# CLOSTRIDIUM DIFFICILE AND ITS TOXINS

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by

Saleem Hafiz

Department of Microbiology

The University of Leeds

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## ABSTRACT

An investigation of *Clostridium difficile* has shown :

1. that the organism is resistant to cresols and phenol, and may be grown from material grossly contaminated with other organisms by culture in media containing these substances; strains isolated before this method was devised are also resistant to cresols and phenol;
2. that the organism divides into four daughter cells;
3. that each strain examined has a different strain specific agglutinogen, and that the strains are otherwise antigenically complex;
4. that the sugar reactions are fairly consistent;
5. that gelatin is liquefied by all available strains in three weeks; and that apart from this there is no proteolytic activity;
6. that the hyaluronidase produced by all strains is antigenically uniform, and so is the lethal toxin;
7. and that the 'haemolysin' is almost certainly complex.

A token of appreciation

to

Professor C.L. Oakley,
C.B.E., D.Sc., M.D.,
F.R.C., Path., F.R.S.

and

my parents

CONTENTS:

## ACKNOWLEDGEMENTS

Whilst the phraseology of formal acknowledgement may sometimes seem to be guided by precedent, my essential concern in prefacing this thesis is to convey if I can, the depth of my personal gratitude to those whose efforts on my behalf have made my work possible.

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I must also record my everlasting thanks to Dr.G.Eaves, who has played his part in enriching my future work by his thorough teaching in the art of 'Electron Microscopy' and upon whose work I have also been able to draw in compiling this thesis.

The period of my research in the department of Microbiology

has been made happy and memorable for me not least because of the cheerful and unstinted spirit of help and co-operation always afforded to me by the staff, my colleagues, technicians of the department, staff at the Leeds Medical School Library and the staff of the office of the Adviser to Overseas Students. Without wishing to particularize, I must specifically mention and thank Dr. R. Freeman, Dr. S.K. Devraj, Mrs. K. Kidd, Mr. R.S. Burrow, Mr. A. Meyer, Mr. M.B.K. Ghory, Mr. A.A. Makkiya, Mr. A.Y. Ahmed and Miss A. Wahid, in this context.

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# PREFACE

"What is new and significant must always be connected with old roots, the truly vital roots that are chosen with great care from the ones that merely survive." This principle, professed by the composer Bela Bartok, is probably applicable as much to my work as to music. Indeed, it seems to me to synthesize the basic problem to be faced in any study of bacteria Clostridium difficile.

It was in 1935 that Hall and O'Toole first isolated this bacterium; the name they gave suggests that its isolation was by no means easy.

It is perhaps because of the difficulties to be faced in isolating and maintaining this bacterium that, although discovered as a microorganism some 40 years ago, most of todays standard works on Microbiology give it either scant reference, or omit reference to it at all. The bibliography to this thesis is therefore not much related to the culture being studied, for little has been written on it. Instead, my readings have necessarily been based upon lines of approach to methods of studying the organisms. It is fair comment to say that even the most exhaustive reading left me still in the situation where the decision on a possible line of progress was to some extent based upon speculation, if not trial and error.

I will be so bold as to admit that at one stage during the course of my work I was sorely tempted to abandon the project, and would perhaps have done so had I not recalled

a phrase used by my father, the gist of which was "When you get into a tight place and everything goes against you until it seems as though you cannot hold for a minute longer, never give up then, for that is just the place and time at which the tide will turn". I do not know whom my father was quoting, but in the event the phrase carried me, through to the point where perseverance paid dividends.

# CHAPTER I

## GENERAL LITERATURE REVIEW

The credit for the discovery of Clostridium difficile goes to Ivan C.Hall and Elizabeth O'Toole who in 1935 while studying the normal intestinal flora of the nursling reported the isolation from the meconium and faeces of newborn infants during the first ten days after birth of a new obligate anaerobic Bacillus from 40 per cent of cases. They gave it the name Bacillus difficilis because of the unusual difficulty that was encountered in its isolation and study. It is quite possible that they may have encountered the organisms in 1934 when they were studying the intestinal flora of newborn infants during the first ten days after birth; they isolated 18 strains of obligate anaerobic bacilli at that time, but they may have overlooked Clostridium difficile, either because they did not incubate the material for a sufficiently long time, or because other organisms may have overgrown this slowly growing organism. Other early workers may have had the same difficulties.

Hall and O'Toole isolated Bacillus difficilis in pure culture from thirteen specimens obtained from four infants under 10 days of age as an obligate anaerobic, actively motile, heavy bodied rod with oval, subterminal or nearly terminal spores of about the same diameter as the rods. In three of the infants the bacillus was associated with "Kopfchenbacterien"; it appeared and apparently disappeared at about the same time and under the same conditions. It produced small amounts of hydrogen sulphide. Neither gelatin nor Loffler's blood serum was liquefied in 15 days. In milk no change or only a small

amount of gas was produced, on prolonged incubation. With suitable dilutions, well separated colonies appeared in deep agar on the second day of incubation; at first they were minute flat opaque disks, later becoming lobulated and reaching a diameter of 1 mm in about three days. No free gas was formed in the medium. Colonies on blood agar slants under alkaline pyrogallol were irregular, flat and nonhaemolytic. Both acid and gas were produced in dextrose, levulose, mannitol, salicin and xylose, but only traces of gas and no acid were produced in galactose, maltose, saccharose, lactose, raffinose, insulin and glycerol. The most remarkable property of Clostridium difficile was its pathogenicity for guinea-pigs and rabbits. Dextrose broth cultures forty-eight hours old were inoculated subcutaneously in doses of 2 ml. Within ten to sixteen hours after inoculation all guinea-pigs showed moderate to marked oedema spreading over the abdomen and thorax from the site of inoculation. They refused to eat, were in evident pain, particularly when handled, and would sit humped up with their hair bristling with spasms that superficially resembled those of tetanus, except that extensor muscles were more markedly affected and the attacks were more transient than those of tetanus. Sixteen of the twenty-two guinea-pigs died within nine days.

Careful search of the literature substantiates Hall and O'Toole's assertion that the species had not been previously described. The early literature recorded organisms morphologically similar, as, e.g. (1) B.polypiformis of Liborius (1886),

(2) B.radiatus of Luderitz (1889), and (3) Bacillus I of Rodella (1902). The former two are differentiated from Clostridium difficile by their rough colony formation in deep gelatin or agar. Rodella's Bacillus I produces skatol, which is not produced by Clostridium difficile.

The most closely related organism on the basis of standard bacteriological tests is Clostridium sticklandii nov. spec. (1956). However, here also there are a number of readily discernible differences among which are size of cell, colony type, fermentation of salicin, pathogenicity to guinea-pigs, nitrate reduction and gelatin liquefaction tests.

After the preliminary work had been done by Hall and O'Toole, the cultures of Bacillus difficilis, were handed over to Mashall L.Snyder for further studies in early 1936, in addition to the four original strains of Bacillus difficilis, Snyder isolated 17 morphologically similar strains; four of these 17 strains were pathogenic, another pathogenic strain was isolated and included in the study of the toxicity of this species. After studying them closely he gave the following description. Large Gram-positive rods, with elongate, subterminal to terminal non-bulging spores. The rods appeared singly, in pairs, or in short segments, and were sluggishly motile, in 24-hour peptone broth cultures.

Culturally all strains were nonhaemolytic, grayish, rough colonies on blood agar slants under alkaline-pyrogallol.

Biochemically all strains failed to attack coagulated blood serum, liquefied gelatin slowly, formed only gas in milk, did

not produce indol, and fermented glucose, levulose, mannose, xylose, salicin, and mannite, but not galactose, lactose, sucrose, raffinose, inulin, dextrin and glycerol. The only significant difference between these results and those of Hall and O'Toole was the liquefaction of gelatin, which Hall and O'Toole did not obtain. It was apparently essential to maintain the gelatin cultures for at least three weeks. But some of the strains isolated later by M.L.Snyder did not liquefy gelatin, so it would seem that these reactions indicate a variability in the liquefaction of gelatin by strains of Clostridium difficile.

Hall and O'Toole earlier suggested that Bacillus difficilis produced a soluble exotoxin, probably a neurotoxin. Snyder keeping this in mind immunized rabbits with increasing subcutaneous doses of 48-hour veal infusion broth cultures, but owing to the toxicity of his strain he found considerable difficulty in building up a satisfactory antibody level. Snyder found that the antiserum prepared against one strain agglutinated to some extent all the strains tested. The four original strains isolated by Hall and O'Toole were about equally agglutinated to a titre of about 640 with his antiserum, and the four pathogenic strains isolated by Snyder himself were agglutinated by his antiserum as well as or to a greater degree than the homologous strain. Furthermore, four non-pathogenic strains were also agglutinated at 1 in 40, the remaining four strains only slightly at 1 in 20. The control test with B.bifermentans was negative in all dilutions. He interpreted these results as showing an antigenic subdivision in this species which would

include the poorly agglutinated strains. Moreover a serum produced against a strain that was agglutinated only poorly by antiserum against the four original strains, agglutinated the homologous strain to a titre of 1 in 640, but failed to cross-agglutinate the original strains.

Therefore because of the morphological and serological similarity between all the strains, Snyder concluded that they should all, whether pathogenic or not, be classified as Bacillus difficilis.

Snyder, while studying the toxins of pathogenic strains, separated the toxins from the cultures by filtering through Berkefeld or Mandler candles; the filtrates produced a gelatinous hemorrhagic oedema and convulsions in guinea-pigs as described by Hall and O'Toole. Furthermore the toxin was found to be inactivated by heating at 60° C for 5 minutes, and antigenic in dog and in rabbits. Thus, the filterability, thermolability, pathogenicity and antigenicity of the principle proved that B. difficilis produced a true exotoxin. Snyder also showed that toxin appeared at 48 hours in veal infusion peptone broth cultures, and was still detectable after 8 days, the peak time of toxin production was four days. The amount of toxin produced by strains of Bacillus difficilis was extremely variable; some strains produced no detectable toxin at all, other strains a toxin with a minimum lethal dose not greater than 0.001 ml. The MLD for most strains lay between 0.1 and 0.01 ml. The accuracy of the determination of the MLD of these toxins was greatly affected by loss of toxicity on storage.

The list of susceptible animals was extended by Snyder to include the cat, rat, dog, and pigeon. On experimentation all animals showed the same gross pathologic changes consisting of gelatinous hemorrhagic oedema around the site of the subcutaneous inoculation. None of the animals, except the guinea-pigs, showed convulsions. Weakness seemed to be the dominant feature. It was possible to produce an effective antiserum in both rabbit and dog, but considerable difficulty was encountered in preparing antiserum because the animals often died when the doses of filtrate injected increase rapidly. Snyder had found that injection of antiserum protected the animals in most instances, even 4 hours after the injection of toxin, as he gave antiserum a short time after injection of toxin to solve this problem. Furthermore he concluded that the toxin of B.difficilis is a single entity.

In 1939 Snyder isolated Clostridium difficile from infants between the ages of two weeks and one year, and found the organism in 15.4 per cent of cases.

In 1959 R.H.McBee isolated Clostridium difficile from the intestinal tract of a Weddell Seal killed in the Antartic.

In 1962, Smith, , and King reported the occurrence of Clostridium difficile in infections of man. Eight strains of Clostridium difficile were isolated from such cases: one from a case of gas gangrene, one from an abscess following a fracture of the femur, one from a blood culture from an infant, two from pleural fluid, two from peritoneal fluid, and one from an abscess in the vaginal vault. Smith, Louis and King suggested

that the most likely source of these strains was the human body, but emphasised that there was no evidence to suggest that their isolates were pathogenic for man.

In 1972 Danielsson, Lambe and Persson studied the immune response in a patient to an infection (a perirectal abscess) with Bacteroides fragilis sub species fragilis and Clostridium difficile, and demonstrated antibodies against Clostridium difficile by the indirect immunofluorescence technique.

Recently Oakley (1970) demonstrated that filtrates of Clostridium difficile produced local swelling in guinea-pigs skin in 24 hours, with central pale purplish necrosis after 48 hours. The guinea-pigs died after 72 hours. The filtrates were injected intracutaneously. Oakley felt that the skin lesions did not seem sufficient cause for death.

The suggestion by Hall and O'Toole that the toxin of Clostridium difficile might be absorbed from the intestinal tract and cause some of the convulsions occasionally seen in infants and children led to experimental studies by Snyder on the absorption of Clostridium difficile toxin from the intestines of animals. The toxin was given by mouth to guinea-pig and rat and directly into the small intestine of the dog. None of the animals showed any trace of illness whereas the control animals given the toxin subcutaneously died. It would seem probable, therefore, that the toxin of Clostridium difficile is not absorbed from the human intestinal tract. It is interesting that Clostridium difficile filtrates injected subcutaneously (Snyder) or intracutaneously (Oakley) cause death of guinea-pigs.

Snyder suggested that Clostridium difficile toxin resembled that of Clostridium tetani in that neither was absorbed from the intestine.

## SOURCES OF STRAINS

When I started three strains were supplied by the department: the organisms were in cooked meat medium. Three freeze-dried strains of Clostridium difficile were received from the American Type Culture Collection, through the International Centre for Information and Distribution of Type Cultures. Their specification is given in Table I.

It was not possible to obtain Clostridium difficile strains from any European or Canadian Collection.

The first three strains, which were received from the department, were subcultured in cooked meat medium and incubated at 37° C; 72 hours were required to obtain a satisfactory growth. The three freeze-dried culture obtained from the American Type Culture Collection were rehydrated aseptically by the addition of 0.3 - 0.5 ml of nutrient broth to the content of the ampoules and mixed well with it; the mixture was then transferred to boiled cooked meat medium and incubated at 37° C. The three strains N4, N5 and N6 exhibited a prolonged lag period, so that 96 hours of incubation were required before a satisfactory growth was observed. To ensure their viability as well as their purity they were immediately plated on fresh and heated blood agar plates.

These six strains i.e. N1, N2, N3, N4, N5 and N6 were extensively studied.

# TABLE I

## SOURCE OF ORIGINAL STRAINS

| Strain | | Abbreviation | Date of preparation | Received |
|---|---|---|---|---|
| No. | Label | | | |
| 1 | G-173 | N1 | not known | 29.10.1970 |
| 2 | G-238 | N2 | not known | 29.10.1970 |
| 3 | G-311 | N3 | not known | 29.10.1970 |
| 4 | ATCC 17857 | N4 | 18.11.1965 | 17.11.1970 |
| 5 | ATCC 17858 | N5 | 15. 2.1966 | 17.11.1970 |
| 6 | ATCC 9689 | N6 | 16.10.1969 | 17.11.1970 |

Twenty six different strains of Clostridium difficile were isolated from different sources, table II gives the complete list of the isolated organisms.

Of the 26 strains isolated from different sources, strains R1 to R8 were isolated from newborn infants under the age of 5 days. One strain N4I was isolated from a rabbit that died from infection with strain N4: it was recovered from the intestinal fluid, agreed in most respects with strain N4 but differed in the greater speed of some reactions, and was found to be more pathogenic to mice. One strain was isolated from an abscess of thumb and was designated as T1, three strains were isolated from soils (agricultural) of Pakistan, two strains were isolated from donkey's dung, two from horse dung, one each from camel and cow dung, four from hay, one each from sand and mud.

The isolation of Clostridium difficile in pure culture was made considerably easier by the finding that it tolerated up to 0.45 per cent of para-cresol; no other organism present in the materials tested will grow at 37°C in the presence of this concentration of para-cresol.

For the isolation of the strains of Clostridium difficile, 0.2 per cent of para-cresol was incorporated in RCM, and the material were mixed in water blanks and inoculated; even when the material was directly inoculated it gave satisfactory results.

The organisms were also isolated by straight culture on RCA, and I found that the colonies could readily be recognized from the pitting of their surface.

TABLE II

PERSONALLY ISOLATED STRAINS

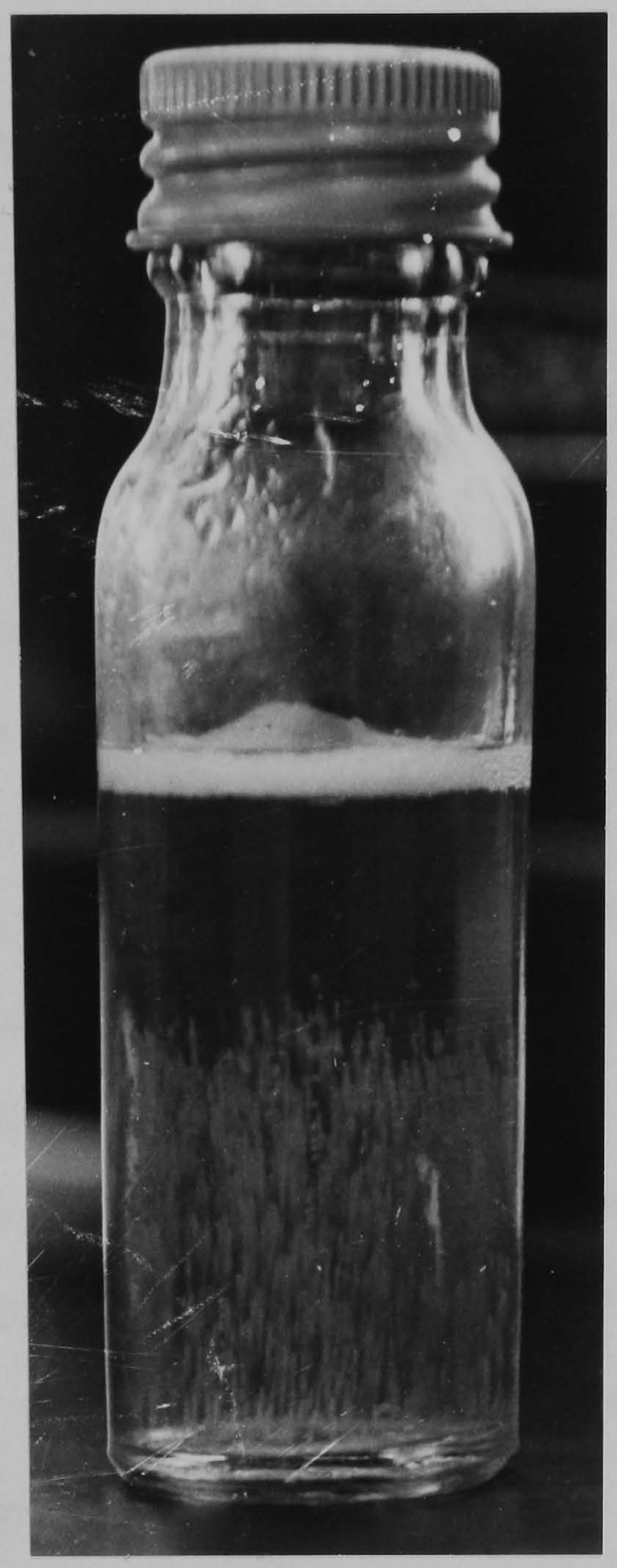
| No | Source of isolation | Date of isolation | Abbreviation |
|---|---|---|---|
| 1 | Stool of newborn baby | 20.11.1970 | R1 |
| 2 | Stool of newborn baby | 20.11.1970 | R2 |
| 3 | Stool of newborn baby | 20.11.1970 | R3 |
| 4 | Stool of newborn baby | 20.11.1970 | R4 |
| 5 | Stool of newborn baby | 21.11.1970 | R5 |
| 6 | Stool of newborn baby | 21.11.1970 | R6 |
| 7 | Stool of newborn baby | 22.12.1970 | R7 |
| 8 | Stool of newborn baby | 22.12.1970 | R8 |
| 9 | Rabbit died from infection with strain N'4 | 27.11.1971 | N'4I |
| 10 | Abscess of thumb | 26. 1.1972 | T1 |
| 11 | Soil (Pakistan) | 25. 4.1972 | T2 |
| 12 | Soil (Pakistan) | 25. 4.1972 | T3 |
| 13 | Soil (Pakistan) | 25. 4.1972 | T4 |
| 14 | Sand (Pakistan) | 25. 4.1972 | T5 |
| 15 | Camel dung (Pakistan) | 25. 4.1972 | T7 |
| 16 | Donkey dung (Pakistan) | 25. 4.1972 | T8 |
| 17 | Donkey dung (Pakistan ) | 25. 4. 1972 | T9 |
| 18 | Horse dung | 27. 4. 1972 | T10 |
| 19 | Hay | 27. 4. 1972 | T11 |
| 20 | Cow dung | 27. 4. 1972 | T12 |
| 21 | Hay | 27. 4. 1972 | T13 |
| 22 | Hay | 27. 4. 1972 | T14 |
| 23 | Mud from the bank of river | 27. 4. 1972 | T15 |
| 24 | Sand | 27. 4. 1972 | T16 |
| 25 | Cow dung | 29. 4. 1972 | T17 |
| 26 | Horse dung | 29. 4. 1972 | T18 |

All the strains received and those isolated by me were Gram-positive rods with oval, subterminal to terminal spores. In smears the rods were usually found singly, in pairs, or in short chains, and were sluggishly motile. Culturally all strains produced nonhaemolytic, grayish, rough colonies on blook agar, failed to attack coagulated serum, liquefied gelatin slowly, formed only gas in milk, did not produce indol, and fermented glucose, levulose, mannose, xylose, salicin, and mannite, but not galactose, lactose, sucrose, raffinose, inulin, dextrin and glycerol when incubated for 72 hours. Strikingly, in Reinforced Clostridial Medium all strains showed well below the surface of the medium a growth of finger-like projections, resembling a corn field; it appeared that each segment is held by air bubbles (Photograph 1).

Tolerance to para-cresol and the typical growth seems to be the most striking and significant characteristic of Clostridium difficile.

PHOTOGRAPH 1

TYPICAL GROWTH OF CLOSTRIDIUM DIFFICILE



# MATERIALS AND METHODS

The materials and methods described here were used throughout the work, whilst the specific methods used for particular experiments have been described under the heading of the experiment.

