11) and as deep as submucosal layer (Fig.12). A single bacillus is shown in the submucosal layer of intestine in figure 12. Acridine orange staining procedure supported these observations on the distribution of the PFO-cells in different layers of ileum.

Summary of the observations on localization of PFO cells is presented in table 4. The organisms were found located at sites such as lumen, adjacent to villus epithelium, the villus core, lamina propria, crypts and submucosa. No organism was detectable in the last layers i.e. muscularis and serosa.

It appears tfrom the above that the poultry feed isolate of Cl. perfringens was invasive in nature.

Fig.8. Ileum infected with Cl.perfringens PFO cells: Innumerable bacilli, erythrocytes and desquamated cells in the lumen. (H&E 7x4).

Fig.9. Ileum exposed to Cl.perfringens PFO cells: Paraffin sections showing orange fluorescing organisms in the inter villus space and core of villus. (Acridine orange stain 15x40).

Fig.8. Ileum infected with Cl.perfringens PFO cells: Innumerable bacilli, erythrocytes and desquamated cells in the lumen. (H&E 7x4).

Fig.9. Ileum exposed to Cl.perfringens PFO cells: Paraffin sections showing orange fluorescing organisms in the inter villus space and core of villus. (Acridine orange stain 15x40).

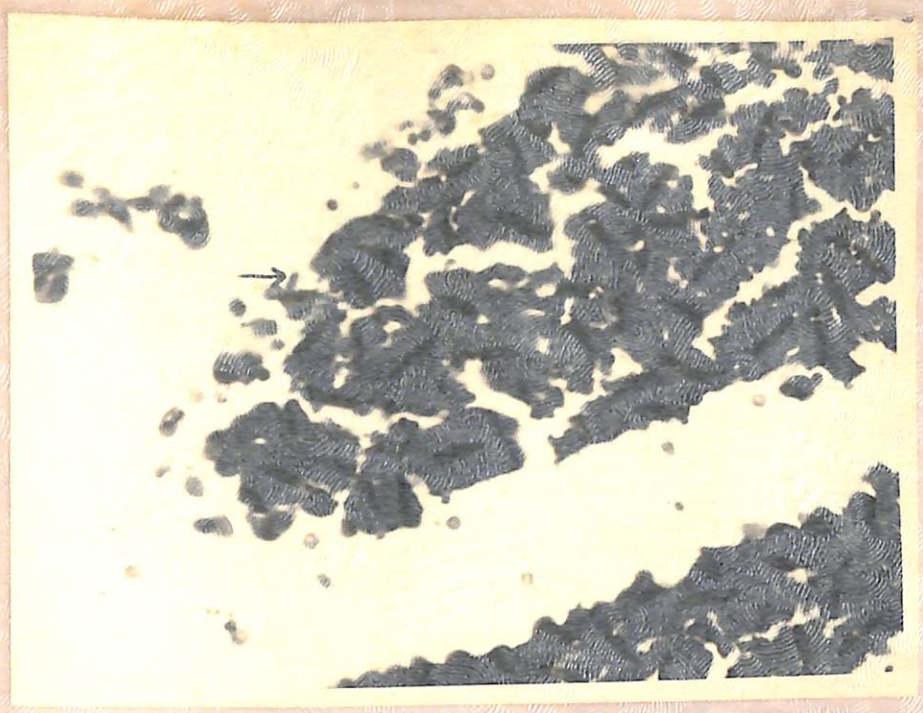

Fig.10. Ileum infected with Cl. perfringens PFO cells: Single bacillus at the side of the villus. (H&E 7 x 40).

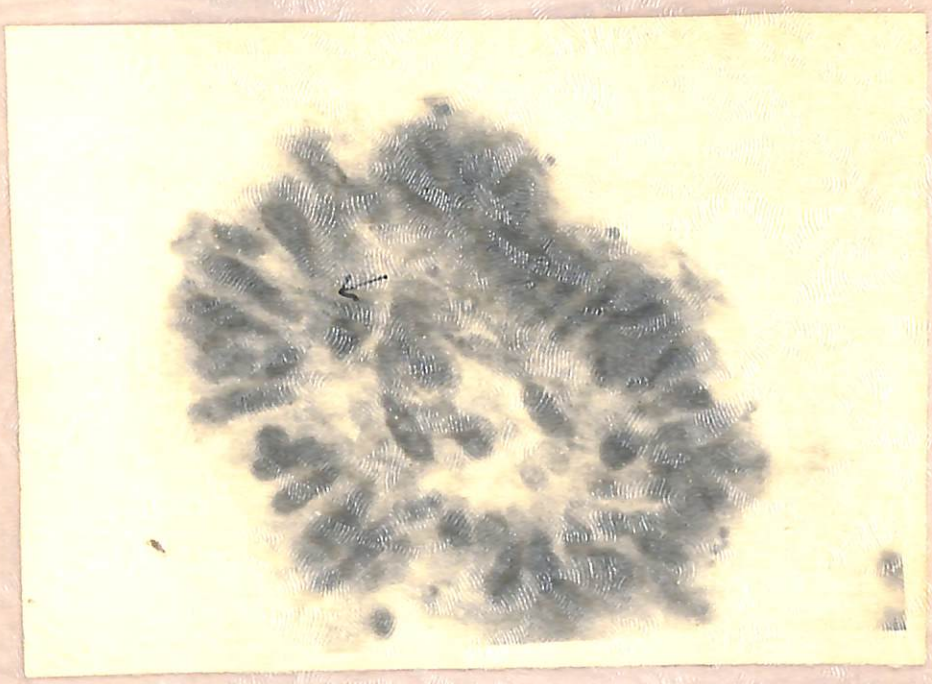

Fig.11. Ileum infected with Cl.perfringens PFO cells: Single bacillus approaching towards the blood vessels in the villus core. (H&E 7x100).

Fig.12. Ileum infected with Cl.perfringens PFO cells: A single bacillus in the submucosal layer of ileum. (H&E 7x40).

Fig.13. Ileum inoculated with NSS: Cross section showing normal histological structure. (H&E 7x100).