For Growth:

The need for an ideal medium for growth of Clostridium difficile led me to try different sorts of media such as: Glucose broth, Cooked meat broth (Robertson), Thioglycollate broth, Reinforced Clostridial Agar, Reinforced Clostridial Medium (Hirsch and Grinsted, 1954); Proteose Peptone broth (Modified), Ellner's medium, Fresh and heated blood agar plates, Reinforced Clostridial Medium plus para-cresol; Wijewanta's (1961) three-step process media were also tried. It is true that slightly modified Wijewanta's media gave fairly good growth, but it was found that when the normal pH of Reinforced Clostridial Agar and of the corresponding Medium was readjusted it was the most suitable and convenient medium for the growth of the organism; Proteose Peptone Broth (Modified) was found to be best for toxin production by it. These two media were used throughout for growth, maintainance and toxin production. The formula and modification of Reinforced Clostridial Medium, Reinforced Clostridial Agar. and Proteose Peptone broth (Modified) are given on the following pages.

## REINFORCED CLOSTRIDIAL AGAR

Formula:

| | | |
|---|---|---|
| Yeast extract (Oxoid L 20)... | 3.0 | grams per litre |
| 'Lab-Lamco' Beef extract .... | 10.0 | " " " |
| Peptone (Oxoid L 37) ..... | 10.0 | " " " |
| Dextrose ..... | 5.0 | " " " |
| Soluble starch ..... | 1.0 | " " " |
| Sodium chloride ..... | 5.0 | " " " |
| Sodium acetate ..... | 3.0 | " " " |
| Cysteine hydrochloride ..... | 0.5 | " " " |
| Agar ..... | 15.0 | " " " |
| Distilled water..... | 1.0 | litre |

pH 6.8

The medium is available in dehydrated form from Oxoid 52.2 grams of the dehydrated medium is dissolved in 1.0 litre of distilled water by soaking and steaming for 30 minutes, bottled and sterilized by autoclaving for 20 minutes at 10 lb. per square inch (115°C).

The medium was slightly modified to suit our purpose, the final pH was adjusted to 7.4 - 7.5, as it gave best results. The media was used for all the viable counts.

## REINFORCED CLOSTRIDIAL MEDIUM

Formula:

| | | |
|---|---|---|
| Yeast extract (Oxoid L 20) ... | 3.0 | grams per litre |
| 'Lab-Lemco' (Beef extract) ... | 10.0 | " " " |
| Peptone (Oxoid L37) ..... | 10.0 | " " " |
| Soluble starch ..... | 1.0 | " " " |
| Dextrose ..... | 5.0 | " " " |
| Cysteine hydrochloride ..... | 0.5 | " " " |
| Sodium chloride ..... | 5.0 | " " " |
| Sodium acetate ..... | 3.0 | " " " |
| Agar ..... | 0.5 | " " " |
| Distilled water ..... | 1.0 | litre |

pH 6.8 (approx.)

The medium is available in dehydrated form from Oxoid, 38 grams of the dehydrated medium is dissolved in 1 litre of distilled water by steaming for 15 minutes, bottled in universal container so that each container has 20 ml of the medium. Sterilize by autoclaving for 20 minutes at 10 lb. per square inch (115°C).

The medium was slightly modified by adjusting the final pH to 7.4 - 7.5, which gave the best growth, and was used for maintaining and growing the organisms for inoculum.

## REINFORCED CLOSTRIDIAL PARA-CRESOL MEDIUM

Formula:

| | | |
|---|---|---|
| Yeast extract (Oxoid L 20)..... | 3.0 | grams per litre |
| 'Lab-Lemco'(Beef extract)..... | 10.0 | " " " |
| Peptone (Oxoid L37) ..... | 10.0 | " " " |
| Soluble starch ..... | 1.0 | " " " |
| Dextrose ..... | 5.0 | " " " |
| Cysteine hydrochloride ..... | 0.5 | " " " |
| Sodium chloride ..... | 5.0 | " " " |
| Sodium acetate ..... | 3.0 | " " " |
| Agar ..... | 0.5 | " " " |
| Para-cresol ..... | 2.0 | " " " |
| Distilled water ..... | 1.0 | litre |

pH... 7.4 - 7.5

The most convenient method is to take 38 grams of Oxoid dehydrated Clostridial medium (RCM) and dissolve it in 1.0 litre of distilled water by steaming the mixture for 30 minutes and then to add 2 grams of para-cresol crystals, steam for another 15 minutes, adjust the pH to 7.4 -7.5, and bottle the medium in Universal containers, so that each container has about 20 ml of the medium. The prepared medium is sterilised at 10 lb. per square inch (115°C) for 15 minutes or it can be used unsterilized as I found that para-cresol inhibits the growth of almost all organisms likely to be present except Clostridium difficile. The medium was used for the isolation of Clostridium difficile from different materials.

## PROTEOSE PEPTONE BROTH (MODIFIED)

Formula:

| | |
|---|---|
| Proteose peptone ..... | 15.0 grams per litre |
| Dipotassium hydrogen phosphate ..... | 5.0 " " " |
| Yeast extract ..... | 5.0 " " " |
| Sodium chloride ..... | 5.0 " " " |
| Glucose ..... | 10.0 " " " |
| Distilled water ..... | 1.0 litre |

Final pH 7.2 - 7.3

The components of the medium are weighed according to the formula, and added to 1.0 litre of distilled water, and mixed by magnetic stirrer for 30 minutes, and then steamed for 5 minutes, and tubed in large tubes or bottles, and sterilized by autoclaving for 10 minutes at 10 lb per square inch (115$^{o}$C). Always freshly prepared medium was used for the toxin production.

# CHAPTER II

# MORPHOLOGY AND CULTURAL CHARACTERISTICS

## Morphology

All the strains of Clostridium difficile were found to be usually long, slender, Gram-positive bacilli, which tend to lose their Gram reaction in cultures more than 96 hours old. At times the shape varied from very short fairly thick bacillus to a large bacillus. In smears the rods were usually found singly, in pairs, in short segments and occasionally in bunches, but when they were found in bunches they were usually very short rods. Spores were produced rarely in older cultures, and were large, oval and subterminal, slightly distending the sporangium. In older cultures the spores appeared to be terminally situated due to loss of a terminal cap of protoplasm from the sporangium. In coverslip preparation of proteose peptone broth medium, the organisms are sluggishly motile. The flagella are few but distributed around the cell.

## Cultural

The strains were grown on various solid media to study the colonial morphology of the organisms. The characteristics of the colonies are described on the following page. The organisms failed to produce any haemolysis on blood agar plates even when they were incubated for 96 hours.

Colonial appearance of Clostridium difficile on nutrient agar plates

All the strains of Clostridium difficile produced colonies between 0.5 and 1.5 mm in diameter, greyish in colour, with an entire margin, opaque, smooth, flat and pitted, and odourless.

b) Colonial appearance of Clostridium difficile on fresh Blood agar (oxalated horse blood) plates

All the strains but N1 produced colonies between 1.0 and 2.5 mm in diameter, greyish, irregular, opaque, smooth, flat and pitted, with a sweet fruity smell; they did not produce haemolysis. The colonies of strain N1 were convex instead of flat; the rest of the characters were the same as in other strains.

c) Colonial appearance of Clostridium difficile on heated blood agar plates

The strains of Clostridium difficile produced colonies between 1.0 and 2.5 mm in diameter, irregular (finely scalloped) to regular, opaque, smooth, flat to convex colonies, which were pitted in the centre and had a sweet fruity odour; there was no proteolysis.

d) Colonial appearance of Clostridium difficile on Reinforced Clostridial Agar medium

The strains of Clostridium difficile produced colonies between 2.0 and 4.0 mm in diameter, greyish, irregular (finely scalloped) to regular, opaque, smooth, flat and pitted colonies with sweet fruity smell.

## Growth in Reinforced Clostridial Medium

Reinforced Clostridial Medium (RCM) is a semi-solid medium designed by Hirsch and Grinstead (1954) for the cultivation and enumeration of clostridia. They showed that the medium gave a more abundant growth and enabled growth to be initiated from inocula smaller than those necessary for growth in five other media tested: 1. Yessair medium (1948); 2. Yessair medium with yeast extract; 3. meat extract liver medium (1937); 4.Corn-liver medium (1934) and 5. peptone-supplemented milk medium (1941). In a further comparison, the highest viable count obtainable was the criterion used, and again, RCM proved superior; I have found that this is true for Clostridium difficile, provided the pH is adjusted to 7.2 - 7.4.

Dehydrated RCM (Oxoid) was found to be most duitable: 38g of the dehydrated medium is dissolved in 1 litre distilled water by heating in the steamer, the pH is adjusted to 7.2 - 7.4 and the medium is filled into Universal containers in volumes of 20 ml, and sterilized by autoclaving for 20 minutes at 10 lb per square inch (115°C).

0.5 ml of the culture is inoculated in each bottle and the inoculation medium is incubated at 37°C, and the growth pattern is observed.

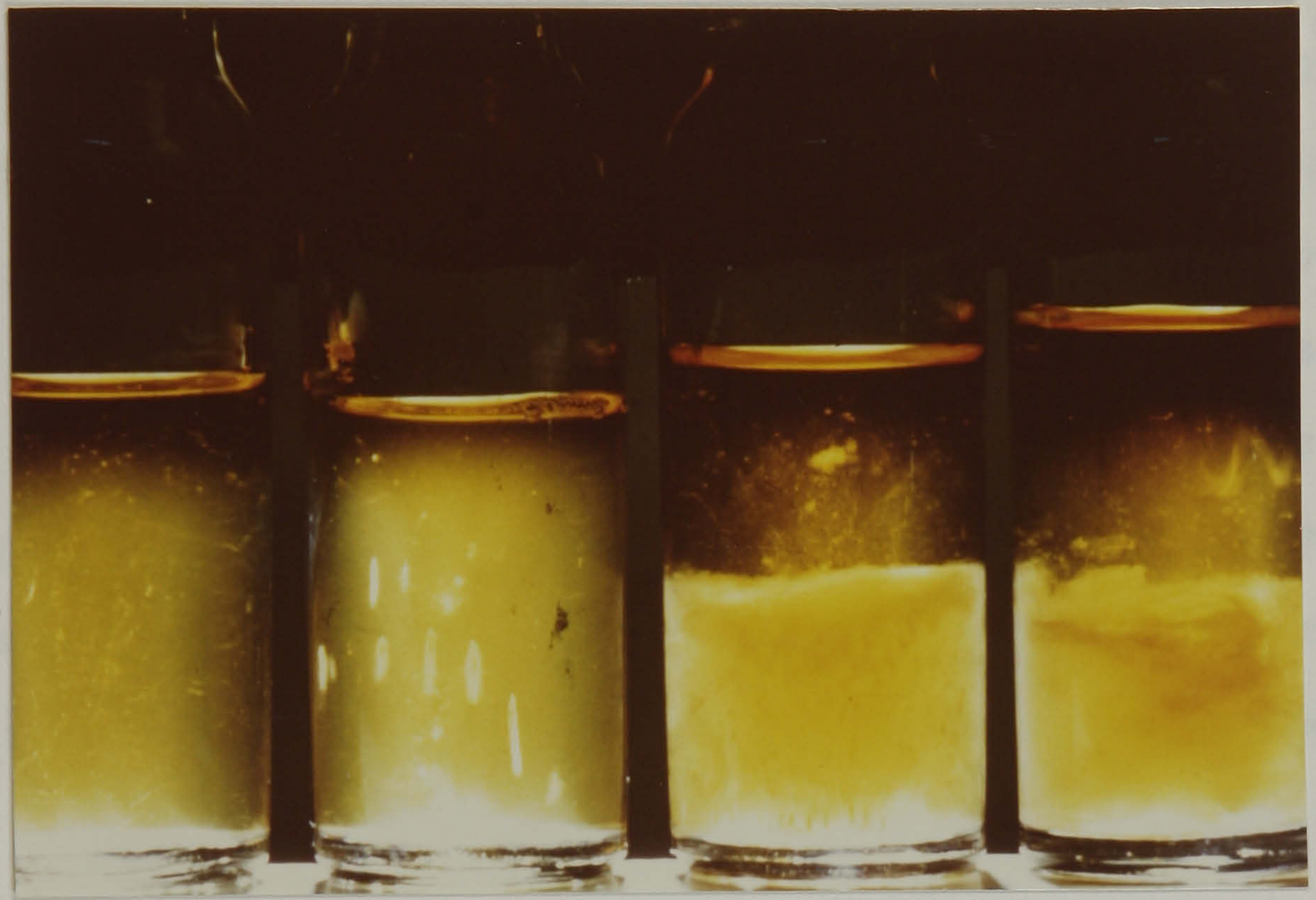
Strangely, it was observed that at first the organisms began to grow as a small "tear drop" at about 1.0 - 1.5 cm from the meniscus of the medium, suggesting that this is the point where growth conditions are optimal. The "tear drop" gradually grow downwards as finger-like projections, these appear to be

held in position by air bubbles, giving the appearance of a 'corn field'. The strings of growth withstand gentle shaking; later the growth becomes more profuse and tends to form a mushroom-like cloud, settling down to the bottom in the final stages, when the supernatant becomes clear and almost sterile. Photographs 2 and 3 depict the different stages of growth.

Further studies have shown that the duration of the characteristic growth-phases of Clostridium difficile varies with the age of the initial inoculum. If the initial inoculum is from the logarithmic phase, the interval between the phases is shorter and less distinct, as the organism passes through the various phases in a very short time, and even when the amount of inoculum is reduced to 0.2, or 0.1 ml, the growth pattern is almost identical with that of a 0.5 ml inoculum. On close observation it has been found that the growth starts as a "tear drop", descending downwards, forming mushroom type cloudy growth within 18 - 24 hours, but the growth settles down to the bottom in 96 - 168 hours. When the initial inoculum is at least a month old, or is obtained from a 72 hour growth on a solid medium, soil, or dung, or faeces of newborn children, the first sign of growth appears in 12 - 18 hours, and the extension of the first growth can be seen after 24 - 36 hours, reaching to the bottom of the Universal container in 48 hours, forming the mushroom-shaped cloudy structure in 96 hours and settling down to the bottom in 168 hours with a clear supernatant.

Bottles of different shape and size (e.g. flattened bottles) have been tested for their capacity to yield the characteristic

PHOTOGRAPHS 2 AND 3 SHOWING THE DIFFERENT STAGES OF GROWTH OF CLOSTRIDIUM DIFFICILE IN REINFORCED CLOSTRIDIAL MEDIUM



growth, but without any appreciable success; this is probably due to the fact that in the Universal type containers the pressure of the medium is just sufficient to yield the characteristic growth.

Survival of Clostridium Difficile in Liquid Medium

Cultures of Clostridium difficile were gorwn in RCM and Cooked meat medium and stored in the cold room at 3°C and at room temperature (16°C) and tested for viability at intervals of 6 months. It was found that cultures in RCM at room temperature remained viable for a period of one year, whereas cultures in Cooked meat medium, both at room temperature and in the cold room, and also in RCM kept in the cold room remained viable even at the end of 3 years. This suggests that the organisms can survive for even longer periods of time, and can be stored conveniently.

## Fermentation

The anaerobic oxidations - reductions are often referred to as 'Fermentations', and in the process the organisms break up the organic compounds in order to obtain energy. As we are aware, bacteria are just as selective as any other form of life for their nutritional requirements, so they are equipped with certain mechanisms to breakdown some specific organic compounds. In most of the cases when the fermentation reactions are studied generally a suitable fermentation medium is selected and after the incorporation of the fermentable substance, the inoculum is added, and incubated at the optimum temperature for 48 hours and the results are finally recorded.

In the course of my studies, the incubation period was prolonged (40 days) and results were recorded at the intervals of 24, 48, 72, 96, 120, 144, 168, 192 hours and after 40 days, and almost a complete picture of fermentation by Clostridium difficile was obtained.

The organisms were grown in Proteose peptone water containing the substrate for fermentation and with phenol red as an indicator. The fermentation tubes for each substrate were incubated along with the control tubes in an anaerobic jar filled with hydrogen by a standard technique and incubated at 37°C for 40 days. The reactions were examined at definite time-intervals; the catalysts were changed regularly and the results recorded. It was found that the fermentation reactions of N1 - N6 of Clostridium difficile and of the other isolates were very uniform. Glucose, dextrose, melezitose, mannite, mannitol, xylose, raffinose and

fructose were fermented with the production of acid and gas; acid is produced during the first 24 - 96 hours of culture, after which gas is produced. Sucrose, maltose, glycogen, soluble starch and sorbitol are fermented by all the strains with the production of acid only; raffinose, starch, rice starch, and DL-methionine are not fermented by any strain. Arabinose is fermented by N1, N3, N4, and N5, and rhamnose fermented by N3, N4, N5 and N6 both with the production of acid and gas. In inulin only gas is produced by all strains. Maize starch is attacked by strains N2, N3 and N5, potato starch by N1 and N2; dulcitol by N1 and N5, and i-inositol by N1, N4 and N6; from all four substrate only acid is produced. Table III gives the complete fermentation picture.

From the present studies it can be concluded that a final conclusion about the capacity of Clostridium difficile to ferment a given sugar requires prolonged incubation.

# TABLE III

## FERMENTATION REACTION OF CLOSTRIDIUM DIFFICILE

## AFTER 40 DAYS INCUBATION

| Substrate | Results of incubation with Cl.difficile strain | | | | | |
|---|---|---|---|---|---|---|
| | N1 | N2 | N3 | N4 | N5 | N6 |
| Glucose | AG( 72) | AG( 48) | AG( 48) | AG( 72) | AG( 72) | AG( 96) |
| Galactose | AG( 72) | AG( 96) | AG(144) | AG(168) | AG( 72) | AG( 96) |
| Levulose | AG( 48) | AG( 96) | AG(120) | AG( 96) | AG( 96) | AG(72) |
| Dextrose | AG(120) | AG( 48) | AG( 72) | AG( 72) | AG( 72) | AG(144) |
| Lactose | - | - | - | - | - | - |
| Sucrose | A ( 72) | A( 48) | A( 72) | A( 72) | A( 72) | A( 96) |
| Maltose | A ( 24) | A( 24) | A( 48) | A( 72) | A( 96) | A( 96) |
| Raffinose | AG( 72) | AG(144) | AG( 72) | AG( 72) | AG(168) | AG( 72) |
| Trehalose | AG( 96) | A( 48) | A( 48) | A( 96) | A(120) | AG( 48) |
| Glycogen | A(144) | A(144) | A(192) | A(240) | A(240) | A(240) |
| Xylose | AG(144) | AG( 96) | AG(168) | AG(144) | AG(168) | AG(168) |
| D(-)Arabinose | AG(120) | - | AG(144) | AG(192) | AG(168) | - |
| L-Rhamnose | - | - | AG( 96) | AG(120) | AG(120) | AG(168) |
| Salicin | A( 24) | AG( 48) | AG( 48) | AG( 48) | AG(120) | A ( 24) |
| Esculin | AG( 24) | AG( 48) | AG( 48) | AG(120) | AG(168) | AG(168) |
| Inulin | G( 72) | G(120) | G(120) | G( 96) | G(120) | G(120) |
| Starch | - | - | - | - | - | - |
| Soluble starch | A( 48) | A( 24) | A( 96) | A( 48) | A( 24) | A( 48) |

TABLE III (Contd.)

| Substrate | N1 | N2 | N3 | N4 | N5 | N6 |
|---|---|---|---|---|---|---|
| Maize starch | - | A( 72) | A( 96) | .. | A( 24) | - |
| Potato starch | A(192) | A( 96) | - | - | - | - |
| Rice starch | - | - | - | - | - | - |
| Fructose | AG( 48) | AG( 48) | AG( 48) | AG( 48) | AG( 72) | AG( 48) |
| Cellobiose | A( 48) | A( 48) | A( 48) | AG( 48) | AG( 72) | A( 24) |
| Mannose | AG( 48) | AG( 72) | AG( 48) | AG( 48) | AG(120) | AG(168) |
| Dextrin | AG( 96) | AG( 96) | AG(144) | - | AG(120) | AG(144) |
| Melezitose | AG( 96) | AG( 72) | AG( 96) | AG( 72) | AG(240) | - |
| Dulcitol | A( 48) | - | - | - | A( 96) | - |
| Glycerol | - | G( 72) | G(144) | G(240) | - | - |
| Mannitol | AG( 72) | AG(120) | AG( 48) | AG( 96) | AG(120) | AG(168) |
| i-Inositol | A( 48) | - | - | A(192) | - | A(144) |
| Sorbitol | A( 48) | A( 24) | A( 72) | A( 24) | A( 24) | A( 48) |
| DL-Methionine | - | - | - | - | - | - |

( ) indicates time in hours when reaction was completed

## Gelatin Liquefaction

The ability of bacteria to liquefy gelatin has been used for long to study the characteristics of bacteria; the method generally used for the liquefaction of gelatin is the stab method, which in a number of cases is not quite reliable. The method was modified by Frazier (1926) to show changes in the composition of the gelatin due to bacteria rather than the detection of liquefaction, and this method has been used for determining the ability of Clostridium difficile to liquefy gelatin.

## Materials and Method

The gelatin agar medium used for this purpose has been prepared according to Frazier's specification, i.e. the gelatin agar medium is prepared by dissolving in 100 ml of distilled water NaCl 5.0 g, $K_2HPO_4$ 1.5 g. 4 g of Bacto gelatin is dissolved in 400 ml of distilled water, and to this solution 0.05 g dextrose and 0.1 g Bacto peptone are added. The two solutions were mixed, heated in a steamer and then mixed with 500 ml of 3 per cent agar, the final pH was adjusted to 7.0 and the medium was bottled in 500 ml bottles and autoclaved. Plates were poured with the sterilized medium and allowed to harden. In each case duplicate plates were inoculated on the surface of the medium at the centre, and incubated in an anaerobic jar under anaerobic conditions, at 37°C for 30 days, as it was found necessary to incubate the plates for 30 days.

After 30 days of incubation one plate was flooded with a 1 per cent solution of tannic acid, whilst the second plate was flooded with an acid solution of mercuric chloride (15 g of $HgCl_2$ dissolved in 20 per cent HCl).

The plate flooded with tannic acid solution gave results varying from a white precipitate around the colony, heavier than the precipitate of gelatin throughout the rest of the plate to clear zone around the colony surrounded by a distinct white ring, indicating considerable decomposition of the gelatin.

The plate flooded with $HgCl_2$ solution, gave a clear zone around the colony surrounded by the cloudy precipitate of unchanged gelatin; the reaction takes about 30 minutes.

It was interesting to note that when the plates were incubated in anaerobic condition in an atmosphere of hydrogen. the zones formed around the colonies were less clear than the zones formed on the plates incubated in the atmosphere of 95 per cent hydrogen and 5 per cent carbon dioxide.

All the strains of Clostridium difficile including the ones isolated by me (table II), liquefied gelatin in 30 days; freshly isolated strains liquefied gelatin in 2 - 3 weeks and an atmosphere of 95 per cent hydrogen and 5 per cent carbon dioxide accelerated the process of gelatin liquefaction, as compared with atmospheres of 100 per cent hydrogen.

Test carried out by punching holes in the gelatin plates and adding filtrates,or concentrated filtrates, failed to produce any gelatin liquefaction.

## Hydrogen Sulphide Production

Some organisms decompose sulphur-containing amino acids, to form hydrogen sulphide. The presence of hydrogen sulphide in a bacterial culture can be demonstrated by exploiting its ability to alter soluble metallic salts to insoluble black metallic salts. For this purpose lead, iron or bismuth may be incorporated in the media. Sometimes the metallic salts inhibit the growth of the organism. In order to avoid this difficulty, a dry sterile strip of filter paper impregnated with a saturated solution of lead acetate was used. The indicator strip was wedged into the top of the test tube with the cotton-wool plug so that about an inch of the strip projected below the plug.

The test was carried out by inoculating cooked-meat medium with Clostridium difficile which had been incubated at 37°C for 72 hours. The filter-paper strip was examined and the results were considered positive when the filter strips turned black.

The results of this qualitative test showed that only one strain N3 produced slight blackening, while all the others gave negative tests, and hydrogen sulphide was not produced.

## Indole Formation

This test demonstrates the ability of certain bacteria to decompose the amino acid tryptophane to indole. The test was carried out by growing the organisms, in Reed and Orr's indole medium, which consisted of Bacto tryptone 20 g, $Na_2HPO_4$ 5 g, glucose 1 g, agar 1, sodium thioglycollate 1 g in 1 litre distilled water. The medium was inoculated with Clostridium difficile and incubated for 48 - 96 hours and tested for indole

formation. The tests were negative in all cases, suggesting that indole is not formed by Clostridium difficile.

Nitrate Reduction Test

Clostridium difficle strains were tested for nitrite production in the following medium.

Bacto tryptone 20 g, $Na_2HPO_4$ 2 g, Glucose 1 g, Agar 1 g, $KNO_3$ 1 g, distilled water 1 litre. The pH was adjusted to 7.6 and sterilized.

The tubes of nitrate medium were inoculated with 24-hour cultures of Clostridium difficile strains, and incubated anaerobically at 37°C, and tested at intervals of 24 hours, 48 hours, 72 hours and 96 hours for the production of nitrite by Tittsler's (1930) method. A pink or red colour was taken to indicate the presence of nitrite.

All strains of Clostridium difficile produced nitrite; the test was positive between 72 and 96 hours.

Litmus Milk

The milk remained unchanged, though a small amount of gas was produced.

None of the strains of Clostridium difficile digested casein, or liquefied coagulated albumin or blood serum.

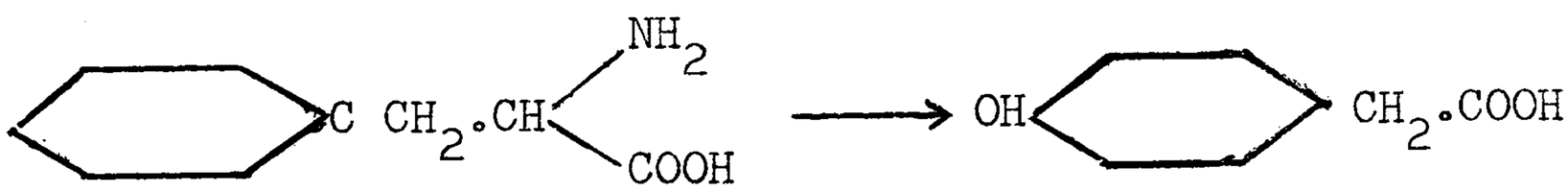
The antibiotic sensitivity tests revealed that all the strains of Clostridium difficile are sensitive to Chloromycetin, Terramycin and Erythromycin, resistant to Penicillin and Sulphadimidine, strains N5 and N6 were sensitive to Streptomycin, the others were resistant. Neomycin (30) has no effect on the strains of Clostridium difficile.

# CHAPTER III

## CRESOLS AND PHENOLS AS SELECTIVE AGENTS FOR THE ISOLATION AND GROWTH OF CLOSTRIDIUM DIFFICILE

A short time after I arrived in the Department of Bacteriology in Leeds, Professor S.D.Elsden, Director of the Food Research Institute, Norwich, wrote to Professor C.L.Oakley, asking him whether he had any strains of Clostridium difficile, as he and a colleague (Dr.Meade) had found that the only strain of Clostridium difficile they had attacked phenylalanine to produce a phenol. Professor Oakley sent Professor Elsden the six strains we had, and soon received a letter stating that all the Clostridium difficile strains behaved in the same way, and that the phenol was paracresol.

Evidently Clostridium difficile first attacks phenylalanine by converting it into parahydroxyphenylacetic acid, as most Clostridia that attack phenylalanine do, and then uniquely among the clostridia examined, decarbooxylates this metabolite to paracresol.



phenylalanine → parahydroxyphenylacetic acid

paracresol

Professor Oakley considered that as Clostridium difficile produced paracresol, a known disinfecting agent, it must be fairly

resistant to it, and suggested that it might be possible to isolate Clostridium difficile by culturing material containing it in paracresol-containing media.

Cresols and Phenols are obtained from the destructive distillation of coal, between the temperatures of 170°C and 270°C, and are powerful antiseptics. Their chief use in a laboratory is for sterilising surgical instruments and discarded cultures, and killing cultures accidentally spilt by the worker. Cresols are generally used in a 0.1 per cent solution for preserving sera and vaccine (Cruickshank 1969). The cresols are much more powerful disinfecting agents than the phenols. Phenols in certain proportions are able to pass into solution in water, but cresols do not do so; when mixed with water they form very fine emulsions.

It has been supposed that phenol acts by forming, in contact with proteins, an insoluble albuminate and other chemical compounds. Reichel (1909) suggested that the action is not so much chemical as physical, and that its disinfectant action results from its penetration into the bacterial cell in the form of a colloidal solution.

The cresols probably act in much the same way as phenol; by virtue of their emulsified state, their particles are absorbed on to the surface of suspended matter, and hence their concentration is increased in the immediate neighbourhood of bacteria.

During the course of the study, I decided to see the effect of paracresol on Clostridium difficile. The results were so astonishing and encouraging that a complete study was done.

Materials and Method

All the strains available were used in the preliminary examination of the effect of paracresols, even those isolated in media containing paracresol. As all the strains behaved in the same way, detailed studies were made with the strain N4. Both liquid and solid medium were used for the studies: the media used were Reinforced clostridial medium, and Reinforced clostridial agar. A range of percentages of para-, meta-, or ortho-cresols or phenol was used.

Method

Reinforced Clostridial Agar (Oxoid) was mixed with distilled water according to the company's directions, and the final pH was adjusted to 7.2 - 7.4. A 10 per cent stock "solution" of paracresol was made. The medium was distributed in bottles so that each bottle contained 100 ml of the medium with the required concentration of paracresol: 0, 0.1, 0.2, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 per cent. One set of the bottles with the medium was sterilized by autoclaving for 20 minutes at 10 lb per square inch (115°C). Plates were poured from both the sets (sterilized and unsterilized) and allowed to solidify, one group of plates from each set was incubated in an anaerobic jar as control, whilst other sets of plates were inoculated with six strains of Clostridium difficile (N1 - N6) by streaking the plates with 24-hour-old cultures. A set of plates from each set was used for the pour-plate method for the six strains; in these tests 0.05 ml of the culture mixed with 12 ml medium was poured and allowed to solidify. The plates were incubated at 37°C in anaerobic jar for

72 hours, and examined after 24, 48, and 72 hours, and the results recorded (Table IV).

From table IV, it can be seen that there was no difference in the behaviour of the medium, whether the medium was used sterilised or unsterilised, with the exception that the control of unsterilised medium showed a few colonies of contaminants. It also shows that with addition of sufficient paracresol the medium became sterilised.

At a concentration of 1.0 per cent paracresol a very thin film of paracresol was visible on the surface of the medium. This film became thicker and thicker with the increase in the concentration of paracresol, indicating that paracresol forms a fine emulsion and is deposited on the surface when the concentration is high. It also indicates that the distribution of paracresol in the solid medium is not uniform so that solid media containing paracresol are unsuitable for experiments to determine the effect of paracresol on the organisms.

Reinforced Clostridial Medium (semi-solid) was prepared according to the instructions of the manufacturer, by adding 38 g of the granules (Oxoid CM 149) to 1.0 litre of distilled water, setting the mixture aside to soak for 15 minutes, steaming it for 30 minutes, and adding enough of the 10 per cent stock solution of para-cresol to give a final concentration of 0.0, 0.1, 0.2, 0.5, 1.0, 2.0, 3.0, 4.0 or 5.0 per cent para-cresol. The pH was adjusted to 7.2 - 7.4, and the medium was bottled in Universal containers, so that each bottle contained 20 ml of the medium (RCM) sterilised by autoclaving for 20 minutes at 10 lb per sq. inch (115°C).

## TABLE IV

## SHOWING THE EFFECT OF VARYING CONCENTRATION OF PARACRESOL ON CLOSTRIDIUM DIFFICILE

| Medium | Strain | Amount of growth on medium containing paracresol (per cent ) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 0.0 | 0.1 | 0.2 | 0.5 | 1.0 | 2.0 | 3.0 | 4.0 | 5.0 |
| Unsterilised | Control | + | - | - | - | - | -* | -* | -* | -* |
| | N1 | ++ | ++ | ++ | ++ | + | - | - | - | - |
| | N2 | +++ | ++ | ++ | ++ | + | - | - | - | - |
| | N3 | +++ | ++ | ++ | ++ | + | - | - | - | - |
| | N4 | +++ | ++ | ++ | ++ | + | - | - | - | - |
| | N5 | +++ | ++ | ++ | ++ | + | - | - | - | - |
| | N6 | +++ | ++ | ++ | ++ | + | - | - | - | - |
| Sterilized | Control | - | - | - | - | - | - | - | - | - |
| | N1 | ++ | ++ | ++ | ++ | + | - | - | - | - |
| | N2 | ++ | ++ | ++ | ++ | + | - | - | - | - |
| | N3 | ++ | ++ | ++ | ++ | + | - | - | - | - |
| | N4 | ++ | ++ | ++ | ++ | + | - | - | - | - |
| | N5 | ++ | ++ | ++ | ++ | + | - | - | - | - |
| | N6 | ++ | ++ | ++ | ++ | + | - | - | - | - |

++ = Abundant growth

\+ = Growth

\- = No growth

* = Formation of film of paracresol

One set which was not autoclaved gave the same results. When the same concentrations of paracresol were made up in Proteose peptone medium it gave the same results as RCM, therefore RCM was used extensively. As no growth was observed in 0.5 per cent paracresol in both RCM and Proteose peptone medium, RCM was made again in such a way that the final concentration of paracresol in the medium was 0.0, 0.1, 0.2, 0.3, 0.4 or 0.5 per cent. 0.5 ml of 24-hour-old culture was inoculated into each container of the medium, the culture was incubated at 37°C and results were observed after 72 hours and recorded. Table V shows the growth of the strains of Clostridium difficile in varying concentrations of paracresol.

From table V it can be concluded that all the strains (N1 - N6) behave in the same way; the organisms are capable of growing in the presence of paracresol up to a concentration of 0.3 - 0.4 per cent. Similar results were obtained with strains of Clostridium difficile isolated without the use of paracresol.

Dilutions of meta- and ortho-cresol, and of phenol were prepared in the same way as for paracresol, and 0.5 ml of the culture was inoculated in each bottle and incubated at 37°C. The results were recorded in a tabular form. From Table VI it can be seen that the position of the substituent in the ring in cresol made no difference to the effect on the organisms, which behaved in the same way as when paracresol was used; with phenol a slight difference was observed, for at any concentration used comparatively more growth was obtained than with the corresponding concentration of cresol, some growth was

seen even at a concentration of 0.5 per cent phenol; indicating that the organisms can tolerate phenol to slightly higher concentrations than cresols.

## TABLE V

## SHOWING EFFECT OF PARACRESOL ON THE STRAINS OF CLOSTRIDIUM DIFFCILE IN RCM

| Strain | Incubation period (hr) | Amount of growth of Cl.difficile in RCM containing paracresol (per cent) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0.0 | 0.1 | 0.2 | 0.3 | 0.4 | 0.5 |
| N1 | 24 | +++ | +++ | ++ | + | - | - |
| | 48 | +++ | +++ | +++ | + | - | - |
| | 72 | ++++ | ++++ | +++ | ++ | ± | - |
| N2 | 24 | ++ | ++ | + | + | - | - |
| | 48 | +++ | +++ | ++ | + | - | - |
| | 72 | ++++ | ++++ | +++ | + | ± | - |
| N3 | 24 | +++ | ++ | ++ | + | - | - |
| | 48 | +++ | ++ | ++ | = | - | - |
| | 72 | ++++ | +++ | +++ | ++ | - | - |
| N4 | 24 | +++ | ++ | ++ | + | - | - |
| | 48 | +++ | +++ | ++ | + | - | - |
| | 72 | ++++ | ++++ | +++ | ++ | ± | - |
| N5 | 24 | ++ | ++ | + | + | - | - |
| | 48 | +++ | +++ | ++ | + | - | - |
| | 72 | ++++ | ++++ | +++ | ++ | ± | - |
| N6 | 24 | +++ | +++ | ++ | + | - | - |
| | 48 | +++ | +++ | ++ | ++ | ± | - |
| | 72 | ++++ | ++++ | +++ | ++ | ± | - |

\- = No growth

± = Slight growth

+,++,+++,++++ = Increasing density of growth

TABLE VI

SHOWING THE EFFECT OF PHENOL, PARA-, META- AND ORTHO-CRESOL ON THE STRAINS OF CLOSTRIDIUM DIFFICILE

| Substance | Strain | Amount of growth in medium containing substance (per cent) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0.0 | 0.1 | 0.2 | 0.3 | 0.4 | 0.5 |
| Phenol | N1 | + | + | + | + | + | - |
| | N2 | + | + | + | + | + | - |
| | N3 | + | + | + | + | + | - |
| | N4 | + | + | + | + | + | - |
| | N5 | + | + | + | + | + | ± |
| | N6 | + | + | + | + | + | - |
| Paracresol | N1 | + | + | + | + | + | - |
| | N2 | + | + | + | + | + | ± |
| | N3 | + | + | + | + | - | - |
| | N4 | + | + | + | + | - | - |
| | N5 | + | + | + | + | - | - |
| | N6 | + | + | + | + | - | - |
| metacresol | N1 | + | + | + | + | + | - |
| | N2 | + | + | + | + | - | - |
| | N3 | + | + | + | + | ± | - |
| | N4 | + | + | + | + | - | - |
| | N5 | + | + | + | + | - | - |
| | N6 | + | + | + | + | - | - |
| orthocresol | N1 | + | + | + | + | - | - |
| | N2 | + | + | + | + | + | - |
| | N3 | + | + | + | + | - | - |
| | N4 | + | + | + | + | ± | - |
| | N5 | + | + | + | + | - | - |
| | N6 | + | + | + | + | - | - |

\- = No growth

± = Slight growth

\+ = Well marked growth

## The growth of bacterial cultures in various concentration of Paracresol and Phenol

The growth of microorganisms is essentially the specific, balanced synthesis of the components of protoplasm from the nutritive substances present in the immediate environment. In addition, the newly synthesized constituents must be assembled and appropriately packaged to yield replicates of the original unit. The presence of any antiseptic substances kills the cells and render them incapable of multiplication. As Clostridium difficile resists paracresol and phenol, I used two methods in order to find out the effect of these antiseptics on the growth of the organisms.

1. Viable count

This was carried out by growing 24-hour-old culture in the various concentrations of paracresol and phenol in RCM and transferring volume of 0.1 ml after every 4 hours to 10 ml of RCA and plating them and counting the colonies on the plates.

2. Bacterial density

The increase in bacterial density in RCM was estimated with a EEL nephelometer against an arbitrary standard of opacity tube.

1. Viable Count

The strain N4 was used for this purpose; the organisms were subcultured in RCM (Reinforced clostridial medium), and incubated at 37°C for 24 hours. 0.5 ml of the 24-hour-old culture was inoculated into each dilution of paracresol and phenol in RCM. The bottles contained 20 ml of the medium, also a control without added antiseptic was inoculated; the

bottles were incubated at 37°C, mixed on the mixer, and 0.05 ml was taken from each bottle immediately, and mixed in 4.95 ml of sterilized saline. This gave a dilution of 1 in 100, further dilutions were made, 0.1 ml of the final dilution was mixed with 10 ml of RCA (Reinforced clostridial agar) which had been sterilized and kept at 45°C. For each subsequent dilution three plates were poured, allowed to solidify and incubated at 37°C in an anaerobic jar under anaerobic condition for 72 hours. The colonies were then counted, and the mean number of colonies for the 3 plates was taken as the estimate of the number of viable organisms present. The procedure was repeated after every 4 hours of incubation up to 72 hours and the dilution of growth used was increased to 1 in 10,000. The number of colonies recorded are given in log. form for convenience (Tables VII, VIII and graphs I, II, and III). From the graphs and the tables it can be seen that maximum viable count was obtained in the control after incubation for 20 hours, whereas in all the dilutions of paracresol and phenol the maximum viable count was delayed till 24 hours. As the concentration of paracresol and phenol was increased, the maximum viable count decreased, and at 0.5 per cent of paracresol and phenol, no viable organisms were detectable after 12 hours incubation; sporadic growth was obtained even after 15 days, indicating that higher concentrations of the antiseptics may be limiting the growth. It is also confirmed that when cresol and phenol are added to the medium the viable count diminishes as compared with that in controls, suggesting that the growth is retarded to some extent.

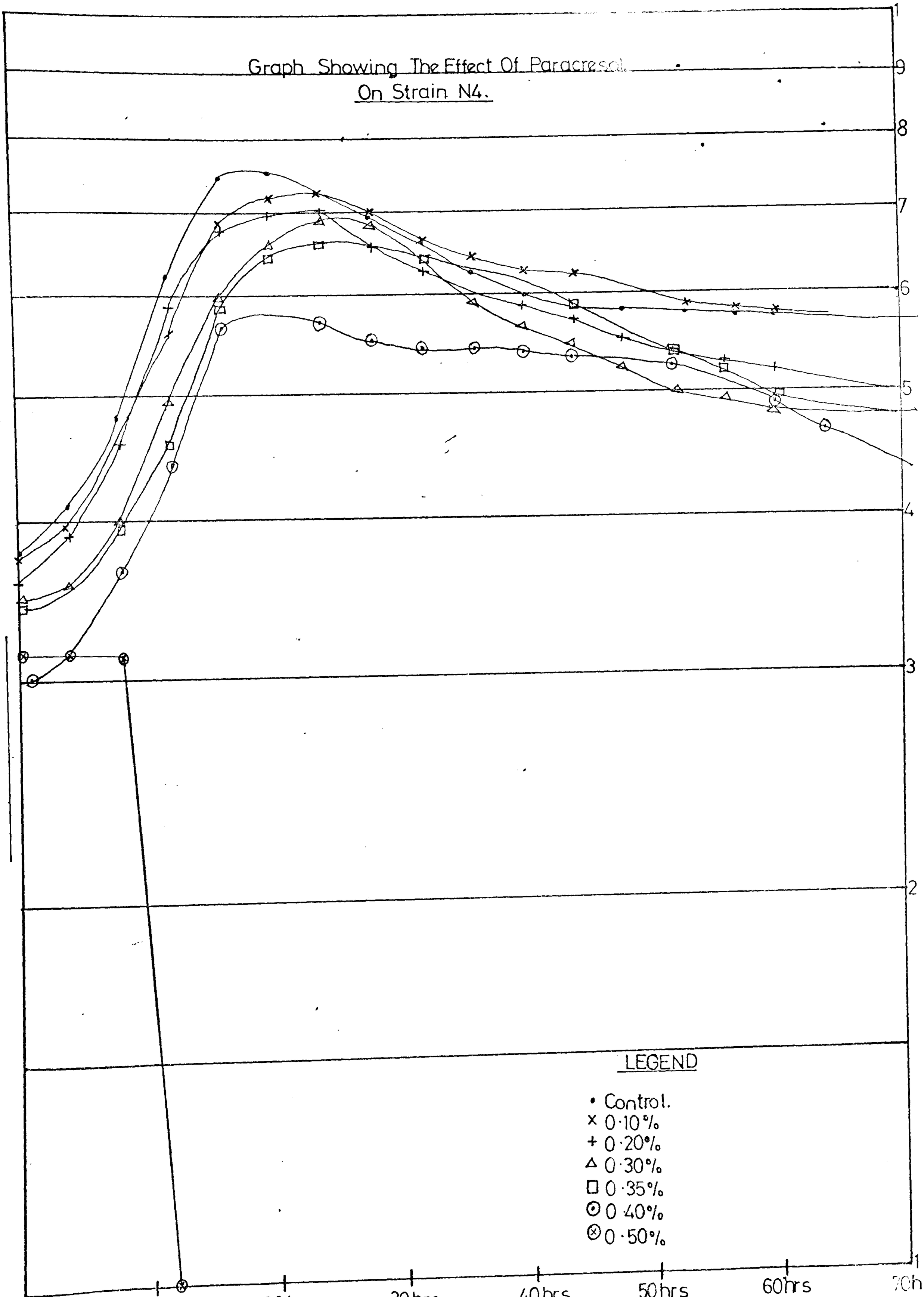
# TABLE VII

## SHOWING THE EFFECT OF PARACRESOL ON VIABLE COUNT OF CLOSTRIDIUM DIFFICILE (N4)

| Time of incubation (hr) | Log viable count of 0.1 ml of culture in medium containing paracresol (per cent) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0.0 | 0.1 | 0.2 | 0.3 | 0.35 | 0.4 | 0.5 |
| 0 | 3.7559 | 3.7160 | 3.5441 | 3.4914 | 3.4150 | 2.9031 | 3.1761 |
| 4 | 4.1271 | 3.9912 | 3.9294 | 3.5798 | 3.5441 | 3.1461 | 3.1761 |
| 8 | 4.8325 | 4.7293 | 4.6129 | 4.0000 | 3.9823 | 3.7076 | 3.1461 |
| 12 | 6.2405 | 5.6232 | 5.9956 | 4.9542 | 4.6021 | 5.6902 | 2.0 |
| 16 | 7.4800 | 6.8573 | 6.7466 | 5.9912 | 5.8976 | 5.9345 | " |
| 20 | 7.5500 | 7.1818 | 6.9956 | 6.5977 | 6.4829 | 5.7782 | " |
| 24 | 7.2634 | 7.2122 | 7.0017 | 6.9085 | 6.6272 | 5.6990 | " |
| 28 | 6.9542 | 7.0000 | 6.5999 | 6.8261 | 6.5832 | 5.5315 | " |
| 32 | 6.6117 | 6.7168 | 6.2989 | 6.4683 | 6.4314 | 5.4771 | " |
| 36 | 6.2788 | 6.4150 | 6.0414 | 5.9445 | 6.4082 | 5.4472 | " |
| 40 | 6.1903 | 6.2923 | 5.8976 | 5.6990 | 6.1875 | 5.4150 | " |
| 44 | 5.8865 | 6.2304 | 5.7160 | 5.4914 | 5.8062 | 5.3802 | " |
| 48 | 5.8573 | 6.1987 | 5.6232 | 5.2788 | 5.6232 | 5.3979 | " |
| 52 | 5.8325 | 5.8865 | 5.3979 | 5.0000 | 5.4150 | 5.2553 | " |
| 56 | 5.7993 | 5.8751 | 5.2788 | 4.9542 | 5.2788 | 4.9031 | " |
| 60 | 5.7853 | 5.7634 | 4.4771 | 4.8451 | 4.9031 | 4.6990 | " |
| 64 | ... | ... | ... | ... | ... | ... | ... |
| 68 | ... | ... | ... | ... | ... | ... | ... |
| 72 | 5.7782 | 5.7634 | 4.6021 | 4.7782 | 4.8451 | 4.6021 | ... |

GRAPH I

Graph Showing The Effect Of Paracresol On Strain N4.