Histopathological Studies

The enteropathy of Cl. perfringens in rabbit ileal segment was studied. The test segments consisted of loops inoculated with cells and CE of Cl.perfringens type A, Hobbs serotype III and the cells of Cl.perfringens PFO. Choleragen inoculated loops served as positive control while NSS inoculated ones as negative controls. Histopathology of ileal segments exposed to test and control materials was studied. Paraffin sections of 5 µ thickness were stained with haematoxyline and eosin.

Figure 13 is the cross section of control segment of ileum showing all the layers i.e. villi, lamina propria, crypts, submucosa, muscularis and serosa which are intact and normal. The histopathology of section of ileal loop exposed to choleragen and manifesting fluid accumulation was also studied. No architectural damage to the ileum was detectable (Fig.14) and the histological observations were indistinguishable from those of NSS exposed loops (Table 5). On the other hand, the loops exposed to HSIII enterotoxin showed congestion and haemorrhage in the lamina propria and submucosa as shown in figure 15. In the same section (not shown in Fig.15) almost all capillaries were found to be haemorrhagic and the capillaries of serosa were highly congested. In

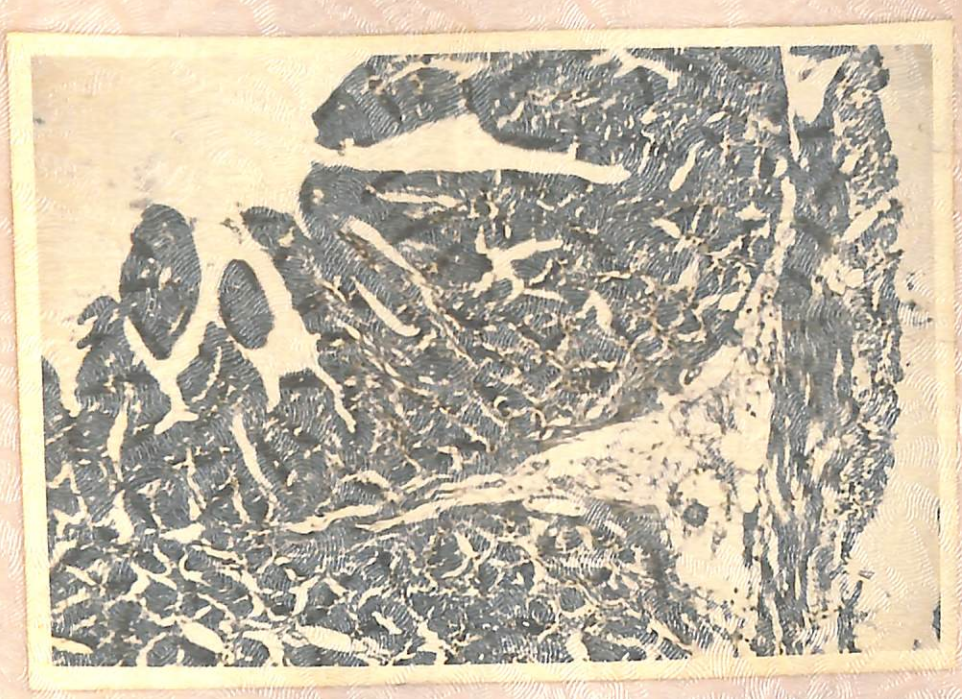

Fig.14. Ileum inoculated with choleragen: Showing no architectural damage. (H&E 7x10).

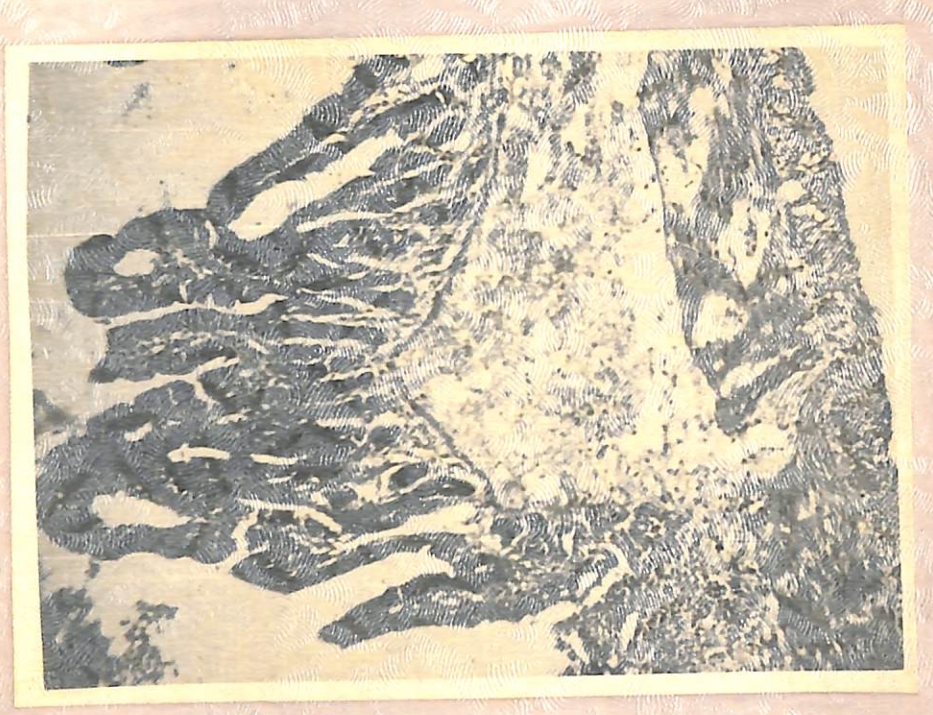

Fig.15. Ileum infected with Cl. perfringens type A HSIII enterotoxin: Showing severe congestion haemorrhage and oedema in submucosa. (H&E 7x10)

Fig.16. Ileum infected with Cl.perfringens type A HSIII enterotoxin: Showing haemorrhage, plasma fluid and few disintegrated PMN's in the lumen, oedema and dialated capillary in submucosa (H&E 7x10).