TIME IN HOURS.

# TABLE VIII

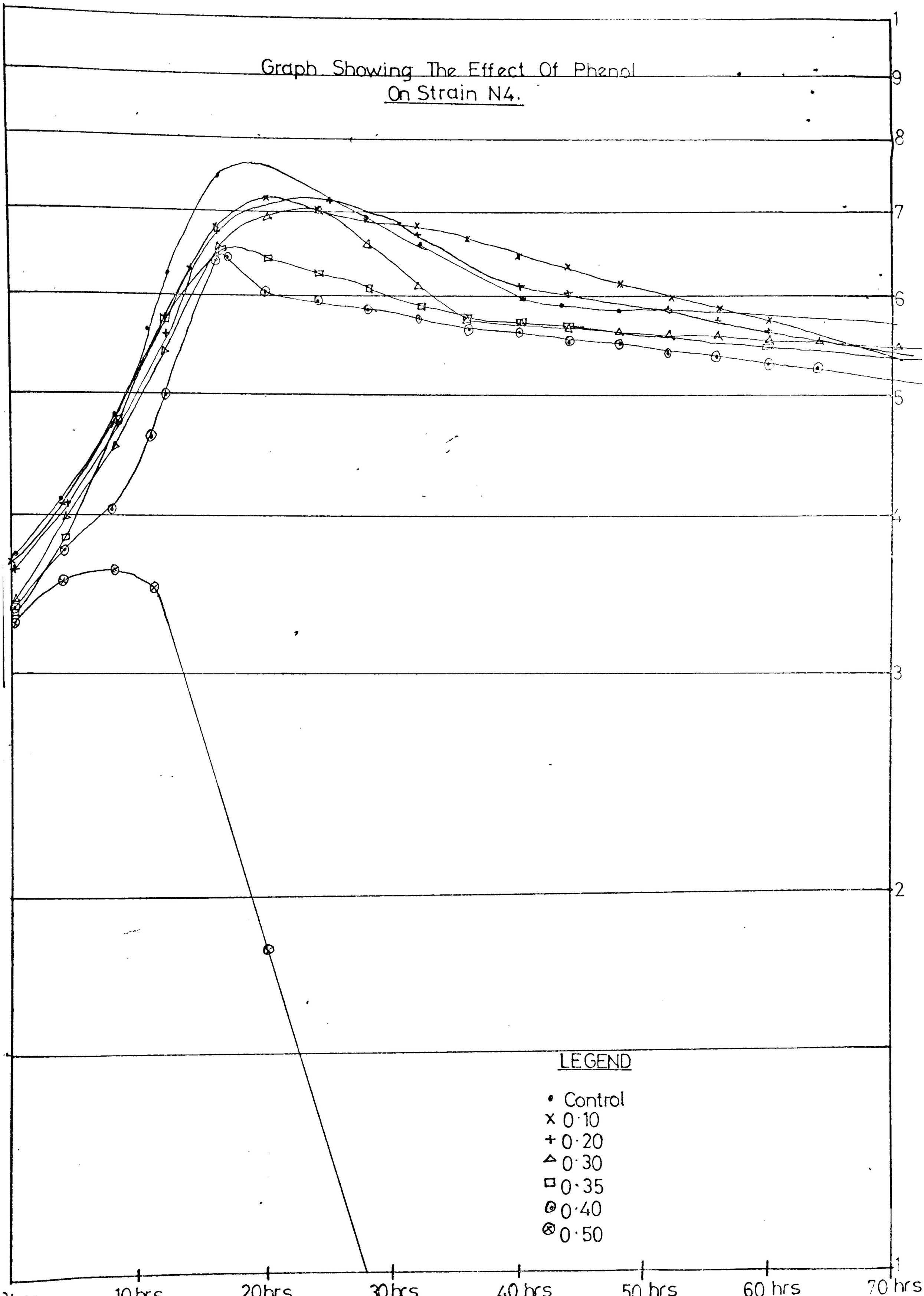
## SHOWING THE EFFECT OF PHENOL ON VIABLE COUNT OF CLOSTRIDIUM DIFFICILE (N4)

| Time of incubation (hr) | Log viable count of 0.1 ml of culture in medium containing phenol (per cent) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0.0 | 0.1 | 0.2 | 0.3 | 0.35 | 0.4 | 0.5 |
| 0 | 3.7559 | 3.7324 | 3.6721 | 3.3222 | 3.3979 | 3.3979 | 3.3010 |
| 4 | 4.1271 | 4.1139 | 4.1072 | 4.0719 | 3.8692 | 3.7782 | 3.5441 |
| 8 | 4.8325 | 4.8287 | 4.8325 | 4.5315 | 4.7226 | 4.0645 | 3.6128 |
| 12 | 6.2405 | 5.7709 | 5.6628 | 5.4914 | 5.7853 | 5.0792 | 3.5761 |
| 16 | 7.4800 | 6.8048 | 6.7709 | 6.5821 | 6.5024 | 6.4346 | - |
| 20 | 7.5500 | 7.1614 | 6.9101 | 6.9533 | 6.4116 | 5.8692 | - |
| 24 | 7.2634 | 7.0212 | 7.1428 | 7.0682 | 6.3502 | 5.9401 | - |
| 28 | 6.9542 | 6.9542 | 6.9538 | 6.6107 | 6.1239 | 5.8021 | - |
| 32 | 6.6117 | 6.8463 | 6.7168 | 6.1072 | 5.8062 | 5.7201 | - |
| 36 | 6.2788 | 6.7782 | 6.3560 | 5.7243 | 5.7782 | 5.6902 | - |
| 40 | 6.1903 | 6.4624 | 6.1106 | 5.6902 | 5.7243 | 5.6021 | - |
| 44 | 5.8865 | 6.3032 | 5.0000 | 5.6128 | 5.7160 | 5.5022 | - |
| 48 | 5.8573 | 6.1139 | 5.9395 | 5.5911 | 5.6021 | 5.4624 | - |
| 52 | 5.8325 | 5.9590 | 5.8921 | 5.5911 | 5.5911 | 5.4201 | - |
| 56 | 5.7993 | 5.8573 | 5.7076 | 5.5798 | 5.5441 | 5.2131 | - |
| 60 | 5.7853 | 5.7709 | 5.6212 | 5.5563 | 5.4914 | 5.1761 | - |
| 64 | ... | ... | ... | ... | ... | ... | ... |
| 68 | ... | ... | ... | ... | ... | ... | ... |
| 72 | 5.7782 | 5.3424 | 5.4914 | 5.5563 | 5.4914 | 5.1761 | - |

- = Less than 2

GRAPH II

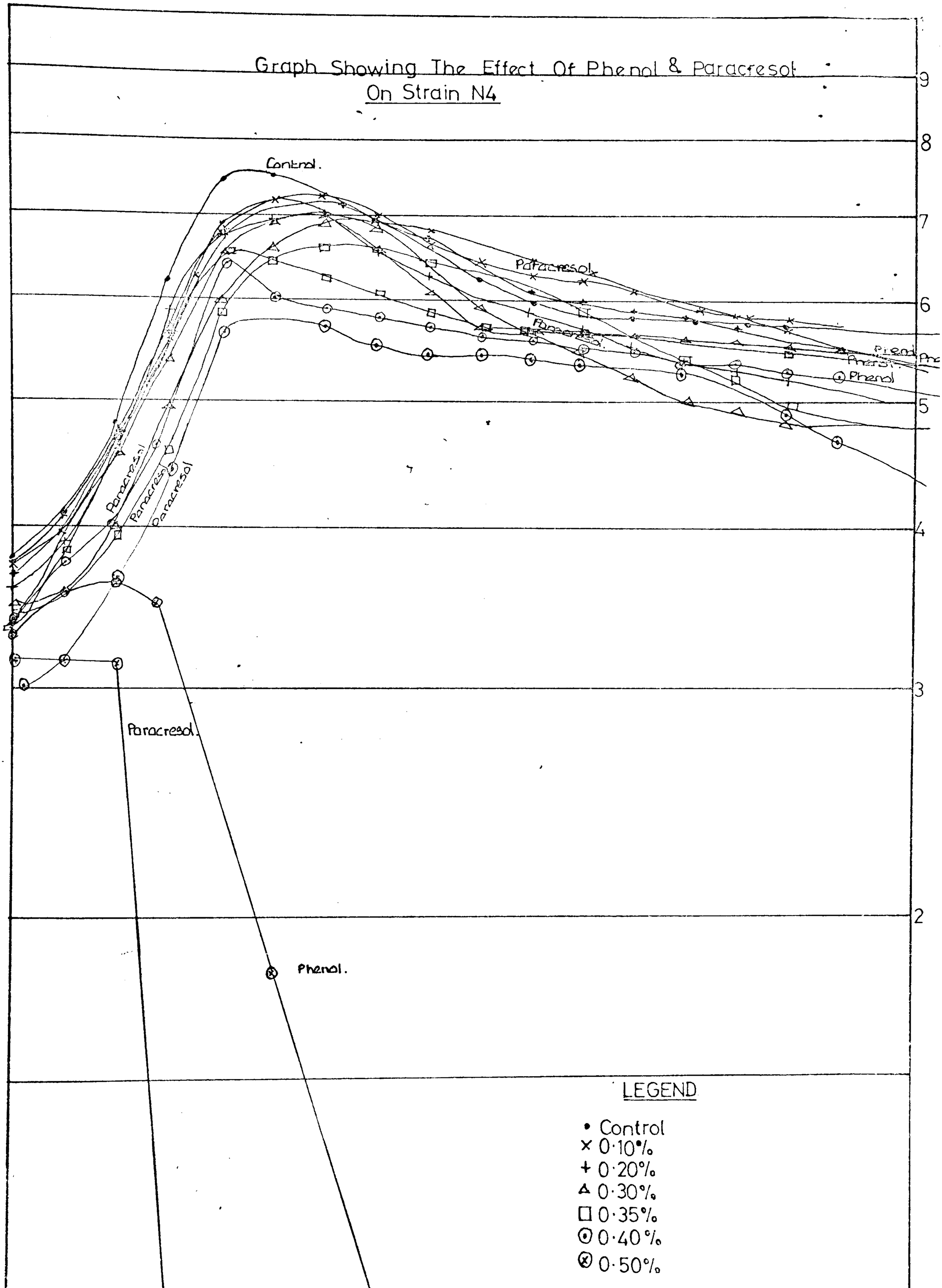
Graph Showing The Effect Of Phenol On Strain N4.




TIME IN HOURS

GRAPH III

## Graph Showing The Effect Of Phenol & Paracresol On Strain N4




TIME IN HOURS

Bacterial Density

Strain N4 was grown in Reinforced Clostridial Medium (RCM), and 0.05 ml of the culture was inoculated into thest tubes (150 x 15 mm) containing 10 ml of different percentages of paracresol or phenol in sterilized RCM. The tubes were sealed with rubber stoppers, and the readings of the bacterial density were taken at 0 hour with a nephelometer against an arbitrary standard opacity tube. Four sets of each dilution of antiseptic were inoculated from the culture and I made sure that the initial readings of all the tubes were almost identical ($\pm$ 0.5); measurements of opacity were made hourly, but for convenience they are shown in the Table IX and X and graph IV., IV and VI for the first five hours at hourly intervals and then after every subsequent five-hour interval.

From the table IX and X and graph VI it can be seen that the organisms grow much more freely in phenol as compared with paracresol as even in 0.3 per cent phenol the growth is more rapid, and at 70 hours almost the same density is attained in 0.2 and 0.3 per cent phenol and 0.1 per cent paracresol. (Almost the same pattern of readings was obtained with ortho- and meta-cresol as in case of paracresol). Growth was almost negligible in 0.4 per cent paracresol or phenol.

From the studies it can be concluded that the organisms thrive in paracresol and phenol, and the presence of these substances does not alter the units or deprive them of their characteristics; but it does retard the rate of growth of the organisms, and the higher concentrations of these substances makes the conditions unfavourable for growth.

## TABLE IX

## SHOWING THE INCREASE IN BACTERIAL DENSITY IN THE PRESENCE OF DIFFERENT CONCENTRATION OF PARACRESOL

| Time of incubation (hr) | Relative opacity of cultures of Cl. difficile in medium containing paracresol (per cent) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0.0 | 0.1 | 0.2 | 0.3 | 0.35 | 0.4 | 0.5 |
| 0 | 14.5 | 14.5 | 14.5 | 14.5 | 14.5 | 14.5 | 14.5 |
| 1 | 15.0 | 15.0 | 15.0 | 15.0 | 15.0 | 15.0 | 14.5 |
| 2 | 16.0 | 16.0 | 15.5 | 16.0 | 15.5 | 15.0 | 15.0 |
| 3 | 19.0 | 17.0 | 16.5 | 16.5 | 16.0 | 15.0 | 15.0 |
| 4 | 21.0 | 18.0 | 17.0 | 17.0 | 16.5 | 15.5 | 15.0 |
| 5 | 24.5 | 19.5 | 18.0 | 17.5 | 17.0 | 15.5 | 15.0 |
| 10 | 47.5 | 27.5 | 22.0 | 19.0 | 18.0 | 16.0 | 15.0 |
| 15 | 60.0 | 34.0 | 24.0 | 19.5 | 18.0 | 16.0 | 15.0 |
| 20 | 70.0 | 40.5 | 25.5 | 20.0 | 18.0 | 16.0 | 15.0 |
| 25 | 72.0 | 42.0 | 28.0 | 20.5 | 18.0 | 16.5 | 15.0 |
| 30 | 72.5 | 42.5 | 30.0 | 21.0 | 18.0 | 16.5 | 15.0 |
| 35 | 73.5 | 44.0 | 32.0 | 21.0 | 18.0 | 16.5 | 15.0 |
| 40 | 75.0 | 45.5 | 33.5 | 21.5 | 18.0 | 16.5 | 15.0 |
| 45 | 76.5 | 47.5 | 34.5 | 21.5 | 18.0 | 16.5 | 15.0 |
| 50 | 78.0 | 48.5 | 36.0 | 22.0 | 18.0 | 16.5 | 15.5 |
| 55 | 79.0 | 51.5 | 38.0 | 22.0 | 18.0 | 16.5 | 15.5 |
| 60 | 80.0 | 55.5 | 40.0 | 22.0 | 18.0 | 16.5 | 15.5 |
| 65 | 81.0 | 58.0 | 42.0 | 22.5 | 18.0 | 17.0 | 15.5 |
| 70 | 82.5 | 61.5 | 44.0 | 23.5 | 18.5 | 17.0 | 15.5 |
| 72 | 84.0 | 62.5 | 45.0 | 23.5 | 18.5 | 17.5 | 15.5 |

GRAPH IV

## GROWTH OF N4 (DENSITY) WITH PARACRESOL CONCENTRATION

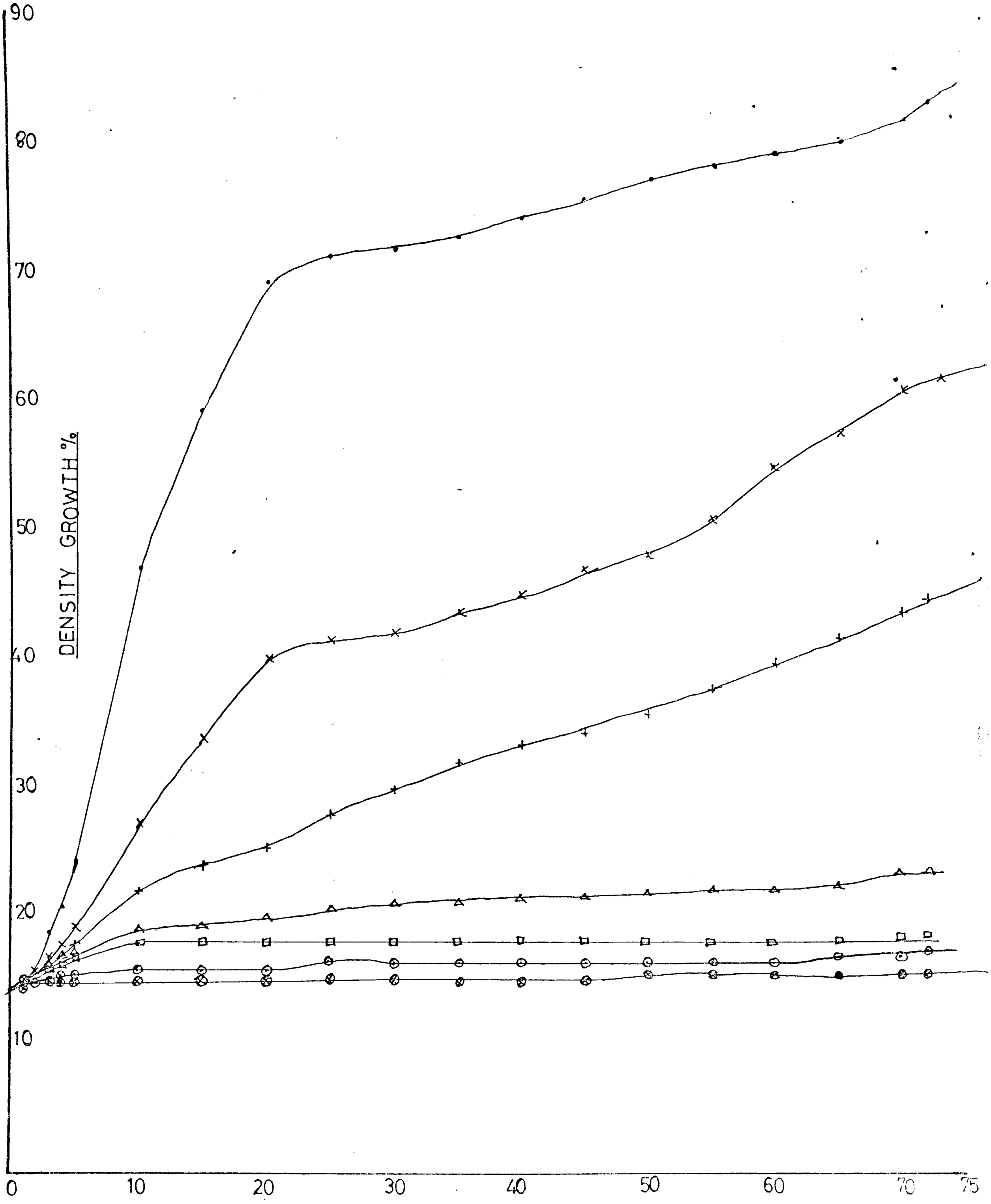
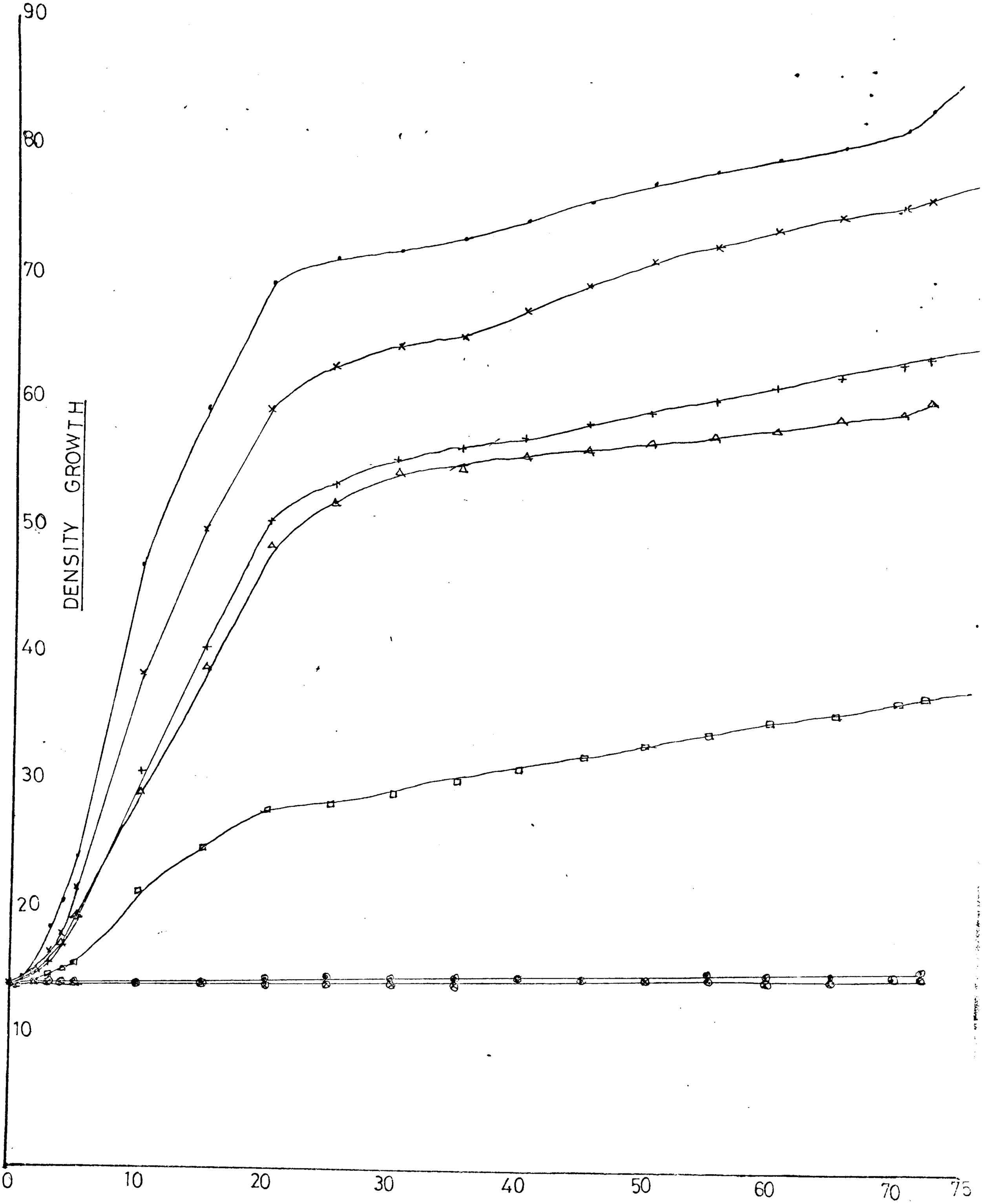