Fig.17. Ileum exposed toCl.perfringens type A HSIII Cells: The submucosa highly haemorrhagic and oedematous.(H&E 7x10).

Table 5

Summary of the histopathological studies on the rabbit ileum infected with *Cl. perfringens* type A Hobbs serotype III, PFO and *V. cholerae*

Material inoculated	Lumen	Lamina propria			Sub-mucosa			Other layers		
	Haemorrhage	Congestion	Haemorrhage	Oedema	Congestion	Haemorrhage	Oedema	Congestion	Haemorrhage	Oedema
Normal saline (control)	(0/12)	(0/12)	(0/12)	(0/12)	(0/12)	(0/12)	(0/12)	(0/12)	(0/12)	(0/12)
V. cholerae	(0/4)	(0/4)	(0/4)	(0/4)	(0/4)	(0/4)	(0/4)	(0/4)	(0/4)	(0/4)
Cl. perfringens										
HS III CE	+(5/12)	+(10/12)	+(2/12)	+(1/12)	+(10/12)	+(2/12)	+(6/12)	+(2/12)	+(3/12)	(0/12)
HS III-cells	+(1/7)	+(4/7)	(0/7)	+(3/7)	+(4/7)	(0/7)	+(2/7)	+(2/7)	+(1/7)	(0/7)
PFO cells	+++(2/2)	+++(2/2)	++(2/2)	+++(2/2)	+(2/2)	+(2/2)	(0/2)	(0/2)	(0/2)	(0/2)

+, ++, +++ indicate mild, moderate and intense positive alterations in the respective column.

Figures in parentheses indicate number of ileal segments positive over total examined.

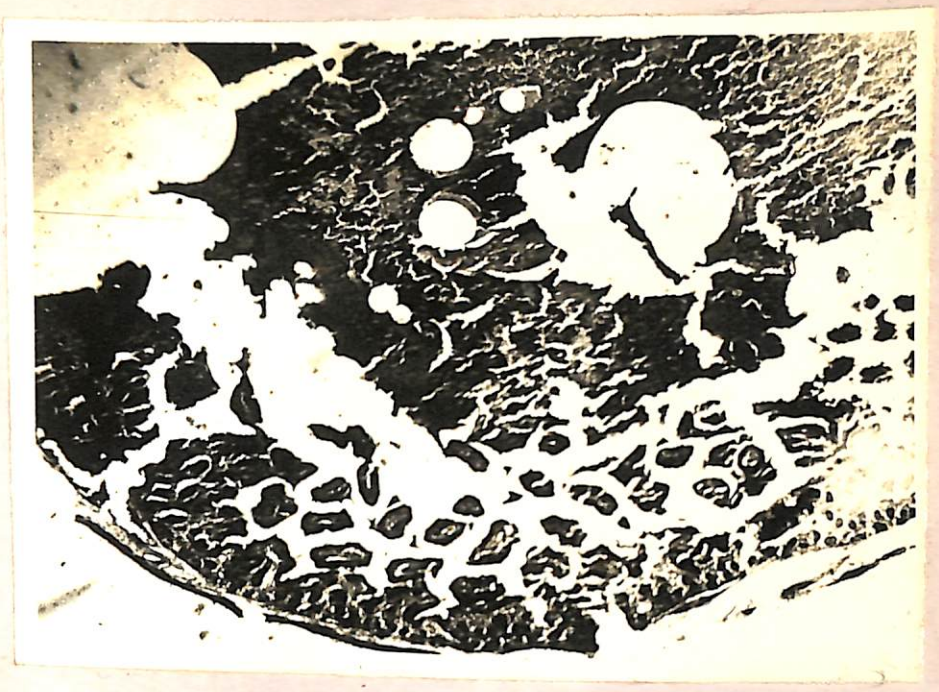

Fig.18. Ileum exposed to Cl.perfringens PFO cells: Large amount of blood and desquamation of epithelium in the lumen. (H&E 7x4).

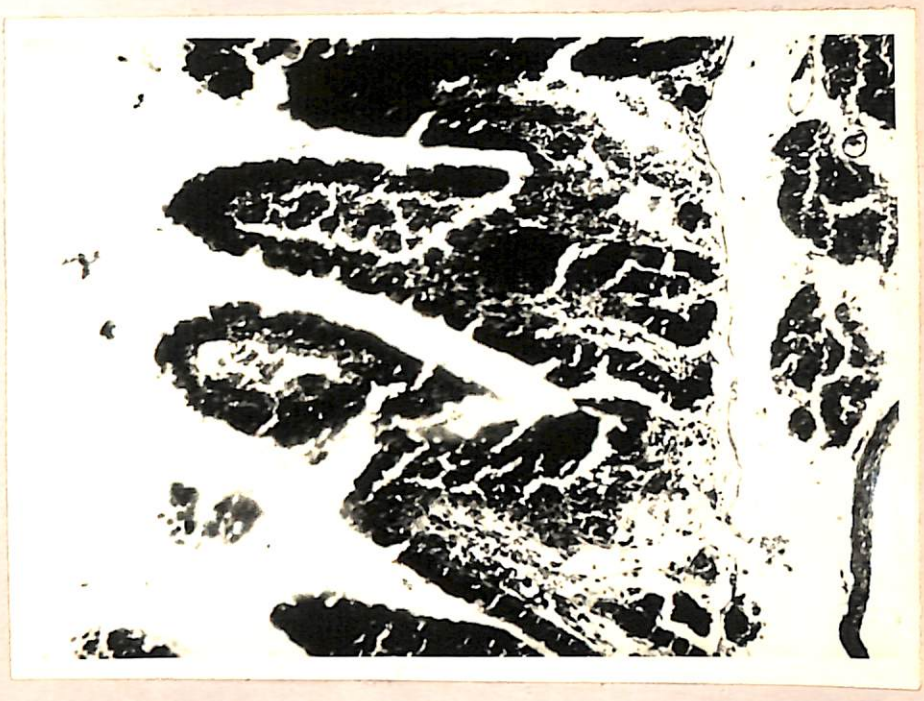

Fig.19. Ileum exposed to Cl.perfringens PFO cells: Showing congested villus core.(H&E 7x20).

technique using imferon and Evans blue, imferon and trypan blue and imferon alone as the intravenous markers. Gross observations of the extravasation of these markers in the fluid accumulated in the test and control loops along with the iron staining in scrapings as well as in paraffin sections of the same were made. The gross observations revealed extravasation of the markers in the lumen of intestine as brown coloured content but the findings were negative by iron staining procedures (Table 6) when stained for both ferric as well as ferrous iron by Gomeri's technique. To check the correctness of the staining technique, spleen sections were stained by the same procedure. Spleen normally contains iron which can be demonstrated with the above staining technique. The spleen sections were found to be positive (Fig.20) for iron.

Enzyme Assay

(Na^+-K^+) ATPase is an important mucosal enzyme which is responsible for the normal maintenance of electrolyte transport across membrane. Its activity in mucosal homogenates was measured as the micromoles of inorganic phosphate liberated per mg protein per 15 minutes. The results of experiments on five different rabbits is presented in table 7. This table shows the

Fig.20. Spleen: Sectionof spleen showing pogitive staining for iron (Gomeri's method 7x10).

Table 6

Alteration of vascular permeability caused by Cl. perfringens type A Hobbs serotype III, V. cholerae (test) and normal saline (control) inoculated in ligated ileum of rabbit

Exp. No.	Marker used	Gross observation		Microscopic observation							
				Test				Control			
				Section		Scraping		Section		Scraping	
		Test	Control	Fe^{++}	Fe^{+++}	Fe^{++}	Fe^{+++}	Fe^{++}	Fe^{+++}	Fe^{++}	Fe^{+++}
1.	Imferon + Evan's blue*	+	-	ND	ND	-	-	ND	ND	-	-
2.	Imferon + Trypan blue**	+	-	ND	ND	-	-	ND	ND	-	-
3.	Imferon + Trypan blue	+	-	-	-	-	-	-	-	-	-
4.	Imferon	+	-	-	-	-	-	-	-	-	-
*** 5.	Imferon	+	-	-	-	-	-	-	-	-	-

\+ Brown coloured loop content
\- Negative
ND Not done
* Caused extensive blueing of entire animal
** Not traceable in the loop content
*** Experiment 5th is repeatation of Exp. 4

KGN-3

Table 7

Effect of enterotoxin of Cl. perfringens type A Hobbs serotype III, Cells of Cl. perfringens PFO and choleragen on mucosal (Na^+ - K^+) ATPase*

Material inoculated	Fluid volume/ cm loop in ml	(Na^+ -K^+) ATPase micromole Pi/mg protein/15 min.
Normal saline (control)	Nil	6.89 ± 0.564
Choleragen	0.45	7.043 ± 1.282
HS III enterotoxin		
800 ug	0.20	17.185 ± 1.590
1600 ug	0.30	3.660 ± 0.650
3200 ug	0.44	1.063 ± 0.371
PFO cells	0.32	8.480 ± 1.40

* Average of data obtained from experiments on five rabbits.

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* Average of data obtained from experiments on five rabbits.

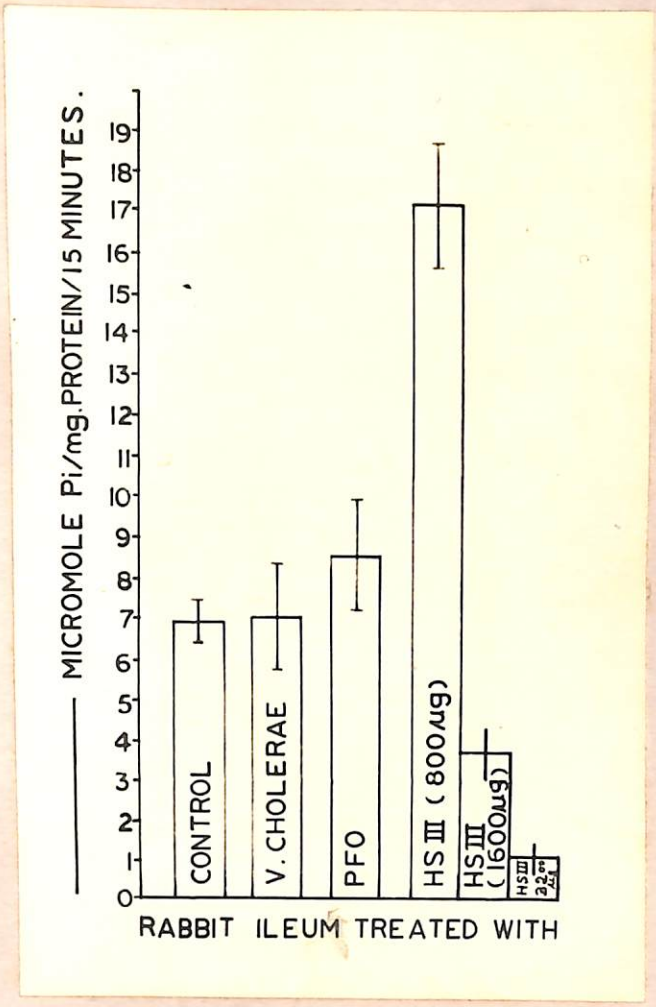


Fig.21. (Na^+-K^+) ATPase activity of mucosa of ileum exposed to NSS (control), V. cholerae (choleragen), PFO cells and three doses of HSIII enterotoxin.