TABLE X

SHOWING THE INCREASE IN BACTERIAL DENSITY IN THE PRESENCE OF DIFFERENT CONCENTRATION OF PHENOL

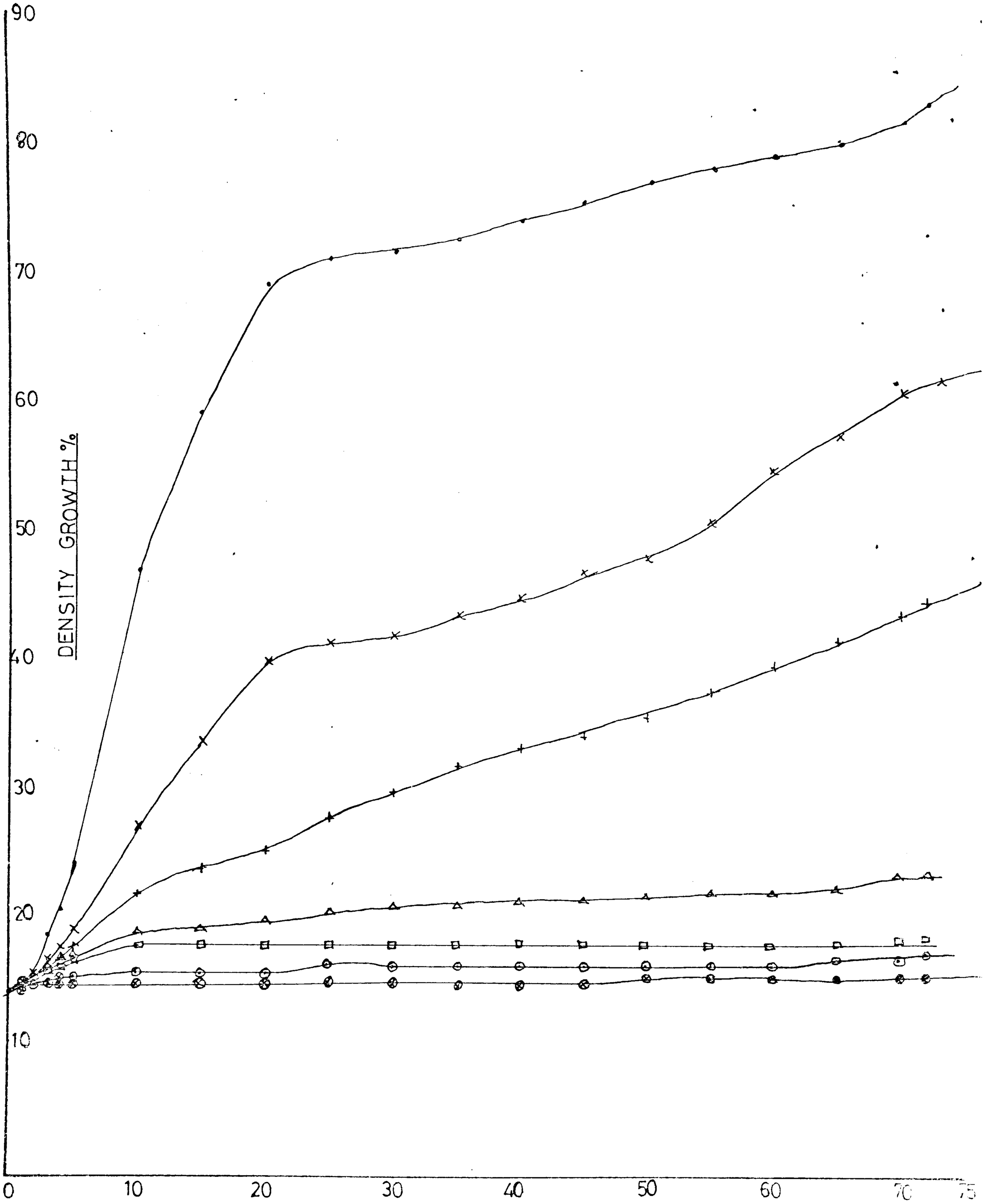
| Time of incubation (hr) | Relative opacity of cultures of *Cl. difficile* in medium containing phenol (per cent) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0.0 | 0.1 | 0.2 | 0.3 | 0.35 | 0.4 | 0.5 |
| 0 | 14.5 | 14.5 | 14.5 | 14.5 | 14.5 | 14.5 | 14.5 |
| 1 | 15.0 | 14.5 | 14.5 | 14.5 | 14.5 | 14.5 | 14.5 |
| 2 | 16.0 | 15.5 | 15.5 | 15.5 | 14.5 | 14.5 | 14.5 |
| 3 | 19.0 | 17.0 | 16.0 | 15.5 | 15.0 | 14.5 | 14.5 |
| 4 | 21.0 | 18.5 | 17.5 | 17.5 | 15.5 | 14.5 | 14.5 |
| 5 | 24.5 | 22.0 | 19.5 | 19.5 | 16.0 | 14.5 | 14.5 |
| 10 | 47.5 | 39.0 | 31.0 | 29.5 | 21.5 | 14.5 | 14.5 |
| 15 | 60.0 | 50.5 | 41.0 | 39.5 | 25.0 | 14.5 | 14.5 |
| 20 | 70.0 | 60.0 | 51.0 | 49.0 | 28.0 | 15.0 | 14.5 |
| 25 | 72.0 | 63.5 | 54.0 | 52.5 | 28.5 | 15.0 | 14.5 |
| 30 | 72.5 | 65.0 | 56.0 | 55.0 | 29.5 | 15.0 | 14.5 |
| 35 | 73.5 | 66.0 | 57.0 | 55.5 | 30.5 | 15.0 | 14.5 |
| 40 | 75.0 | 68.0 | 58.0 | 56.5 | 31.5 | 15.0 | 15.0 |
| 45 | 76.5 | 70.0 | 59.0 | 57.0 | 32.5 | 15.0 | 15.0 |
| 50 | 78.0 | 72.0 | 60.0 | 57.5 | 33.5 | 15.0 | 15.0 |
| 55 | 79.0 | 73.0 | 61.0 | 58.0 | 34.5 | 15.5 | 15.5 |
| 60 | 80.0 | 74.5 | 62.0 | 58.5 | 35.5 | 15.5 | 15.5 |
| 65 | 81.0 | 75.5 | 63.0 | 59.5 | 36.0 | 15.5 | 15.0 |
| 70 | 82.5 | 76.5 | 64.0 | 60.0 | 37.0 | 15.5 | 15.5 |
| 72 | 84.0 | 77.0 | 64.5 | 61.0 | 37.5 | 16.0 | 15.5 |

INCREASE IN N4 (DENSITY) WITH PHENOL CONCENTRATION



GRAPH VI

GROWTH OF N4 (DENSITY) WITH PARACRESOL CONCENTRATION



It is quite possible that this is due to the difference in the osmotic concentration of the cell contents and the surrounding medium. and the cells undergo a period of resting. This is confirmed by the sporadic growths obtained even after long exposures of the cells to these substances.

# CHAPTER IV

## PATHOGENICITY FOR LABORATORY ANIMALS

Mice and guinea pigs were used for experiments on the pathogenicity of Clostridium difficile. Both crude cultures and filtrates were used in order to determine and compare the rate of toxin production by Clostridium difficile in different fluid media. The organisms were grown in Cooked meat medium, Reinforced clostridial medium, and Proteose peptone medium; 3 - 5 day growths were used. Further observations were also made on mice by intraperitoneal inoculation of washed cell suspensions of Clostridium difficile. During immunization with washed cells of strain N4 injected intravenously, one rabbit died. In all cases dead animals were opened aseptically and internal organ changes were noted, and where there were obvious abnormalities the whole organ was cultured in Reinforced clostridial medium containing 0.2 per cent paracresol.

### Experiments on Mice

Strains N1, N4 and R1 were selected for experiments on mice.

I. Crude Culture

0.5 ml of crude culture was injected intraperitoneally.

a (i) Strain N1 (cultured in Cooked meat medium)

Death occurred after 30 hours; there was localised gelatinous oedema of the skin and the peritoneum at the site of injection, intestinal necrosis, congestion of the spleen and the kidneys.

a (ii) Strain N1 (cultured in Reinforced clostridial medium)

Death occurred after 24 hours; the findings were the same as in a (i).

a (iii) Strain N1 (cultured in Proteose peptone medium)

Death occurred in 24 hours; the findings were the same as in a (i)

b Strain N4

The animal that received this strain died sooner than any mice receiving other strains.

b (i) Strain N4 (cultured in Cooked meat medium)

Death occurred in 12 hours. There were lysis and necrosis of some parts of the intestine, and congestion of the lungs; fluid was present in the pleural cavity, and there was enlargement of the kidneys.

b (ii) Strain N4 (cultured in Reinforced clostridial medium)

Death occurred in 6 hours, post-mortem examination did not show any striking findings, with the exception of accumulation of some gas in the stomach and the upper part of the intestinal tract, and congestion and enlargement of the spleen.

b (iii) Strain N4 (cultured in Proteose peptone broth)

Death occurred in 4 hours; there were no striking findings with the exception of congestion of the lungs, and some accumulation of gas in the stomach.

c (i) Strain R1 (cultured in Cooked meat medium)

Death occurred in 18 hours. There were intestinal haemorrhage and necrosis, haemorrhagic spots on the lungs, and congestion of the spleen and kidneys.

c (ii) Strain R1 'cultured in Reinforced clostridial medium)

Death occurred in 8 hours, with lysis and necrosis of some parts of the intestine, congestion of the lungs and kidneys.

c (iii) Strain R1 (cultured in Proteose peptone medium)

Death occurred in 8 hours; there was lysis and necrosis of some parts of the intestine, congestion of the lungs and kidneys, and enlargement of the spleen.

The mice receiving strain N4 died in a shorter time than mice receiving other strains, and the crude culture from Cooked meat medium took slightly more time to kill mice than cultures in Reinforced clostridial medium and Proteose peptone medium. From this we can conclude that the strain N4 is more toxigenic than other strains, particularly when it is grown in RCM or Proteose peptone.

II. Washed Cell Suspension of Clostridium difficile

Strains N1, N4 and R1 were grown in Reinforced clostridial medium and 48-hours-old cultures of the strains were washed four times with sterilized normal saline; the turbidity was matched with the 5th tube of the standard turbidometric opacity tube series and 0.5 ml of the suspension in saline was injected intraperitoneally into mice. All three strains caused the death of the mice within 12 - 24 hours.

III. Inoculation of Mice with the filtrate and supernatant

Strain N1, N4 and R1 were grown in Reinforced clostridial medium and Proteose peptone medium; the culture were incubated for 24, 48, 72 and 96 hours, the growth was centrifuged at 3,500 r.p.m. for 30 minutes; the supernatant was tested for sterility, half of it was filtered through a Seitz filter, and 0.5 ml was injected intraperitoneally into the mice. Mice receiving filtrate and supernatant of 24-hour-old cultures did not die in 7 days,

whereas mice receiving filtrate and supernatant of 48-hour-old cultures of N4 and R1 died on the 4th day after the inoculation, whilst the onen receiving the filtrate and supernatant of N1 had not died as late as the 7th day, and those receiving the 72-hour-old culture supernatant and filtrates died in 8 - 24 hours. The same results were obtained with 96-hour-old cultures. This indicated that the peak of toxin production of Clostridium difficile occurs at 72-96 hours of incubation.

Loss of Toxicity by exposure of light and heat

The strains of N1, N4 and R1 were grown in Proteose peptone medium, incubated for 72 hours, centrifuged and heated in a waterbath at 60°C for 5 minutes and injected into mice; the same filtrate were exposed to daylight for 2 days and injected in the mice. In all cases the animals survived.

Intramuscular Injection of guinea-pigs

Two strains were used in this experiment, strains N1 and N4 were grown in Proteose peptone medium for 72 hours at 37°C, and the culture was centrifuged and the supernatant was injected intramuscularly. 0.5 ml of the supernatant was injected into guinea-pigs weighing about 400 g.

a) A guinea-pig into which N1 strain of Clostridium difficile had been injected showed signs of limping about 14 hours after inoculation, with its injected leg affected (probably a partial paralysis). During this time the animal was irritated by touch and noise; 48 hours after the time of inoculation the animal returned to its normal state, but it died on the 8th day after inoculation.

b) A guinea-pig given an injection of N4 supernatant intramuscularly, became agitated and restless after the injection; 10 hours after injection it became very sensitive to sound, light and contact, and 16 hours after the injection convulsions of the hind extremities were noticed. The animal died after 24 hours of inoculation; before death it showed generalised convulsions.

Intravenous injection of Clostridium difficile in rabbits

Strain N4 of Clostridium difficile was grown in RCM and, after incubation for 48 hours, the culture was centrifuged at 3500 r.p.m. for 30 minutes, the sediment was washed 4 times with sterilized saline and the turbidity was adjusted to that of the 5th of standard opacity tubes (1500 million organisms per ml). One ml of saline suspension was injected intravenously into a rabbit, and the injection was repeated after 7 days; the rabbit died 7 days after the 2nd injection.

On autopsy it was found that, the external appearance of the rabbit was normal, there was no discharge, and the animal's coat was in good condition.

| | | |
|---|---|---|
| Heart | - | normal; blood collected and inoculated in RCM and paracresol. |
| Liver | - | toxic effects visible, definite signs of toxaemia. |
| Lungs | - | congested |
| Kidney | - | slightly swollen |
| Small intestine | - | burst |
| Large intestine | - | intact with some gas. |

The autopsy therefore showed effects and general hyperaemia, the presence of an enormous amount of gas in the stomach, fatty degeneration of the liver, and excess of peritoneal fluid. Blood was collected from heart; fluid from the large intestine, peritoneal fluid, intestinal fluid and faeces samples were cultured in RCM, and RCM containing 0.2 per cent paracresol. A typical growth of Clostridium difficile was obtained and the organisms also grew in the medium containing paracresol from all the clinical material. The organism was therefore certainly pathogenic for the rabbit.

# TOXIN

In the early days of bacteriology the word 'toxin' meant an antigenic substance of high molecular weight produced by bacteria. Two groups were distinguished: exotoxins, secreted by the organisms into the medium in which they were growing, and endotoxins, which formed part of the body of the organisms, and were liberated only when destruction of the cell occurred. The exotoxins were heat-labile, highly antigenic, and easily converted to toxoid by treatment with formaldehyde.

More recently the distinction between the two classes of toxins has grown less sharp, since substances having the properties of exotoxins can be obtained in high concentration by allowing certain bacteria to autolyse or by extracting with certain solvents. It has also been found that the toxic activities of a bacterial filtrate are sometimes due to a number of different substances. Because of these increases in our knowledge, Oakley (1954) suggested that the word "toxin" should be replaced by 'soluble bacterial antigen', although he admitted that the word "toxin" is so throughly embedded in the literature and has so romantic a sound that is not likely to be given up.

The toxins that have been investigated, apart from the endotoxins of Gram negative organisms, all seem to be proteins, since they are:-

1. inactivated by treatments which denature proteins;
2. nondialysable;
3. precipitated by the usual protein precipitants;
4. insoluble in organic solvents;

5. destroyed by proteolytic enzymes, and

6. antigenic.

I would like to agree with Oakley in suggesting that "toxin" does not necessarily mean that the substance is toxic.

Toxins or should we call them "soluble bacterial antigen" being proteins, and antigenic, will produce antibodies if injected into an animal, unless the animal is too highly susceptible to the toxic action. There is no particular reason to think that the toxic group in a toxin are necessarily identical with those which act as determinants when it functions as an antigen. The antibodies to toxin will neutralize toxin, and hence are called antitoxins, but their action might be due to a blocking of the toxic group, i.e. the latter might simply be covered up by the antitoxin molecules, although these were attached to other groups.

In those toxins that have been studied, the toxicity seems to be an activity of an integral part of the molecule, not dependent on any prosthetic group. In several cases it has been found that the toxicity can be destroyed, without violent alteration of the molecule otherwise, by the action of formaldehyde and other agents.

"Toxins" have been classified by Van Heyningen (1954) as follows:-

1. Competitive inhibitors

2. Neutrotoxins

3. Dehydrogenase inhibitors

4. Blood coagulants

5. Anticoagulants

6. Shock-producing substances

7. Spreading factors, i.e. hyaluronidases

8. Polymolecular toxins (Phospholipide-polysaccharide protein complexes); the endotoxins of Gram negative bacteria are of this type.

9. Neurotoxins.

Some organisms produce more than one sort of toxin. It was observed that on storage toxins might lose part of their toxicity while retaining their power to neutralize antitoxin. Treatment with certain chemicals had a similar effect. Ramon (1923) probably preceded by Glenny) found it possible to destroy the toxic properties completely, without appreciable loss of antigenic qualities, by incubating toxin with formaldehyde. Ramon called this product "Anatoxine", but the name "Toxoid" is more common in the English language literature.

There is no appreciable change in the amount of nitrogen in one flocculating unit when toxin is converted to toxoid. There have been some experiments indicating that in-vivo combining power has been somewhat affected.

Formaldehyde apparently acts directly on the toxic group without affecting the other parts of the toxin molecule that are concerned with antigenicity and combining activity. It is natural to suppose that the effect is due to combination with the free amino groups of the toxin, but Hewitt (1930) pointed out that the reaction between toxin and formaldehyde to form toxoid is slow and irreversible, while that between formaldehyde and the free amino groups of protein, polypeptides, and amino acids is rapid and reversible. The amount of formaldehyde required

for toxoid formation is also less.

Toxoid is a more stable product than toxin. It does not appear to lose its antigenic value with ageing, and it is more resistant to heat than toxin. Eaton (1938) pointed out that the fact that toxin and toxoid have the same optical rotation suggests that the optically active atoms adjacent to the peptide linkages may not have been affected.

Clostridium difficile has been shown to produce exotoxin, by the following:

Hall and O'Toole (1935) first suggested the production of a soluble exotoxin, and added further that the toxicity of the filtrate lay between 100 and 1,000 minimum lethal doses per ml. Boiling for one minute completely destroyed the toxicity. Subcutaneous inoculation of either cultures or filtrates in 2 ml doses killed small rabbits in from 2 - 6 days. Intravenous injections also were lethal, with marked congestion and oedema of the lungs.