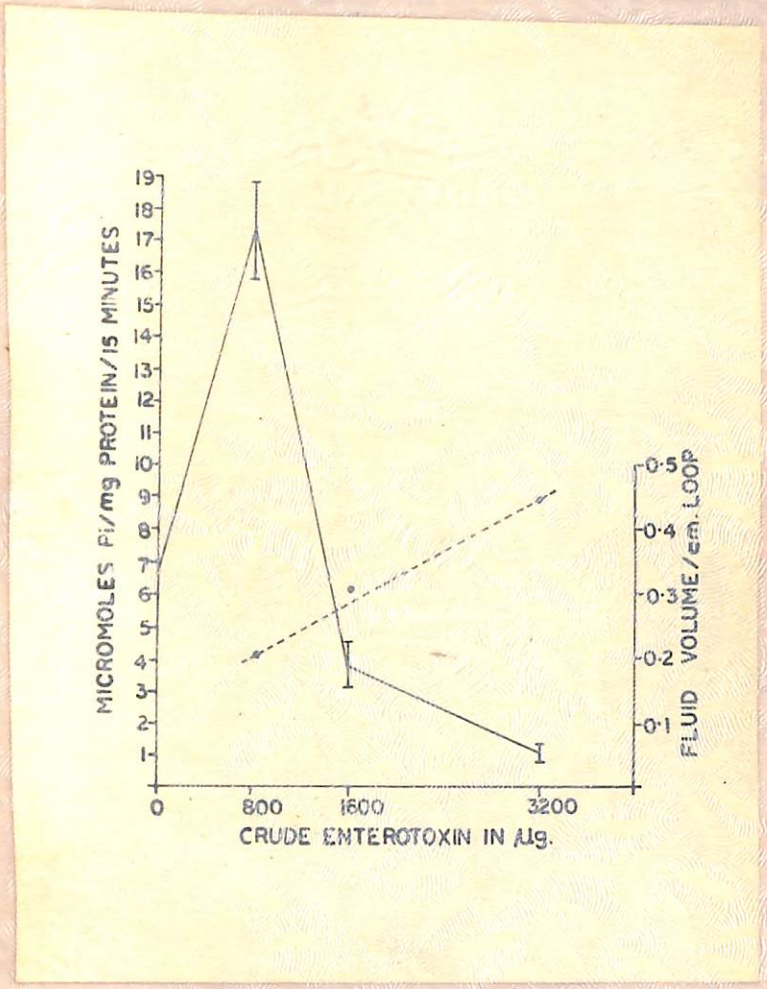


Fig.22. The relationship of (Na^{+}-K^{+}) ATPase activity (continuous line) and corresponding fluid accumulated (dotted line) per cm of loop length with three different doses of Cl. perfringens type A, HSIII enterotoxin.

Fig.23. Ileum exposed to Cl.perfringens type A HSIII enterotoxin showing succinic dehydrogenase activity in the crypts (SDH staining of frozen section 7x10).

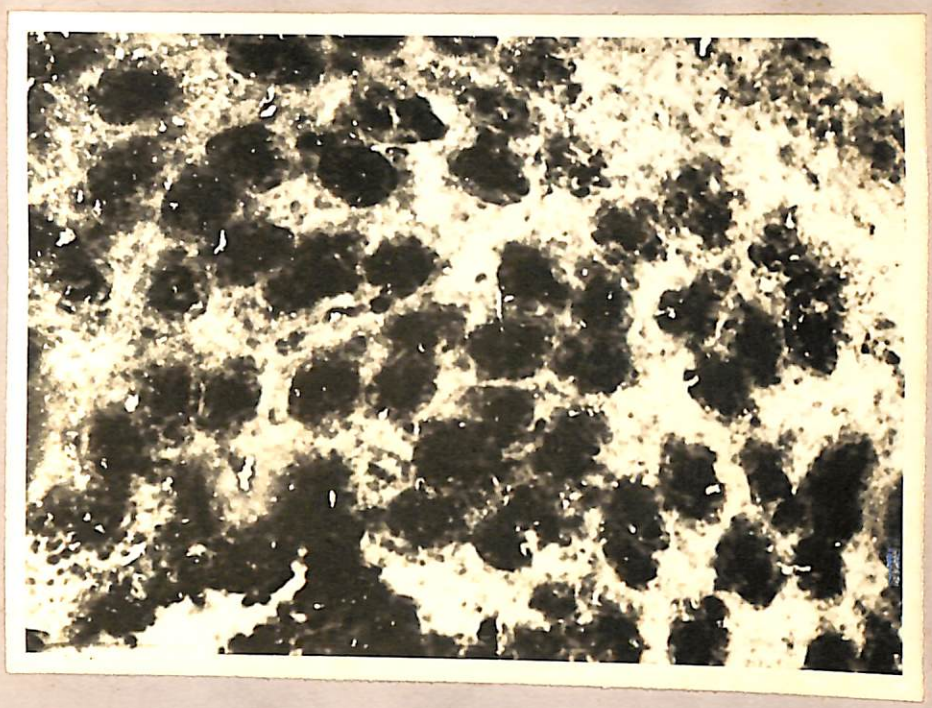


Fig.24. Crypts of ileum exposed to Cl.perfringens type A, HSIII enterotoxin showing SDH activity at the apical region, of epithelial cells of crypts (SDH staining of frozen section 7x40).

Fig.25. Ileum exposed to NSS (control), showing SDH activity at the villus tip (SDH staining of frozen section 7x20).

Table 8

Histochemical studies on succinic dehydrogenase and cytochrome oxidase in rabbit ileum exposed to enterotoxin and cells of *Cl. perfringens* type A Hobbs serotype III, cells of *Cl. perfringens* PFO and *V.cholerae*

Ileum exposed to	Succinic dehydrogenase			Cytochrome oxidase		
	Villus	Crypts	Other layers	Villus	Crypts	Other layers
Normal saline (control)	+++	+	++	-	++	-
HS III-cells	+	+++	++	-	-	-
HS III-enterotoxin	+	+++	++	-	-	-
PFO cells	-	+	++	-	+	-
V.cholerae cells	+	+	+	-	++	-

+, ++, +++ indicate mild, moderate and intense positive reaction, respectively.

- Negative

to normal. The cytochrome oxidase activities were inhibited by HSIII cells and enterotoxin while a moderate activity in crypts was observed in NSS and V. cholerae treated loops. A mild activity was observed in other layers of loop exposed to PFO cells.

DISCUSSION

DISCUSSION

In order to understand the pathogenic mechanism of Cl. perfringens type A enteropathy a two way approach was attempted. The role of bacterial cells, spores and enterotoxin of Cl. perfringens type A and the associated physiopathological and physio-anatomical alterations were studied.

The site of localization of cells, spores and enterotoxin was traced in ligated ileal loops of rabbit inoculated with vegetative cells of Cl. perfringens type A Hobbs serotype III along with sporulation medium. Similarly, localization of enterotoxin in loops inoculated with it was also studied. The ligated ileal loops of rabbits inoculated in either way showed accumulation of fluid. In the frozen sections examined with IFAT and acridine orange, bacterial cells and spores were observed in close proximity of villi. These could not be demonstrated in any of the layers of ileum either with IFAT or with acridine orange, Gram's or haematoxyline-eosin staining in frozen and paraffin sections. However, by IFAT, it was possible to demonstrate enterotoxin absorbed to the villus epithelium. Similarly with ileal loops which had been exposed to enterotoxin, the same was found by IFAT to be localized to the villus epithelium.

The demonstration of the enterotoxin absorbed to the villus epithelium of loops exposed to it and to the cells suspended in sporulation medium suggested that the interaction of enterotoxin with some component of the villi was an important event in the pathogenesis of Cl. perfringens enteropathy. As the cells suspended in the sporulating medium synthesized enterotoxin in situ, the fluid accumulation appears to be enterotoxin mediated. Further the histopathological damage to ileum caused by enterotoxin appears to be similar to those when cells suspended in sporulating medium were inoculated into ligated ileal loops. The enteropathogenic role of cells and spores of Cl. perfringens type A appears to be limited to the in vivo production of enterotoxin only.

The cells of Cl. perfringens type A Hobbs serotype III were non-invasive as evidenced by: (i) their absence from the sites of histopathological alterations observed in ileum exposed to cells suspended in sporulation medium, (ii) similarities between the histopathological damage observed in ileum exposed to enterotoxin and cells suspended in sporulating medium, and (iii) simultaneous demonstration of pathological changes and enterotoxin absorbed to ileal villi but not the bacterial cells.

The present study confirmed the earlier observations made by Chakrabarty and Narayan (1978) who reported in vivo

synthesis of enterotoxin and sporulation by Cl.perfringens type A Hobbs serotype III in the ligated ileum of rabbits. Their failure to demonstrate the organism in the cross section of ileum was also substantiated.

The histopathological changes in the ileal loop of rabbits, brought about by the enterotoxin of Cl. perfringens type A, Hobbs serotype III were mild. The observations included congestion, oedema in submucosa and lamina propria and rarely haemorrhage in lumen of ileum. The cells of this strain suspended in sporulating medium induced similar histopathological changes upon inoculation in ileal loops of rabbits. Others (McDonel, 1974; McDonel and Asano, 1975) also observed only slight histological damage to the mucsoa but the animal model used by these workers was rat. In the rabbits, desquamation of epithelial cells was observed by Chakrabarty (1977) who used the same strain (HS III) of Cl. perfringens type A. His observation, however, was based on a limited number of rabbits.

The observation that enterotoxin of Cl. perfringens type A, Hobbs serotype III absorbed to villus epithelium, to which enteropathy is possibly associated, suggested inhibition of the activity of some epithelial cell membrane bound enzymes. Of the membrane bound enzymes (Na^+-K^+) ATPase is known to regulate the electrolyte

absorption and excretion in the intestine. In the present study it was found that with the increasing dose of enterotoxin and concomittant increase in fluid volume accumulated per cm of ileal loop, the (Na^+-K^+) ATPase activity decreased. There appeared to be an inverse relationship between the enzymic activity and the dose of enterotoxin and fluid secretion. This reduced enzymic activity might be because of reduction in the availability of ATP which is the substrate of this enzyme. In other words, the supply of ATP (i.e. energy), which is required by (Na^+- K^+) ATPase to maintain the (Na^+-K^+) flow across the membrane in the intestine, is reduced under the influence of enterotoxin. McDonel (1974) and McDonel and Asano (1975) suspected a depletion of energy supply to explain the inhibition of glucose uptake and secretion of fluid and electrolyte by rat ileum treated with enterotoxin of Cl. perfringens type A.

In order to reveal the possibility of depletion of energy supply under the influence of enterotoxin, the effect of the latter on the enzymes of the oxidative metabolism was studied. In the present study it was found that in the loops exposed to cells and enterotoxin of HS III the succinic dehydrogenase (SDH) activity in the crypts areas was enhanced while the cytochrome oxidase (CO) activity was completely inhibited. Complete inhibition

of cytochrome oxidase and a simultaneous increase in succinic dehydrogenase is possible. With the inhibition of cytochrome oxidase there is more of reduced ubiquinone and nicotinamide adenine dinucleotide (NADH) which in turn brought about the hyper activity of succinic dehydrogenase (Gutman et al., 1971). Complete inhibition of cytochrome oxidase may initiate an accumulation of metabolic intermediates in the oxidative pathway which bring about a feed back inhibition of the metabolism and, therefore, stop generation of energy. The reduced activity of (Na^+-K^+) ATPase and also the earlier observations on physiopathology cited above are thus amply explained. It appears that the enterotoxin exhibited a toxic effect on the enzymes of respiratory chain of intestinal epithelial cells which lead to the arrest of oxidative metabolism. This enterotoxin has been reported to cause a reduction in oxygen consumption by rat ileum (McDonel and Asano, 1975) and by rat liver mitochondria (McDonel and Duncan, 1977).