Snyder (1936 and 1939) confirmed the suggestions of Hall and O'Toole. The toxins were separated from the cultures by filtering through Berkefeld or Mandler candles; the sterile filtrates produced a gelatinous haemorrhagic oedema and convulsions in guinea-pigs. Further proof of its nature as a true exotoxin was obtained by testing against heat and for antigenicity. The toxin was found to be inactivated in five minutes at 60°C, and it was possible to immunize a dog and rabbit. Thus, the filterability, thermolability, pathogenicity, and antigenicity of the principle indicated that Bacillus difficilis produced a true

exotoxin. Hall and O'Toole (1935) described the toxin of Bacillus difficilis as lethal for the guinea-pig and rabbit. The list of susceptible animals was extended by Snyder to include cat, rat, dog and pigeon. There was no indication of species immunity to Bacillus difficilis. Injection of the organism ( 8 strains) into guinea-pigs was followed by slight to moderate swelling at the site of inoculation and the death of animals in 2 - 8 days. Little change from normal was noted at the post-mortem examination of the animals. There was no haemorrhage or other evidence of neurotic action, and Clostridium difficile could not be regularly isolated from the site of inoculation in animals that survived more than a few days. Apparently, the death of the guinea-pigs was brought about by the toxin introduced with the inoculum.

Wilson and Miles (1966) suggests that the bacteria are antigenically heterogeneous, the toxin apparently antigenically homogeneous. Some strains produce a filterable, thermolabile, antitoxinogenic toxin, which induces local oedema and convulsions in guinea-pigs. The toxin is lethal on injection into the cat, dog, rat, guinea-pigs, rabbit and pigeon, but has no effect by mouth in the rat, guinea-pig and dog.

A.T.Willis (1969) states that the organisms produce a lethal toxin that can be demonstrated in culture-filtrates. On subcutaneous injection into guinea-pigs, toxic filtrates cause local oedema, convulsions, respiratory arrest and death. The toxin is produced in largest amount in ordinary nutrient broth without added glucose, maximum yields being obtained 4 days after inoculation of the medium. Culture filtrates are comparatively

weakly toxic, however, with a minimum lethal dose (guinea-pigs) of the order 0.01 - 0.001 ml. It is a heat-labile substance, inactivated in a few minutes at 60°C. Strains of Clostridium difficile appear to be serologically homogenous.

In Bergey's manual it is suggested that glucose-broth culture-filtrates of Clostridium difficile kill guinea-pigs and rabbits in 24 - 36 hours. Subcutaneous inoculation induces marked oedema. Death may occur in from 1 - 9 days.

Oakley (1970) reported that filtrates of Clostridium difficile injected intradermally produced local swelling in guinea-pigs in 24 hours, with central pale-purplish necrosis after 48 hours. The guinea-pigs died after 72 hours, but the skin lesions did not seem sufficient cause for death.

As is clearly seen from the survey of literature, Clostridium difficile produces a soluble exotoxin, and the toxins of different strains appear to be indistinguishable.

Toxin Production

For detailed study of the toxin, the strains of Clostridium difficile used included strains N1, N2, N3, N4, N5 and N6; strain N4 was extensively used as it was found to be more toxic than the others.

The medium used for toxin production was mainly Proteose peptone broth, although Reinforced clostridial medium was also used; both the media gave almost identical results.

The stock culture was subcultred in RCM and incubated at 37°C for 72 hours, it was again subcultered in RCM and the 24-hour-growth was used as the inoculum for toxin production.

The toxin production medium i.e. Proteose peptone broth, was used in an anaerobic jar de-oxygenated in the usual way filled with hydrogen and incubated at 37°C for 72 hours. When RCM was used for toxin production screw-top bottles filled with the medium and incubated at 37°C for 72 hours. After 72 hours incubation, the culture was centrifuged at 3,500 r.p.m. for 30 minutes; the supernatant was collected and filtered through a Seitz filter. The filtrate was used for inoculation into mice and guinea-pigs.

Titration of Clostridium difficile Filtrates in Mice

In order to determine the lethality of the toxins, strains N1, N2, N3, N4, N5 and N6 were used. The filtrate was prepared as described earlier. The route of injection was intravenous. Mice were warmed before injection, and 0.5 ml of the filtrate was injected into the lateral tail vein of the mice; where the amount of filtrate was less than 0.5 ml the volume was adjusted to 0.5 by the addition of sterilized proteose peptone broth. The mice were kept under observations for 7 days, and the results recorded.

Table XI shows that mice receiving 0.5 ml of N1, N2, N3, N4 or N5 filtrate died within 24 hours, whilst the mice receiving N6 did not die even in 7 days, indicating that either the strain is non-toxic or that the amount of toxin produced was insufficient to cause the death of the animal in practical doses. Mice receiving 0.3 ml of N1, N2, N3, N4 or N5 filtrate died in 48 - 72 hours, whilst the mice receiving 0.2 ml of the toxin did not die even in seven days indicating that 0.2 ml of the filtrate was not sufficient to kill the mice.

TABLE XI

TITRATION OF CLOSTRIDIUM DIFFICILE

FILTRATES IN MICE

| Dose of material (ml) | Time of death (hr) of mice receiving filtrates from cultures of strain | | | | | | control material |
|---|---|---|---|---|---|---|---|
| | N1 | N2 | N3 | N4 | N5 | N6 | |
| 0.5 | 24 | 24 | 24 | 24 | 24 | -* | - |
| 0.4 | 24 | 48 | 48 | 24 | 48 | - | - |
| 0.3 | 48 | 72 | 72 | 48 | 72 | - | - |
| 0.2 | - | - | - | - | - | - | - |
| 0.1 | - | - | - | - | - | - | - |

* - indicates that the mice survived till the end of the experiment ( 7 days )

Determination of LD50

Filtrates from cultures of strain N4 were used for determination of the LD50 of Clostridium difficile filtrates. Toxin was prepared in Proteose peptone broth as described earlier.

The amount of filtrate for inoculation was prepared in the following way.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Amount of filtrate (ml) | 1.6 | 1.2 | 1.1 | 1.0 | 0.9 | 0.8 | 0.0 |
| Proteose peptone broth | 0.4 | 0.8 | 0.9 | 1.0 | 1.1 | 1.2 | 2.0 |
| Amount of filtrate in 0.5 ml inoculum | 0.4 | 0.3 | 0.275 | 0.250 | 0.225 | 0.20 | 0.0 |

Two mice were used for each dilution of the toxin, and were inoculated intravenously, by first warming the mice and then injecting the filtrate in the lateral tail vein; the volume of inoculum was constant. The inoculated mice were kept under observation for 7 days, after which the survivors were discarded. The dose of filtrate at which one mice died and the other survived was taken as the LD50.

Table XII gives the results obtained from inoculation of various dilutions of N4 toxin. Results obtained shows that 0.25 ml of the toxin kills one mice out of two; thus 0.25 ml is the LD50 for N4 strain of Clostridium difficile.

## TABLE XII

## TITRATION OF CLOSTRIDIUM DIFFICILE STRAIN N4 FILTRATES IN MICE

| Dose of filtrates(ml) | Time of death (hr) | Number of deaths | Number of survivals |
|---|---|---|---|
| 0.4 | 24 | 2 | - |
| 0.3 | 48 | 2 | - |
| 0.275 | 72 | 2 | - |
| 0.250 | 72 | 1 | 1 |
| 0.225 | -* | - | 2 |
| 0.20 | - | - | 2 |
| 0.00 (Control) | - | - | 2 |

*- indicates that the mice survived till the end of the experiment (7 days)

## PRECIPITATION OF CLOSTRIDIUM DIFFICILE TOXIN

Precipitation of toxins has been used for almost over a century for the purification and concentration of protein toxins. Ammonium sulphate was used as the precipitating material.

Strain N4 of Clostridium difficile was used for this purpose. 7 ml of a 24-hour culture of N4 was inoculated into 250 ml of Proteose peptone broth in the flask, incubated in an anaerobic jar for 72 hours. Also 14 - 15 ml of the culture was inoculated into Reinforced Clostridial medium in a 500-ml screw-capped bottle and incubated for 72 hours. After 72 hours of incubation the culture was centrifuged at 5,000 r.p.m. for 30 minutes, and the supernatant was filtered through a Seitz filter, the filtrate was collected and used for the precipitation of the "toxin", which was carried out in the following way:-

1. To 900 ml of filtrate of N4, 10 per cent peptone dissolved in 100 ml of distilled water and sterilized was added.
2. 700 g of ammonium sulphate was added gradually to the above mixture, with constant stirring.
3. The mixture was left in the cold room for 48 hours for complete precipitation.
4. After 48 hours, it was interesting to note that there was a layer of precipitate floating on the surface of the mixture and that a layer of precipitate has settled at the bottom. The floating precipitate and the precipitate that had settled were carefully collected separately in petri plates. The plates were kept slanting to allow "sweating" and removal of saturated ammonium sulphate solution, and part of the precipitated

material was freeze-dried in ampoules and the rest was dried in vacuum and stored in screw-cap bottles.

It was also seen that when both the precipitates were collected together in universal bottles with the liquid and centrifuged at 350 r.p.m. for 3 minutes, a certain amount of the precipitate settled at the bottom and some formed a layer at the surface of the bottle. The yield is 100 mg from 80 ml of filtrate.

5. For inoculation of the precipitated "toxin", 100 mg of the precipitated material was dissolved in 10 ml of sterile saline, and from this stock solution appropriate dilutions were made, and 0.5 ml of each dilution was injected intravenously in the lateral tail vein of pre-warmed mice.

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Amount of stock toxin (ml) | 2.0 | 1.6 | 1.2 | 0.8 | 0.4 | 0.3 | 0.2 | 0.0 |
| Amount of saline | 0.0 | 0.4 | 0.8 | 1.2 | 1.6 | 1.7 | 1.8 | 2.0 |
| Amount of toxin in 0.5 ml of the dilution in mg | 5.0 | 4.0 | 3.0 | 2.0 | 1.0 | 0.75 | 0.5 | 0.0 |

The results obtained are recorded in Table XIII.

## TABLE XIII

## TITRATION OF CLOSTRIDIUM DIFFICILE STRAIN (N4) PRECIPITATED FILTRATES

| Dose of precipitated Filtrate in mg. | Time of death (hr) of the mice given an dissolved | | | |
|---|---|---|---|---|
| | Floating pptd filtrate | Fresh pptd filtrate | Dried pptd filtrate stored for 3 months | Freeze dried pptd filtrate stored for 3 months |
| 5.0 | -* | 6 | - | 6 |
| 4.0 | - | 12 | - | 18 |
| 3.0 | - | 24 | - | 24 |
| 2.0 | - | 48 | - | 48 |
| 1.0 | - | 48 | - | 72 |
| 0.75 | - | - | - | - |
| 0.50 | - | - | - | - |
| 0.0 (Cpntrol) | - | - | - | - |

* - indicates that the mice survived till the end of the experiment ( 7 days )

From the results it can be concluded that 1.0 mg of the precipitated "toxin" that had settled is sufficient to kill the mice, whereas the floating precipitate was non-toxic.

The precipitated toxins were stored in screw-capped bottles, and in freeze-dried ampoules in the deep freeze ( - 5°C) and injected into mice after 3 months of storage; the results of the injection are given in the Table XIV. It can be concluded from the results that the vacuum-dried precipitate in the screw-capped bottles became non-toxic, whilst the freeze-dried precipitate in the ampoules remained toxic.

Lesions produced by intradermal injection of Clostridium difficile filtrates

The method for demonstrating bacterial toxin and generally recommended by workers,such as Glenny, Llewellyn-Jones and Mason (1931), and Oakley (1970) is the intradermal inoculation of the toxins. During the experiments I adopted the method and technique used by Oakley, and I am in full agreement with Oakley that phctographing the lesion to reproduce the exact colours is not satisfactory as the colour films are not sensitive enough to produce the exact coloration seen by the worker.

The method was used on guinea-pigs. The hairs of the guinea-pigs are clipped with fine barber's clippers and the final traces of hair were removed by applying a barium sulphide paste (composition: Barium sulphide 35 g, flour 35 g, talcum powder 35 g and powdered soap 5 g) for a timed period of three minutes; the paste was then washed off with warm water, and the guinea-pig was dried with a towel and rested for one hour before use.

Filtrates were prepared for strains N1, N2, N3, N4, N5, N6, N4I, R1, T1, T4, T5 and T18 as described earlier, and 0.2 ml of each filtrate injected into guinea-pigs intradermally by the method suggested by Oakley. In all the cases it was observed that the filtrates produced a local swelling and a small local nodule in the guinea-pig's skin in 24 hours, with a central purplish necrosis in the guinea-pigs receiving N1, N3, N4, N6, R1 and T1 filtrates, while astonishingly the guinea-pig receiving N4I filtrate showed spreading necrosis over large areas, while the guinea-pigs receiving N2, N5, T4, T5 or T18 filtrate showed excessive reddening more of lilac colour in 48 hours. 72 - 96 hours after inoculation the guinea-pigs receiving N1, N3, N4, N4I, R1 and T1 died. The other guinea-pigs recovered, and the control guinea-pig receiving 0.2 ml of proteose peptone broth did not show any visible reaction and survived.

Further studies were done by using varying amounts of N4 filtrates, and also by using the precipitated filtrates of N4. The reactions were almost the same in all guinea-pigs, with the only difference that the guinea-pigs receiving precipitated filtrates showed more swelling and slightly more necrosis than the ones receiving the crude filtrates; the time of death also varied slightly, as is quite clear from Table XIV. It can be concluded that the toxicity of the filtrate is retained in the precipitates and can be preserved by freeze drying them.

TABLE XIV

RESULTS OF INJECTING VARYING AMOUNTS OF CLOSTRIDIUM DIFFICILE (N4) FILTRATES INTRADERMALLY IN GUINEA PIGS

| Dose of filtrate (ml) | Time of death (hr) of the guinea pig given an intradermal injection of | | |
|---|---|---|---|
| | filtrate | precipitated filtrate | freeze-dried filtrate (stored for 3 months) |
| 0.3 | 60 | 48 | 48 |
| 0.25 | 72 | 48 | 60 |
| 0.2 | 72 | 60 | 60 |
| 0.15 | 96 | 72 | 72 |
| 0.1 | survived | 96 | 96 |
| 0.05 | survived | survived | 168 |
| 0.025 | survived | survived | survived |
| 0.0 (Control) | survived | survived | survived |

# HYALURONIDASE AND DEOXYRIBONUCLEASE

## ESTIMATION OF HYALURONIDASE

Hyaluronidases are enzymes, many of them produced by bacteria that cause a marked and immediate increase in the permeability of the tissue and increases the extent of infection produced by organisms, Duran - Reynals (1933) reported that filtrates from invasive strains of staphylococcus and streptococcus contain a soluble factor that causes a marked and immediate increase in the permeability of tissue and enhances the infection by these organisms. McClean (1936) found a factor that causes a merked and immediate increase in the permeability of the tissues in culture-filtrates of Clostridium welchii; this factor is active in high dilution. Robertson, Ropes and Bauer (1940) described an enzyme which they had derived from culture filtrates of Clostridium welchii and some other organisms which hydrolysed synovial fluid. In their studies on the diffusing factors McClean and Hall (1941) found that the hyaluronidase of bacterial culture filtrates increase tissue permeability and can be completely neutralised by appropriate antisera. In 1943 McClean developed a method for assaying hyaluronidase that depends upon the destruction by the enzyme of the power of the substrate protein complex to form a typical "mucin clot" on addtion of acetic acid.

Burnet (1948) devised the acid-congo red-alcohol (ACRA) test for the demonstration of hyaluronidase in culture filtrates. Oakley and Warrack (1951) modified Burnet's ACRA test to estimate the hyaluronidase and deoxyribonuclease activity of culture filtrates. Princewill and Oakley (1972) devised an agar plate

technique for testing for hyaluronidase and antihyaluronidase.

The method used in these tests was described by Oakley and Warrack (1951). The test depends on the fact that mixtures of human, horse, or bovine synovial fluid and aqueous congo red, will under suitable conditions, form a compact blue lenticular "blob" when a drop of the mixture is allowed to fall into acid alcohol (1 per cent concentrated HC1 in 70 per cent alcohol). Progressive reduction of the amount of hyaluronic acid in the mixture, either by dilution or by enzymatic digestion with hyaluronidase, leads to progressive changes in the blob formed in the test; it becomes less cohesive, with less distinct edges; then it becomes annular, and at sufficient dilution the drop of synovial fluid and congo red spreads freely into the acid alcohol.

Materials and Methods

Strains; the strains used for the determination of hyaluronidase were N1, N2, N3, N4, N5 and N6 and also the isolates N4I, T1, T2, T3, T4, T7, T8, T9, T10, T11, T12, T14, T15, T16, T17 and T18. Filtrates of the 72 hours growth was found to be most satisfactory and were used.

Substrate; bovine synovial fluid was used as the substrate. The substrate was tested every time before the test, and in all tests 8 indicating doses of the synovial fluid were used.

Diluent; Borate buffer saline was used throughout as the diluent. B.B.S. was prepared by first dissolving 3,0 g of NaC1 in 1.0 litre of distilled water, then dissolving in the

solution 3.657 g borax, 5.207 g boric acid and 6.136 g of NaC1; the final pH should be 8.04.

Concentration of congo red; the concentration of congo red used was 0.5 per cent.

The diameter and height of the capillary nozzle; throughout the experiment standard Pasteur pipette of 1 mm internal diameter were used, and the liquid was dropped from 1 cm above the acid alcohol.

The depth of acid alcohol layer; a petri plate was filled to a measured depth of 4.5 mm with acid alcohol.

Preliminary test for substrate; doubling dilution of bovine synovial fluid were prepared in 0.5 ml volumes in Lambeth tubes, and in each tube 1.5 ml B.B.S. was added and mixed by inversion of the tubes over non-absorbent paper and incubated in a water bath and immediately cooled them in chilled water, 0.4 ml of 0.5 per cent Congo red was added in each tube and mixed by inversion, and allowed to stand in the chilled water for five minutes and then tested as described below.

A petri plate was levelled and acid alcohol was added to a measured depth of 4.5 mm. A suitable quantity of the mixture was removed from each tube and added drop by drop from each dilution. The results were directly read from the appearance of the patterns formed. For the purpose the tube containing the smallest amount of substrate giving a good cohesive blob was taken as the end point, and was taken as 1 indicating dose. For the estimation of hyaluronidase 8 indicating doses were used.

Test for Clostridium difficile hyaluronidase

Doubling dilutions of the supernatant to be tested were prepared in 1.0 ml volumes, to it 0.5 ml of B.B.S. and 0.5 ml of bovine synovial fluid diluted in B.B.S. to contain 8 indicating doses were added and then proceeded in the same way as in case of substrate. Positive and negative controls using buffer and substrate only were always included in the tests. The whole scheme is quite clear from the flow sheet on the next page.

From the Table XV it can be concluded that all strains of Clostridium difficile produce hyaluronidase, and that the amount produced fairly consistent, i.e. in the range of 1 in 16 to 1 in 32, with the exception of N1 strain which showed hyaluronidase in the range of 1 in 4 to 1 in 8. The maximum amount of hyaluronidase was produced by strains N4, N4I, T3 and T7, i.e. in the range of 1 in 32 to 1 in 64.

The test has been done with supernatant from cultures of different age and it has been found that the maximum amount of hyaluronidase is produced between 48 and 72 hours.

In order to obtain more accurate results, dilution were prepared at 10 per cent differences from N1, N2, N3, N4, N5 and N6 filtrates. First a stock dilution of the filtrate was made (for example N1 supernatant was diluted to 1 in 4 in B.B.S., N2 to 1 in 16 and N4 to 1 in 32), then tubes of dilutions at 10 per cent differences were prepared according to the following scheme and the results obtained have been recorded in table XV.

## FLOW SHEET FOR HYALURONIDASE TEST

For Substrate

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Dilution of synovial fluid | 1:1 | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 | 1:128 | Cont. |
| Amount of diluted synovial fluid in ml | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.0 |
| B.B.S.in ml | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 2.0 |
| Congo red 0.5 per cent in ml | Incubate at 37°C for 1 hr, cool in chilled water and add 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 |
| Allow to stand in chilled water for 5 minutes and test for blob formation in acid alcohol and record the results | | | | | | | | | |

For Clostridium difficile supernatant

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Dilution of supernatant in ml. | 1:1 | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 | 1:128 | Control +ve -ve |
| Amount of diluted supernatant in ml. | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 0.0 0.0 |
| B.B.S. in ml. | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.0 0.0 |
| Synovial fluid containing 8 indicating doses in ml | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 2.0 0.0 |

Rest is the same as in the case of substrate,

TABLE XV

PRODUCTION OF HYALURONIDASE BY STRAINS OF CLOSTRIDIUM DIFFICILE

| Filtrate of strain | Results of test's on filtrate | | | | | | | | Results of control tests on material | |
|---|---|---|---|---|---|---|---|---|---|---|
| | un-diluted | diluted 1 in | | | | | | | | |
| | | 2 | 4 | 8 | 16 | 32 | 64 | 128 | +ve | -ve |
| N1 | + | + | + | - | - | - | - | - | + | - |
| N2 | + | + | + | + | + | - | - | - | + | - |
| N3 | + | + | + | + | - | - | - | - | + | - |
| N4 | + | + | + | + | + | + | - | - | + | - |
| N5 | + | + | + | + | + | - | - | - | + | - |
| N6 | + | + | + | + | + | - | - | - | + | - |
| N4I | + | + | + | + | + | + | - | - | + | - |
| R1 | + | + | + | + | + | - | - | - | + | - |
| T1 | + | + | + | + | + | - | - | - | + | - |
| T2 | + | + | + | + | + | - | - | - | + | - |
| T3 | + | + | + | + | + | + | - | - | + | - |
| T4 | + | + | + | + | - | - | - | - | + | - |
| T7 | + | + | + | + | + | + | - | - | + | - |
| T8 | + | + | + | + | - | - | - | - | + | - |
| T9 | + | + | + | + | + | - | - | - | + | - |
| T10 | + | + | + | + | + | - | - | - | + | - |
| T11 | + | + | + | + | + | - | - | - | + | - |
| T12 | + | + | + | + | + | - | - | - | + | - |
| T13 | + | + | + | + | + | - | - | - | + | - |
| T15 | + | + | + | + | - | - | - | - | + | - |
| T16 | + | + | + | + | - | - | - | - | + | - |
| T17 | + | + | + | - | - | - | - | - | + | - |
| T18 | + | + | + | + | - | - | - | - | + | - |

\+ indicates spreading

\- indicates blob formation

Scheme for 10 per cent dilutions

| Tube No | Stock solution | B.B.S. (ml) | Dilution produced by diluting mixture 1 in | | | |
|---|---|---|---|---|---|---|
| | | | 4 | 8 | 16 | 32 |
| 1 | 1.0 | 0.0 | 4 | 8 | 16 | 32 |
| 2 | 0.91 | 0.09 | 4.39 | 8.79 | 17.58 | 35.16 |
| 3 | 0.83 | 0.17 | 4.81 | 9.63 | 19.25 | 39.75 |
| 4 | 0.75 | 0.25 | 5.33 | 10.66 | 21.33 | 42.66 |
| 5 | 0.68 | 0.32 | 5.85 | 11.76 | 23.52 | 48.52 |
| 6 | 0.62 | 0.38 | 6.45 | 12.90 | 25.80 | 51.61 |
| 7 | 0.56 | 0.44 | 7.14 | 14.28 | 28.57 | 57.14 |
| 8 | 0.51 | 0.49 | 7.84 | 15.68 | 31.37 | 62.74 |
| 9 | 0.46 | 0.54 | 8.69 | 17.39 | 34.78 | 69.56 |

The diluted filtrate was used for the hyaluronidase test.