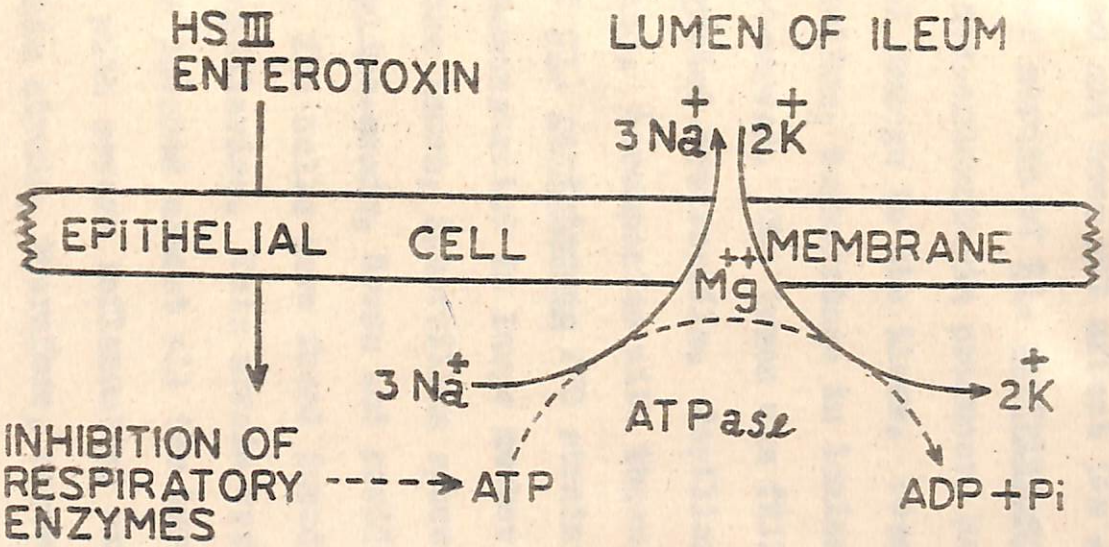
Of the many concepts which have been propounded to explain the fluid accumulation in enteropathies, Norris and Majno (1968) considered the following to be important: (a) mucosal invasion, (b) epithelial denudation, (c) inhibition of sodium pump and (d) alteration of vascular permeability.

Studies conducted on Cl. perfringens type A Hobbs serotype III, ruled out the possibility of mucosal invasion and denudation of epithelium with cells. No conclusive evidence on the alteration of vascular permeability could be brought out, although the staining technique employed to demonstrate the iron was satisfactory.

In brief the mechanism of Cl. perfringens type A, Hobbs serotype III enteropathy is enterotoxin mediated. The enterotoxin appears to have cytotoxic effect on the intestinal epithelium, as also observed by Keusch and Donta (1975). The present observations suggest that the cytotoxic effect is directed to depress the enzymes of respiratory chain. Possibly this leads to a chain of events in the oxidative metabolism ultimately leading to its complete inhibition. The supply of energy is possibly stopped. This is manifested by the reduction in the (Na^+-K^+) ATPase activity. The sodium and potassium ion pump model of Post (1977) is inhibited, as shown on page 67. The mechanism of electrolyte transport across the membrane of intestinal epithelium is thus completely lost. Probably this leads to uncontrolled fluid secretion.

The mechanism of Cl. perfringens type A enteropathy is similar, in part, to those V. cholerae. Both organisms multiply in the intestinal lumen and elaborate enterotoxin in situ. The enteropathy is ascribed to

The effect of Clostridium perfringens type A enterotoxin on the Na^+ and K^+ ion pump.



enterotoxin in both the cases. However, the mechanism of action of the two enterotoxins is different. While enterotoxin of Cl. perfringens type A is cytotoxic those of V. cholerae is cytotonic. Cl. perfringens enterotoxin inhibits (Na^+-K^+) ATPase, choleragen stimulates adenyl-cyclase and does not affect (Na^+-K^+) ATPase.

The other strain of Cl. perfringens, PFO which was found to be non-enterotoxin producer, caused extensive architectural damage to the ileum. Desquamation of villus epithelium, haemorrhage in lamina propria and lumen were observed. The lumen was filled with erythrocytes and disintegrated neutrophils. Capillaries in the submucosa were congested. Accompanied with these destructive changes the cells of Cl. perfringens PFO strain were also seen. These were demonstrated in large number in the haemorrhagic exudate in the lumen, intervillus space and lamina propria by haematoxyline-eosin, Grams and acridine orange staining techniques. The cells were found invading submucosa and also some capillaries. This strain was non-enterotoxin producer and invaded almost all the layers of intestine accompanied with severe inflammatory and destructive changes. This strain, therefore, appeared to be invasive in nature and the enteropathic manifestation may be ascribed to invasion only. Little is known of the mechanisms whereby non-enterotoxin elaborating enteropathogens evoke fluid secretion in the small intestine. The pathogenic

mechanism of this strain seems to be similar to those described for Shigella (La Brec et al., 1964) and Salmonella typhimurium, strain TML (Gianella et al., 1973). Whether this invasive strain of Cl. perfringens synthesized some toxin in vivo, was not tested. However, the effect of PFO infection on the (Na^+-K^+) ATPase, SDH and CO was not significant. Therefore, till more studies are conducted with this strain, the enteropathy is considered to be brought about by invasion only.

SUMMARY

SUMMARY

The mechanism of Cl. perfringens enteropathy was studied in rabbits. Food poisoning strain FH/8588/66 belonging to Hobbs seroype III of Cl. perfringens type A, a laboratory isolate of Cl. perfringens from poultry feed, PFO and V. cholerae Inaba 569B were used in the study.

Cl. perfringens type A, Hobbs serotype III was observed to be non-invasive. The enterotoxin was found to localize at the epithelial cells of ileal villi, whereas the vegetative cells and spores in the spaces adjoining the villi and the lumen. Histological damage caused was relatively mild. The Cl. perfringens type A enteropathy appeared to be enterotoxin mediated. The enterotoxin inhibited the cytochrome oxidase and simultaneously enhanced the activity of succinic dehydrogenase. The (Na^+-K^+) ATPase activity was inversely related with the dose of enterotoxin while the amount of fluid accumulated per cm ileal loop length was directly related. Inhibition of respiratory enzymes possibly lead to reduction in the amount of energy generated and thereby inhibition of the (Na^+-K^+) ATPase activity.

Another strain of Cl.perfringens, PFO, was found to be non-enterotoxin producers but invasive. It caused severe destruction of ileal mucosa. It did not have any effect on the respiratory enzymes and the (Na^+-K^+) ATPase activity.

Choleragen did not produce any histological damage to

the ileum. It did not affect the activities of cytochrome oxidase, succinic dehydrogenase and (Na^+-K^+) ATPase.

The mechanism of Cl. perfringens type A enteropathy appeared to be different from those of V. cholerae.

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APPENDICES

APPENDIX A

Fractionation of serum globulin

The goat antisera raised against normal rabbit serum was fractionated by following the technique of Goldman (1968).

To 40 ml of antisera 40 ml of saturated ammonium sulfate (542 gm/liter) was added slowly with continuous stirring in an ice-batch to achieve globulin precipitation. This suspension was then centrifuged at 4C and the precipitate obtained was dissolved in 40 ml of 0.01M phosphate buffer saline (PBS), pH 7.5. Saturated ammonium sulfate solution was again added and the process was repated twice. Final precipitate was re-dissolved in 40 ml PBS and dialysed at 4C against PBS followed by normal saline (NSS). The NSS was repeatedly changed and checked for sulfate ions using barium chloride. The contents of dialysis bag were considered free of ammonium sulfate when the dialysate showed no reaction with barium chloride. The dialysis process was completed in about 48 hours. The content of the dialysis bag were centrifuged and supernatant stored at 4C in suitable aliquotes.

Protein content was estimated by method of Lowry et al.(1951) and was found to be 21.5 mg/ml.

Labelling of serum globulin, with fluorescein isothyocyanate

The procedure for fluorescein labelling was basically similar to that of Clark and Shepard (1963) as described by Goldman (1968).

Buffered globulin solution obtained above was placed in a dialysis bag and suspended in fluorescein isothyocyanate (FITC) solution. This solution was prepared by dissolving 40 mg FITC in 40 ml of 0.5 M carbonate bicarbonate buffer (pH 9.5), and PBS was added to make the final volume of 400 ml. The container was covered with aluminium foil to retard evaporation. Dialysis was continued for overnight at 4C with constant stirring.

Absorption with tissue preparation

For reducing the non-specific staining the fluorescein conjugate was absorbed with acetone-precipitated rabbit liver powder (Coons and Kaplan, 1950).

Rabbit liver was homogenized in saline with the help of a pestle and mortar, filtered through cotton gauze to remove coarse particles and then washed with acetone to precipitate, and later dried. The precipitated protein was washed in saline before being dried in order to remove soluble materials that might otherwise contaminate the conjugates during the absorption process.

For absorption, the tissue powder, 50 mg/ml of the conjugate was used, incubated two times in succession for one hour at 37C with occasional shaking. The

conjugate was recovered by centrifugation at 3000 rpm for 15 minutes. To avoid loss in volume at each absorption, the tissue powder was soaked in PBS before use.

The final absorbed fluorescein conjugate was passed through a Sephadex G-25, column to remove excess of FITC and carbonates. Elution was done with 0.01 M PBS, pH 7.6 and eluate was concentrated by using 20 per cent polyethylene glycol at 4C. The preparation was distributed in small aliquotes and stored at 4C.

APPENDIX B

Gomeri technique for iron staining (Lillie, 1965).

The scrappings and sections were flooded for an hour with potassium ferrocyanide ($K_4Fe(CN)_6 3H_2O$) solution in 0.06 N HCl when tested for ferric iron and potassium ferricyanide ($K_3Fe(CN)_6$) for ferrous iron. After an hour the slides were washed with one per cent acetic acid and counter stained for two to three minutes in 0.5 per cent basic fuchsin in one per cent acetic acid, washed, dehydrated and mounted in DPX.

Ferric iron was demonstrated as dark blue prussian blue and ferrous iron as dark blue Turnbull blue colour. As a positive control, a section of spleen was also stained along with the intestinal sections by this method.

APPENDIX C

Preparation of standard curve for inorganic phosphorus

Standard stock solution of 10 µg/ml of KH_2PO_4 (M.W. 136) was prepared by dissolving 4.38 mg of it in 100 ml of distilled water. Dilutions of the stock solutions were made starting from 0.5 µg/ml to 4 µg/ml in distilled water. To this 2.8 ml of working solution was added mixed well and the tubes were incubated at 45C for 20 minutes. Yellowish blue colour developed which was read at 820 mu using red filter with Spectronic-20. The optical density values were plotted against the concentrations to obtain a standard curve.

APPENDIX D

Preparation of inocula for ligated ileal loop test

Vegatative cells of Hobbs serotype III raised in cooked meat medium for three to four hours were washed and suspended in NSS. Bacterial counts per ml of the suspension was determined in sulphite iron agar medium (Narayan and Takacs, 1966). In the present study 1×10^6 cells were used for the gut loop test. The vegetative cell stock containing 1×10^6 cells were taken in test tubes and centrifuged at 770 g for 45 minutes at 4C. The cell mass was pelleted and supernatant fluid was discarded. The cell mass of each tube was suspended in 0.8 ml of DS medium and NSS separately and kept ready at 4C for intraluminal inoculation.

Lypholised culture of strain of V.cholerae Inaba 569B was regenerated and choleragen prepared by following the method described by Oza and Dutta (1963). Inoculum per loop consisted of 3×10^{11} cells of overnight grown culture suspended in 0.8 ml NSS or 1.68 mg of choleragen.

APPENDIX E

Preparation of loops for frozen section

Intact ileal loops were suspended in aluminium-foil cylinder containing two per cent carboxy methyl cellulose in 0.15 M saline. The tissue and supporting media were frozen in liquid nitrogen. The frozen loop along with the supporting media was removed from the aluminium foil cyclinder. It was placed on the block holder by means of a forecep after placing few drops of water on the block holder and releasing carbon-di-oxidd gently until the tissue freezed completely. The tissue was shaved down to establish a smooth level surface on which to begin sectioning. Then by a rapid motion of the knife, sections of five to ten μ were cut. The sections were removed from the knife with the help of camel hair brush and placed in petri-dish containing chilled saline. The sections so obtained were subjected to different histochemical and immunohistochemical procedures.