From the Table XVI it can be concluded that the titre of hyaluronidase (i.e. the number of indicating doses of hyluronidase per ml filtrate) produced by the strains is as follows:-

N1 5.85; N2 25.80; N3 10.66; N4 35.16; N5 21.33 and N6 17.58.

## TABLE XVI

## PRODUCTION OF HYALURONIDASE BY STRAINS OF CL. DIFFICILE WITH A DIFFERENCE OF 10 PER CENT

| Filtrate of strain | Stock dilution 1 in | Dilution tubes with a difference of 10 per cent | | | | | | | | | Control material | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1* | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | +ve | -ve |
| N1 | 4 | + | + | + | + | ± | ± | ± | - | - | + | - |
| N2 | 16 | + | + | + | + | + | + | ± | ± | - | + | - |
| N3 | 8 | + | + | + | + | ± | ± | - | - | - | + | - |
| N4 | 32 | + | + | ± | ± | ± | - | - | - | - | + | - |
| N5 | 16 | + | + | + | + | ± | ± | - | - | - | + | - |
| N6 | 16 | + | + | ± | ± | ± | - | - | - | - | + | - |

* See scheme for dilution

\+ = complete spreading (test positive)

± = partial spreading (test negative)

\- = blob formation

## ESTIMATION OF DEOXYRIBONUCLEASE

Deoxyribonuclease are enzymes produced by some organisms that have the ability to damage the nuclei fixed or otherwise damaged of leukocytes and other cells.

It has been known for a long time that some organisms produce enzymes that have the capacity to degrade highly polymerised deoxyribonucleic acid. Eisenberg (1907) demonstrated that cultures and filtrates of Cl. septicum and Cl. chauvoei contain a thermolabile substance that damaged washed leukocytes incubated with them. Butler (1942, 1943) reported a case of Cl. welchii infection following an abortion; during the examination of a post-abortion cervical smear, she noticed that in severe infections heavily capsulated bacilli were present and that a large proportion of the leukocytes in the smears showed severe damage. Several other authors reported the production of deoxyribonuclease (DNase). I decided to see whether Clostridium difficile produce deoxyribonulease, and employed the method of Oakley and Warrack (1951), i.e. the ACRA test.

Strains; N1, N2, N3, N4, N5 and N6 strains of Clostridium difficile were used.

Substrate; Sodium salt of deoxyribonucleic acid extracted from calf thymus, supplied by Koch Light Laboratories was made up to 0.15 per cent in M/40 veronal buffer pH 7.5, containing 0.003M magnesium (McCarty, 1945 - 46) by gentle shaking in a water bath at 37°C.

Diluents; veronal buffer was used for diluting substrate, 1.5 per cent neopeptone in 1 per cent saline for diluting filtrates.

Test for substrate

Two-fold dilutions of the substrate were prepared in 0.5 ml volumes of veronal buffer; 1.5 ml of 1.5 per cent neopeptone was added to each tube, and mixed in by inversion; the mixture was incubated in a water bath at 37°C for one hour. The tubes were removed from the water bath and placed in chilled water, then 0.4 ml of 0.5 per cent congo red was added in each tube and mixed in. The tubes were placed in chilled water for 5 minutes and the rest of the procedure was the same as in case of test for synovial fluid. The blob formed in this case was smaller than the blob formed by the synovial fluid. The sodium deoxyribonuclease used gave a very good viscous blob at 0.0625 per cent, and spread at 0.312 per cent. A dilution of 0.15 per cent was made and gave a good viscous blob and so this concentration was used in the tests.

Test for Clostridium difficile deoxyribonuclease

The organisms were grown in Proteose peptone medium and RCM. and after 72 hours of incubation, the growth was centrifuged at 3000 r.p.m. for 30 minutes. The supernatant and filtrate were diluted in 1.5 per cent neopeptone in 1 per cent saline and tested for spreading of the blob. None of the supernatant or filtrates produce any spread even in undiluted form, thus it was concluded that the test was negative; even supernatants from the fresh isolates failed to produce any spread.

## HAEMAGGLUTINATION

Haemagglutination may be defined as 'The aggregation of erythrocytes by specific antibody (through antigens on the erythrocyte surface) or by viruses or bacteria or plant proteins (through receptors on the erythrocyte surface)'. This aggregation may be prevented (haemagglutination inhibition) by free antigen or by antiviral antibody respectively. In direct haemagglutination, antigens naturally present on the erythrocytes are involved, in indirect, conditioned or passive haemagglutination, soluble antigens are attached to the erythrocytes artificially.

Erythrocytes are provided with certain receptors on the surface, and if these receptors are exhausted the erythrocytes lose their ability to settle down in definite and regular way known as button formation. Some bacteria possess enzymes which have the ability to destroy the receptor sites of the erythrocytes, and these enzymes are termed as "Receptor destroying enzyme" (RDE).

Hirst (1942 and McClelland and Hare (1941) were the first to clearly demonstrate that the agglutination of chicken erythrocytes in the presence of influenza-virus constitutes a valuable basis for the quantitative estimation of influenza virus haemagglutinin and its corresponding antibody. Salk (1944) suggested a simple method for titrating influenza haemagglutinin, and its antibody. For experimentation purposes Salk's method was used.

### Materials and Methods

Strains; the strains used for haemagglutination wer N1, N2, N3, N4, N5 and N6 strains of *Clostridium difficile*.

Media; the medium used for the cultivation of the organisms was RCM. The organisms were grown for 24-48 hours in

the medium, the growth was centrifuged at 4000 r.p.m. for 30 minutes, and the supernatant was collected and tested for sterility and used immediately.

Erythrocytes; for experimental purposes, human, rabbit, horse and sheep erythrocytes were used.

a) Human blood, fresh human blood was obtained from the vein. 10 ml of the blood was mixed with 1 - 2 ml of sodium citrate solution.

b) Rabbit blood, rabbits were bled from the ear vein and approx. 10 ml of the blood was mixed with 1 - 2 ml of 5 per cent sodium citrate solution.

c) Horse blood, oxalated blood supplied by Burroughs Wellcome Laboratories was used.

d) Sheep blood, oxalated blood supplied by Burroughs Wellcome Laboratories was used.

R.B.C. Suspension

The cells were separated from the blood by centrifuging for 10 - 15 minutes at 1500 rpm, and washed three times with quantities of physiological saline equal to 5 - 10 times the volume of packed cells. The washed cells were then resuspended in fresh 0.85 per cent NaCl, transferred to a 15-ml graduated tube and centrifuged for exactly 10 minutes at 1500 rpm. The volume of packed cells could be readily estimated and a 10 per cent stock suspension was made by adding sufficient 0.85 per cent NaCl solution to reach a final volume of 10 times that of packed cells. 0.25, 0.5, 1.0 and 2.0 per cent suspension was made from the stock solution and tested for haemagglutination. 1.0 per cent proved to be best and so 1.0 per cent R.B.C. suspensions were used throughout the experiment.

Method

Small round-bottomed tubes with an internal diameter of 10 mm and 75 mm long were used; the experiment can also be performed on an agglutination slab, Serial two-fold dilutions ranging from 1 in 10 to 1 in 2560 of the supernatant were made in 0.85 per cent NaCl. To 0.5 ml of each dilution, 0.5 ml of 1.0 per cent suspension of R.B.C. was added. The mixture was mixed by shaking and kept at room temperature until settling was complete usually in 1 - 2 hours. Distinct patterns were formed by the sedimented cells, depending upon the amount of haemagglutinin present in the mixture. In the control tube containing 0.5 ml of saline solution and 0.5 ml of 1 per cent R.B.C. the cells roll to the bottom and settle as a central, sharply demarcated red disc, and where agglutination is maximal, a uniform reddish film covered the entire hemispherical bottom of the tube, giving the impression that the clumps of the cells have been dispersed rather than aggregated, intermediate reactions were associated with a halo of finely aggregated or unagglutinated cells. The results were read by viewing the tubes from the bottom. Results were recorded as plus (+) meaning complete agglutination, plus-minus (±) for partial or slight agglutination and minus indicating no agglutination.

It was found that in the tube which contained undiluted supernatant, some haemolysis occurred, but when the supernatant was dialysed against saline for 24 hours, haemolysis did not occur, and it did not alter the titre of haemagglutination.

The results obtained for haemagglutination are summarized in the Table XVII.

The end point was taken at the highest dilution of the supernatant producing maximal agglutination.

From the table it can be seen that the titre of haemagglutination varied markedly among the strains, strains N1, N5 and N6 showed poorest haemagglutinating capacity, and agglutinated only Rabbit erythrocytes; N2 also agglutinated poorly, but it agglutinated Rabbit and Horse RBC's; N3 agglutinated RBC's of all four species, agglutinating the Rabbit RBC's to the highest dilution (1 in 320); N4 failed to agglutinate Horse RBC's, but it agglutinated Rabbit RBC's to a titre of 640.

# TABLE XVII

## SHOWING THE ABILITY OF THE SUPERNATANT OF CLOSTRIDIUM DIFFICILE TO HAEMAGGLUTINATE

| Supernatant of strain | Limiting dilution of supernatant that agglutinate erythrocytes of | | | |
|---|---|---|---|---|
| | Man | Rabbit | Horse | Sheep |
| N1 | - | 1 in 20 | - | - |
| N2 | - | 1 in 40 | 1 in 20 | - |
| N3 | 1 in 20 | 1 in 320 | 1 in 20 | 1 in 80 |
| N4 | 1 in 10 | 1 in 640 | - | 1 in 20 |
| N5 | - | 1 in 80 | - | - |
| N6 | - | 1 in 40 | - | - |

- indicates no effect even by undiluted supernatant

## HAEMOLYSIS

Haemolysis is defined as 'The lysing or laking of erythrocytes, the release of haemoglobin and clearing of the suspension' and the substance responsible for this action is termed as 'Haemolysin'. A variety of bacteria produce haemolysins, substance which bring about the dissolution of the red blood cells of higher animals. The bacterial haemolysins are of two types, the so-called filterable haemolysins, which are extracellular and may be separated from the bacterial cells by filtration, and the haemolysins, which are demonstrated by cultivation of bacteria on solid media containing whole blood.

In case of filterable haemolysins, the haemolytic activity is demonstrated by the addition of filtrate or whole culture to a suspension of washed erythrocytes in 0.85 per cent NaC1 solution; and after a period of incubation the red cells are laked, and haemoglobin appears free in solution.

Clostridium difficile failed to produce any haemolytic activity on the solid meda, so the liquid haemolysis method was tried.

The method for liquid haemolysis has been tried in case of the filtrates of bacteria by Bamforth and Dudgeon (1952), Smith (1963), Snyder and Koch (1966) quite successfully, so a method based on similar principle was tried for filtrates of Clostridium difficile and quite interesting results were obtained.

### Materials and Methods

Strains; Six strain of Clostridium difficile, i.e. N1, N2, N3, N4, N5 and N6 were used for this purpose.

Media; Two media were used, they were Proteose peptone medium, and RCM; Proteose peptone medium was found to be more satisfactory so it was used for haemolysin production. The medium was tried with and without M/20 cysteine hydrochloride, and it was found that the addition of cysteine hydrochloride reduced the haemolytic titre to about 50 per cent.

Erythrocytes; Horse erythrocytes were used, oxalated blood was centrifuged and washed in 0.85 per cent NaCl, and 2 per cent, solution of the packed RBC was made from the 10 per cent, stock solution (details on page 86).

Preparation of haemolysin; The haemolysin was prepared by inoculating 1.0 ml of a 24-hour growth of the strain of *Clostridium difficile* in RCM into large tubes containing 25 ml of Proteose peptone medium. The tubes were incubated at $37^{o}C$ in the anaerobic jar filled in the usual way with hydrogen for 24 - 30 hours. The growth was centrifuged at 3500 rpm for 30 minutes, and filtered through a membrane filter; the filtrate was collected and tested for haemolytic activity.

Assay of haemolysin

Two-fold dilutions of the filtrate were made in 0.85 per cent, NaCl. One ml each dilution of the filtrate was taken in small test tubes of internal diameter 8mm and 1.0 ml of a 2 per cent, suspension of horse RBC was added to each tube; the mixture was mixed by shaking and incubated in water bath at $37^{o}C$ for two hours. If the limiting dilution of filtrate that produced 50 per cent haemolysis was 1 in x, the haemolytic titre of the filtrate was recorded as x. Positive and negative controls were

run, the positive control included 1 ml of RBC suspension plus 1 ml of 4 per cent ammoniated water (4 per cent ammonium hydroxide), this gave complete haemolysis, while the negative control tube included 1 ml of proteose peptone medium and 1 ml of 2 per cent, RBC solution. Table XVIII gives the results of the haemolytic activity of the filtrates of Clostridium difficile.

From the table it can be concluded that the filtrates of Clostridium difficile produces haemolysins and the titre of haemolysins can be taken as 1 in 16, in another experiment 1.1. fold dilutions were made from a stock dilution of 1 in 16 of each filtrate, and the table XIX gives the results obtained, and 50 per cent haemolysis was taken as the haemolytic dose, the control included 1 in 16 dilutions of the filtrate and taken as positive control, while the negative control included saline and RBC suspension.

From the table it can be observed that the haemolytic titre of the filtrates lies in the region of 16 to 25.80, most haemolytic filtrates being that of N4 and N6.

Experiments were repeated to see the effect of Clostridium difficile strain N4 filtrate on human, rabbit and sheep erythrocytes. Erythrocytes of all species were haemolysed, almost equally, the only difference which was that human RBC were haemolysed to a titre of 19.4.

TABLE XVIII

HAEMOLYSIS OF 2 PER CENT HORSE RBC BY FILTRATES OF CULTURES OF CLOSTRIDIUM DIFFICILE

| Filtrate of strain | Degree of haemolysis produced by Clostridium difficile filtrate | | | | | | | Control | |
|---|---|---|---|---|---|---|---|---|---|
| | un-diluted | diluted 1 in | | | | | | +ve | -ve |
| | | 2 | 4 | 8 | 16 | 32 | 64 | | |
| N1 | + | + | + | + | + | - | - | + | - |
| N2 | + | + | + | + | + | - | - | + | - |
| N3 | + | + | + | + | ± | - | - | + | - |
| N4 | + | + | + | + | + | - | - | + | - |
| N5 | + | + | + | + | + | - | - | + | - |
| N6 | + | + | + | + | + | - | - | + | - |

\+ indicates complete haemolysis

± indicates 50 per cent haemolysis or more

\- indicates no haemolysis or less than 50 per cent

TABLE XIX

HAEMOLYSIS OF 2 PER CENT HORSE RBC BY 1.1-FOLD DILUTIONS OF FILTRATES OF CULTURES OF CLOSTRIDIUM DIFFICILE

| Filtrate of strain | Degree of haemolysis produced by filtrates diluted 1 in | | | | | | | | Control | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 16.0 | 17.6 | 19.4 | 21.3 | 23.4 | 25.8 | 28.3 | 31.2 | +ve | -ve |
| N1 | + | + | ± | - | - | - | - | - | + | - |
| N2 | + | + | + | ± | - | - | - | - | + | - |
| N3 | ± | - | - | - | - | - | - | - | + | - |
| N4 | + | + | + | + | ± | ± | - | - | + | - |
| N5 | + | ± | - | - | - | - | - | - | + | - |
| N6 | + | + | + | + | + | ± | - | - | + | - |

\+ indicates complete haemolysis

± indicates 50 per cent haemolysis or more

\- indicates no haemolysis or less than 50 pet cent

# CHAPTER V

## IMMUNIZATION OF RABBITS

Immunization has been widely used for many purposes, and one of them being to find the response of an animal to a particular substance, and to test a variety of strains of the bacteria.

In the course of study a number of antigens were used to immunize the rabbits. It is well known that the route of injection has a marked affect on the production of antibodies. But in the course of our studies for immunization purposes we adhered to one route of injection as much as possible, and mainly the animals were immunized by the intravenous route, with the only exception that when toxins (toxoid) were injected the first injection given was intramuscular, but all the subsequent injections were given intravenously.

### Materials and Methods

Strains; N1, N2,N3, N4, N5 and N6 strains of Clostridium difficile were used for the immunization of rabbits, besides this the toxin of N4 strain was precipitated and rabbits were immunized both by the floating precipitate, and the settled precipitate. Also the N4 strain of Clostridium difficile was disintegrated, and rabbits were immunized with the stroma and the extracts of the cells. In one case washed growth was used for immunization; in the first instance the rabbit died so the immunization was repeated and it was successful in the 2nd case.

Preparation of Material for Immunization: Material for immunization was prepared in different ways.

a. Whole Cells; Strains N1,N2,N3,N4, N5, and N6 were grown in RCM and a 24-hour growth was used as inoculum for the preparation of the material. 2 ml. of the 24-hour growth of the strains was inoculated into screw-cap bottles containing 80 ml of RCM and incubated at $37^{o}$C for 48 hours. The growth was centrifuged at 3,000 rpm for 30 minutes, the organisms were washed four times with 0.85 per cent NaCl solution, and then they were resuspended in 0.85 per cent NaCl. The concentration of the organisms was adjusted to about $3 \times 10^{8}$ per ml, and the suspension was heated in a water bath at $100^{o}$C for 1 hour,and this was used for intravenous injections of the rabbits.

b. Living Cells; Strains N4 of Clostridium difficile was grown in the same way as described earlier, the organisms were washed four times with 0.85 per cent. NaCl, and the growth was suspended in 20 ml of saline, and kept at room temperature for one week, and then the organisms were collected by centrifuging the saline suspension, and washing twice with saline. The organisms were resuspended in saline to a concentration of of $3 \times 10^{8}$ per ml, and used for intravenous injection in rabbits.

c. Precipitated Toxin; The toxin of strain N4 was used for this purpose. The toxin was precipitated by

Salination with Ammonium sulphate, and the floating and settled precipitates were collected separately. One g of each precipitate was dissolved in distilled water and the volume was made up to 10.0 ml. The dissolved toxin was kept at room temperature in a dark place for 1 week, and tested for toxicity by injecting into mice. It was found to be non toxic, and was used for injections into rabbits.

d. Disintegrated Cells; 48- hour growth in RCM was centrifuged, the cells were washed three times in saline, and a thick suspension was made by adding 10.0 ml of distilled water; the organisms were disintegrated with an ultrasonic disintegrator.

The suspension was taken into a wide-mouthed sterile universal container, placed in a beaker containing crushed ice, and the cells were disintegrated with a 'Soniprobe' sonic disintegrator, at a speed of 6 - 7 at 1.8 - 2.0 amp. for 20 minutes. The disintegrated cells were examined for viability by making a slide film for direct examination, and inoculating in RCM; and incubating for 72 hours; there was never any growth. The disintegrated cells were centrifuged at 5,000 rpm for 45 minutes, and the supernatant was filtered through a membrane filter and the filtrate collected, and used for the immunization of the rabbit. The sediment of the disintegrated cells were washed

3 times with saline, and a suspension was made in matching the turbidity of 3 x $10^8$ per ml and was used for the immunization of rabbits.

Process of Immunization

1. All the rabbits to be immunized were bled first, and the sera obtained was tested by agglutination and precipitation test for the presence of natural antibodies for each strain to be used for immunization. (All the results of these tests were negative, indicating that the rabbits did not possess any natural antibodies.)

2. The rabbits to be immunized were divided in two groups, one group receiving the intact organisms, which included N1,N2,N3 N4,N5, and N6, and the other group receiving the precipitated toxins, disintegrated cells and the supernatant of the disintegrated cells. Both the groups were immunized in the same way, i.e. by the intravenous route, with the only exception that the 2nd group received the first injection intramuscularly.

(a) The first group of rabbits received a first injection of 0.5 ml of the intact organisms intravenously, the 2nd injection of 1.0 ml intravenously on the 8th day,and subsequently 1.0 ml of the material on every 3rd day. After a months immunization the rabbit was rested for a week and bled and tested for the formation of antibodies by agglutination tests. It was found necessary to continue immunizing the rabbits for 3 months to get a high titre of antibody. After 3 months the animals were killed, and all the blood was collected and left at room temperature for two hours. Then the clots were broken down and the

blood was left in a cold room overnight. The next day all the sera was collected with the aid of the centrifuge and transferred into screw-capped bottles, tested for their antibodies by the agglutination test against the specific strains and stored in the deep freeze until needed. The schedule of immunization is given in the table XX.

(b) The second group of rabbits received a first injection of 0.5 ml of the appropriate filtrates mixed with 0.5 ml of Freund's adjuvant (incomplete)intramuscularly, and a second similar injection intravenously after one month. The animal was then rested for three months; after the rest it received a 3rd injection, and was then bled and tested for antibodies, it was found necessary to give two more injections to the animals to get a higher titre of antibodies. The animals were killed and blood was collected in the same way as described above and stored. The schedule of injections is given in the table XXI.

TABLE XX

SCHEDULE OF INJECTIONS OF INTACT CELLS OF CLOSTRIDIUM DIFFICILE IN RABBIT

| Injection | Day | Route | Amount | Bleeding No. |
|---|---|---|---|---|
| | | | | 1st |
| 1 | 1st | I/V | 0.5 ml | |
| 2 | 8th | " | 1.0 ml | |
| 3 | 11th | " | 1.0 ml | |
| 4 | 14th | " | 1.0 ml | |
| 5 | 17th | " | 1.0 ml | |
| 6 | 20th | " | 1.0 ml | |
| 7 | 23rd | " | 1.0 ml | |
| 8 | 26th | " | 1.0 ml | |
| 9 | 29th | " | 1.0 ml | |
| | 36th | - | - | 2nd |
| 10 | 37th | I/V | 1.0 ml | |
| 11 | 42nd | " | 1.0 ml | |
| 12 | 47th | " | 1.0 ml | |
| 13 | 52nd | " | 1.0 ml | |
| | 59th | - | - | 3rd |
| 14 | 60th | I/V | 1.0 ml | |
| 15 | 65th | " | 1.0 ml | |
| 16 | 70th | " | 1.0 ml | |
| 17 | 75th | " | 1.0 ml | |
| | 82nd | - | - | 4th |
| 18 | 83rd | I/V | 1.0 ml | |
| 19 | 88th | " | 1.0 ml | |
| 20 | 93rd | " | 1.0 ml | |
| | 100th | - | - | 5th<br>Animal killed and bled out |

## TABLE XXI

## SCHEDULE OF INJECTIONS OF PRECIPITATES, CELL EXTRACTS AND STROMA OF CLOSTRIDIUM DIFFICILE IN RABBITS

| Injection No | Day | Route | Amount of injected material + F.A. | | Bleeding No |
|---|---|---|---|---|---|
| | | | | | 1st |
| 1 | 1st | I/M | 1.0 | + 0.5 | |
| 2 | 30th | I/V | 1.0 | + 0.5 | |
| 3 | 120th | I/V | 1.0 | + 0.5 | |
| | 127th | - | - | - | 2nd |
| 4 | 150th | I/V | 1.0 | + 0.5 | |
| | 157 | - | - | - | 3rd |
| 5 | 180th | I/V | 1.0 | + 0.5 | |
| | 187th | - | - | - | 4th |
| | 190th | - | - | - | Animal killed and bled out |

## INJECTION OF STRAIN N4 INTACT CELLS AND PRECIPITATED TOXIN IN PREGNANT RABBITS

Three pregnant rabbits were taken and on the 25th day of pregnancy, all were given an intravenous injection, one of 1.0 ml of strain N4 intact cells, and the second 1.0 ml of the precipitated toxin, whilst the third (control) received 1.0 ml of saline. The rabbits receiving the intact cells and toxin precipitate aborted on the 28th day of pregnancy, whilst the control delivered itself normally.

The experiment was again repeated on two other female rabbits. They were mated and when the pregnancy was confirmed, one rabbit was given an intravenous injection of intact cells, and the other precipitated toxin, on the 24th day of the pregnancy; again the rabbits aborted on the 27th day.

These tests of the effect of the toxin or the strain of Clostridium difficile on pregnant rabbits is not sufficient to allow for any conclusions, but it is interesting to note that in all four cases the rabbits aborted on the 3rd day after the injections.

## ANTIGEN-ANTIBODY REACTIONS

By definition an antibody must react with its specific antigen in some measurable manner. The nature of the reaction between antigen and antibody has been of great interest since the phenomenon was originally described.

## BACTERIAL AGGLUTINATION

The reaction of agglutination is in principle like that of precipitation, both are aggregative, but in this case particulate rather than soluble, antigens are brought together by antibodies.

If the blood or serum of an animal previously immunized against a bacterium be mixed with a suspension of the micro-organisms, the latter become immobilized and in a short time aggregate to form large clumps of cells. In the test tube these clumps settle out, and the turbid bacterial suspension is cleared with the formation of a precipitate- like mass of clumped cells in the bottom of the tube. This phenomenon is termed agglutination, and the bacterial cells are said to be agglutinated. The bacteria are not killed by agglutination, and will, in fact, grow in immune serum although with altered morphology and the formation of long chains of bacillary forms. Living bacteria need not be used for the agglutination reaction, for dead bacteria are agglutinated readily as the viable forms.

In principle, the test consists of setting up a series of tubes containing progressively increasing dilutions of serum and adding a saline suspension of living or dead bacteria to each tube. After a suitable incubation period, the tubes are

examined for evidence of visible clumping, and if the limiting dilution of the material that gives agglutination is 1 in x, the titre is recorded as x.

Materials and Method

Antisera against strains N1, N2, N3, N4, N5, and N6 were taken, and tested against Clostridium difficile strains N1, N2, N3, N4, N5, and N6. 0.9 per cent NaCl was used as the diluent for the serum and the antigen.

Procedure; The serums were diluted in 0.9 per cent NaCl, the first dilution being 1 in 10, and subsequently by the the two-fold dilutions to give a series from 1 in 10 to 1 in 5120. 1.0 ml of each dilution was taken in agglutination tubes, and to it 1.0 ml of the appropriate suspensions of organisms was added, the mixture were shaken and incubated at $55^{0}C$ for 4 hours, agglutination results were read, and then the tubes were left in the cold room for overnight and the final results recorded. Table XXII gives the titre of each serum against the specific strain of Clostridium difficile against which serum was made, and it is interesting to note that the titre of antibodies produced against each strain ranged from 1280 to 2560; sera against strains N1, N2,N4, and N6 gave a titre of 2560, whilst N3 and N5 gave a titre of 1280.

Cross-agglutination reaction were also performed by using the same serum against all the six strains to obtain the titre of each serum against the other antigens. Table XXIII has been summarized to give the titre of each serum against the different strains of Clostridium difficile; it also includes the agglutinating titre of the antiserum made against the precipitates

## TABLE XXII

## SHOWING THE AGGLUTINATION REACTIONS OF DIFFERENT STRAINS OF CLOSTRIDIUM DIFFICILE AGAINST THE SPECIFIC ANTISERUM

| Strain | Degree of agglutination of strain by specific serum diluted 1 in | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 10 | 20 | 40 | 80 | 160 | 320 | 640 | 1280 | 2560 | 5120 | 00 |
| N1 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | ++ | + | - | - |
| N2 | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | ++ | ++ | + | - | - |
| N3 | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | +++ | + | - | - | - |
| N4 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | ++ | + | - | - |
| N5 | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | ++ | + | - | - | - |
| N6 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | ++ | + | - | - |

\+ indicates degree of agg utination (Test positive)

\- indicates no agglutination (Test negative)

TABLE XXIII

SHOWING THE CROSS-AGGLUTINATION TITRES OF VARIOUS ANTI-CL. DIFFICILE SERA AGAINST DIFFERENT STRAINS OF CL. DIFFICILE

| Serum against strain or substance | Titre of serum against the strains | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | N1 | N2 | N3 | N4 | N5 | N6 | N4I | T1 |
| N1 | 2560 | 320 | 640 | 320 | 320 | 80 | 320 | 640 |
| N2 | 320 | 2560 | 160 | 640 | 320 | 640 | 320 | 320 |
| N3 | 640 | 320 | 1280 | 320 | 320 | 320 | 320 | 320 |
| N4 | 640 | 320 | 320 | 2560 | 320 | 640 | 2560 | 160 |
| N5 | 320 | 640 | 320 | 320 | 1280 | 640 | 320 | 320 |
| N6 | 160 | 320 | 320 | 640 | 640 | 2560 | 320 | 640 |
| N4S | 80 | 40 | 20 | 80 | 40 | 40 | 160 | 20 |
| N4D | 80 | 80 | 80 | 1280 | 80 | 80 | 1280 | 80 |
| N4P | 160 | 160 | 80 | 1280 | 320 | 160 | 1280 | 80 |
| N4F | 160 | 80 | 40 | 320 | 40 | 40 | 320 | 40 |

and fractions of N4 strains.

The cross-agglutination tests confirm that all the strains have some antigens in common as all sera produced showed significant cross-agglutination against the other strains of Clostridium difficile.

Sera against N4P and N4D gave a titre of 1280 against N4 and N4I, and the serum against N4D gave a cross-agglutination titre of 80 against N1, N2, N3, N5, N6 and T1, indicating that probably the stroma of all the strains of Clostridium difficile is made up of a common antigenic material, and that the strain against which serum was produced has some additional antigens.

Agglutination absorption tests were performed with all the antisera and the strains against which the serum was produced. The absorption was done by mixing equal amounts of serum diluted 1 in 5 with the bacterial suspension in saline, incubating the mixture at 37°C for 2 hours, and centrifuging it at 3,000 rpm for 30 minutes; the supernatant was then tested for agglutinins. Two absorptions were necessary to remove all the agglutinins to the absorbing strain.

Table XXIV shows the results of absorption tests, from the results obtained it is clear that all strains absorb the agglutinins to some extent, confirming that the antigenic pattern is common in all the strains. The serum produced against the stroma of N4 cells shows that all the strains absorbed the agglutininins almost totally with the exception of those against N4 and N4I in which case they were reduced to 25 - 50 per cent.

TABLE XXIV

AGGLUTINATION ABSORPTION OF SERA BY

DIFFERENT STRAINS OF CL. DIFFICILE

| Serum against | Absorbed with | Titre of absorbed serum against strain | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | N1 | N2 | N3 | N4 | N5 | N6 | N4I | T1 |
| N1 | Nil | 2560 | 320 | 640 | 320 | 320 | 80 | 320 | 640 |
| | N1 | - | - | - | - | - | - | - | - |
| | N2 | 640 | - | 80 | - | - | - | - | 50 |
| | N3 | 640 | - | - | 20 | - | - | 40 | - |
| | N4 | 1280 | 40 | 160 | - | 20 | - | - | 160 |
| | N5 | 640 | - | - | - | - | 20 | - | 40 |
| | N6 | 1280 | 40 | 160 | 40 | 160 | - | 40 | 320 |
| N2 | Nil | 320 | 2560 | 160 | 640 | 320 | 640 | 320 | 320 |
| | N1 | - | 640 | - | 80 | - | 320 | 80 | - |
| | N2 | - | - | - | - | - | - | - | - |
| | N3 | - | 640 | - | 80 | - | 80 | 80 | 20 |
| | N4 | 40 | 640 | - | - | 40 | 80 | - | 40 |
| | N5 | - | 640 | - | - | - | 40 | - | - |
| | N6 | 40 | 640 | - | - | - | - | - | - |
| N3 | Nil | 640 | 320 | 1280 | 320 | 320 | 320 | 320 | 320 |
| | N1 | - | - | 160 | - | - | - | - | 20 |
| | N2 | 40 | - | 320 | 20 | - | - | - | 20 |
| | N3 | - | - | - | - | - | - | - | - |
| | N4 | 80 | 20 | 640 | - | 20 | - | - | 80 |
| | N5 | 40 | - | 320 | - | - | 20 | - | - |
| | N6 | 20 | - | 160 | - | - | - | - | - |

Table XXIV continued

| Serum against | Absorbed with | Titre of absorbed serum against strain | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | N1 | N2 | N3 | N4 | N5 | N6 | N4I | T1 |
| N4 | Nil | 640 | 320 | 320 | 2560 | 320 | 640 | 2560 | 160 |
| | N1 | - | - | - | 320 | - | - | 320 | - |
| | N2 | 80 | - | 20 | 640 | - | 40 | 640 | 20 |
| | N3 | 80 | 20 | - | 640 | 40 | - | 640 | - |
| | N4 | - | - | - | - | - | - | - | - |
| | N5 | 40 | - | - | 640 | - | 20 | 640 | - |
| | N6 | - | - | - | 320 | - | - | 640 | - |
| N5 | Nil | 320 | 640 | 320 | 320 | 1280 | 640 | 320 | 640 |
| | N1 | - | 80 | 20 | 20 | 320 | 40 | 20 | 40 |
| | N2 | - | - | - | - | 160 | 40 | - | 20 |
| | N3 | 20 | 40 | - | - | 320 | 20 | 20 | - |
| | N4 | 40 | 40 | 20 | - | 320 | 80 | - | 40 |
| | N5 | - | - | - | - | - | - | - | - |
| | N6 | - | 80 | - | - | 160 | - | - | - |
| N6 | Nil | 160 | 320 | 320 | 640 | 640 | 2560 | 320 | 640 |
| | N1 | - | 40 | 80 | 40 | 20 | 1280 | 20 | 160 |
| | N2 | - | - | 20 | 20 | 40 | 640 | 20 | 40 |
| | N3 | - | - | - | 40 | 40 | 640 | - | - |
| | N4 | - | - | - | - | - | 320 | - | - |
| | N5 | - | - | - | - | - | 320 | - | - |
| | N6 | - | - | - | - | - | - | - | - |

Table XXIV continued

| Serum against | Absorbed with | Titre of absorbed serum against strain | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | N1 | N2 | N3 | N4 | N5 | N6 | N4I | T1 |
| N4S | Nil | 80 | 40 | 20 | 80 | 40 | 40 | 160 | 20 |
| | N1 | - | 10 | - | 20 | - | - | 80 | - |
| | N2 | 20 | - | - | 40 | 10 | - | 80 | - |
| | N3 | 40 | - | - | 40 | 10 | 10 | 80 | - |
| | N4 | - | - | - | - | - | - | - | - |
| | N5 | 10 | .. | - | 20 | - | - | 40 | 10 |
| | N6 | - | - | - | 40 | - | - | 80 | 10 |
| N4D | Nil | 80 | 80 | 80 | 1280 | 80 | 80 | 1280 | 80 |
| | N1 | - | - | - | 320 | - | .. | 640 | - |
| | N2 | .. | - | - | 640 | - | - | 640 | - |
| | N3 | - | - | - | 320 | - | - | 640 | - |
| | N4 | - | - | - | - | - | - | - | - |
| | N5 | - | - | - | 320 | - | 10 | 320 | - |
| | N6 | - | - | - | 320 | 20 | - | 320 | - |
| N4P | Nil | 160 | 160 | 80 | 1280 | 320 | 160 | 1280 | 80 |
| | N1 | - | - | - | 640 | 40 | - | 320 | - |
| | N2 | 20 | - | - | 320 | - | - | 320 | 10 |
| | N3 | 20 | 20 | - | 640 | 80 | 20 | 320 | - |
| | N4 | - | - | - | - | - | - | - | - |
| | N5 | - | - | - | 160 | - | - | 320 | .. |
| | N6 | - | - | 10 | 640 | .. | - | 640 | - |

Table XXIV continued

| Serum against | Absorbed with | Titre of absorbed serum against strain | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | N1 | N2 | N3 | N4 | N5 | N6 | N4I | T1 |
| N4F | Nil | 160 | 80 | 40 | 320 | 40 | 40 | 320 | 40 |
| | N1 | - | - | - | 20 | - | - | 20 | - |
| | N2 | - | - | - | - | - | - | - | - |
| | N3 | - | - | - | - | - | - | - | - |
| | N4 | - | - | - | - | - | - | - | - |
| | N5 | 20 | - | - | 40 | - | - | 20 | - |
| | N6 | - | - | - | 80 | - | - | 80 | - |

It can be concluded from the absorbtion tests that there is a strain specific antigen which causes the production of a strain specific antibody in the animal which is not completely removable from the serum by any other strain. In fact it seems likely that it is not so removable at all.

## IMMUNOFLUORESCENT ANTIBODY TECHNIQUE

The technique of immunofluorescent antibody was introduced by Coons et al. in 1941 and this method was further extended by Coons and Kaplan (1950). There are two methods for applying the technique - direct and indirect; in the direct method, the specific labelled antibody is applied to the preparation, whilst in the indirect technique the preparation is first reacted with a specific unlabelled antiserum, washed free from uncombined antibody and then treated with a specific fluorescent anti-globulin serum. In my tests the indirect method was used.

### Materials and Method

Prepared sera was used and the strains of Clostridium difficile were washed and used at a concentration of about $1 \times 10^8$.

1. Labelling of antisera with fluorescein isothiocyanate; The method was originally suggested by Riggs et al (1958) and modified by Marshall et al (1958), who used Rivanol precipitation for the removal of albumin and the alpha- and beta- globulins (Horsjai and Smentha, 1956), leaving a pure immunoglobulin preparation.

a) The pH of the antiserum was adjusted to 8.5 with 0.1M-NaOH, and 9 ml of the serum was taken;

b) 32 ml of 0.4 per cent Rivanol ( 2 - ethoxy 6,9-diamino acridine lactate ) in distilled water was added gradually with constant stirring; a heavy yellow precipitate was formed.

c) The supernatant was collected by centrifuging the mixture, 0.49 g of activated charcoal was added, and stirred

vigorously to remove excess of Rivanol.

d) The mixture was filtered through a No. 42 filter paper on a Buchner funnel under vacuum; the filtrate obtained was colourless.

e) To the filtrate an equal volume of 70 per cent. Ammonium sulphate was added gradually, the mixture was stirred constantly, and then left at $4^{o}C$ overnight.

f) The precipitate was collected by centrifugation and the supernatant was discarded.

g) The precipitate was dissolved in 3.0 ml of distilled water and transferred to Visking cellulose tubing, and dialysed against phosphate buffered saline pH 7.5 for 72-hours on a magnetic stirrer. The phosphate buffer saline was changed frequently.

2. <u>Staining by indirect method</u>

a) A smear was prepared from the suspension of organisms in phosphate buffered saline pH 7.5 and dried in air.

b) The smear was fixed in acetone for 10 minutes, and allowed to dry in air.

c) The smear were covered with the diluted sera. The slides were left at room temperature in a petri plate containing a moist filter paper, for 30 minutes, then washed for 2 minutes.

d) The preparation was stained with antibody dye ( sheep anti-rabbit-gamma-globulins) made fluorescent with fluorescein isothiocyanate for 30 minutes at room temperature in a moist petri plate.

e) The preparation was washed with phosphate buffer saline

pH 7.5 in the following way

( (i) rinsed for 5 seconds

( ii) the preparation was allowed to stand in phosphate buffer saline for 2 minutes

(iii) rinsed for 5 seconds

( iv) rinsed in distilled water.

f) The slides were dried between blotting papers and mounted in mounting fluid (90 per cent glycerol in PBS ).

The slides were examined in a Zeiss fluorescence microscope; the results are given in Table XXV. It is obvious from the table that the titre of serum obtained with IFL was much higher as compared with the agglutination tests, but the same conclusions are drawn from the results obtained, as in case of agglutination tests.

## TABLE XXV

### RESULTS OF INDIRECT IMMUNOFLUORESCENCE TESTS OF CLOSTRIDIUM DIFFICILE AND ITS SERUMS

| Serum against strain | Titre in indirect immunofluorescence tests of serum against strain | | | | | |
|---|---|---|---|---|---|---|
| | N1 | N2 | N3 | N4 | N5 | N6 |
| N1 | 5120 | 1280 | 1280 | 1280 | 1280 | 640 |
| N2 | 1280 | 5120 | 960 | 1280 | 1280 | 1280 |
| N3 | 1280 | 960 | 5120 | 1280 | 1280 | 1280 |
| N4 | 1280 | 1280 | 1280 | 7680 | 1280 | 1280 |
| N5 | 1280 | 1280 | 1280 | 1280 | 3840 | 1920 |
| N6 | 960 | 1280 | 1280 | 1920 | 1920 | 5120 |
| N4S | 160 | 80 | 60 | 640 | 80 | 80 |
| N4D | 640 | 640 | 640 | 3840 | 640 | 640 |
| N4F | 960 | 960 | 960 | 1280 | 960 | 960 |
| N4P | 320 | 320 | 320 | 1920 | 320 | 320 |

## THE PRECIPITATION REACTION.

The precipitation reaction takes place between a soluble antigen and a solution of its homologous antibody. The reaction is manifested by the formation of a visible precipitate at the interface of the reactants, but may be inhibited by an excess of either antigen or antibody. As is well known, if the antiserum is derived from a rabbit, the precipitate is soluble in excess of antigen only, and in consequence, precipitation occurs over a wide range of antigen concentrations.

In this kind of serological reaction the antibody functions as a percipitin and the antigen that provokes the formation is known as precipitinogen. The physical state of antigen used for immunization is not of particular importance; antibacterial serum, for example, will give precipitates when mixed with preparations of the soluble cell substance of microorganisms, and precipitins may almost always be demonstrated in lytic, antitoxic, opsonic and agglutinating antiserums.

The precipitation test is carried out with undiluted, or only slightly diluted serum, but the antigen solution is diluted in series. There has been a number of methods to perform precipitation tests they include (1) The ring test (2) Agar diffusion method and (3) Ouchterlony method.

For the purpose of studying the precipitation reaction during the studies of Clostridium difficile, the following methods were used

I. Oakley - Fulthrope method (Double diffusion in one dimension )

In this method an equal amount of antibody is incorporated in 2 per cent agar, and the agar is allowed to solidify; then a

column of 1 per cent agar is superimposed, and again allowed to solidify and the filtrate of antigen solution is placed over it and allowed to diffuse for 48 hours at 37°C; if the appropriate antibody is present in sufficient concentration in the serum a band of precipitate is formed in the layer of agar.

II. Double diffusion in two dimension (Ouchterlony method)

The method is based on pouring a 5 mm layer of 1 per cent agar in a petri plate. The various reactants are placed in the well, which can be cut out with a cork borer, and the plates are incubated at 37°C for 48 hours; a band of precipitate is formed. The method was utilized by Bjorkland and Berengo (1954), and it was also used by Ellner and Green (1963) to study the filtrates of clostridia. The same method was used in this study.

Materials and Method

Serum prepared against the whole bacterial cell, and also sera prepared against the precipitated toxins, the disintegrated cells and the cell extract were used. The organisms were grown in RCM for 72 hours and centrifuged, and the filtrate from the supernatant was used as the antigen. 0.85 per cent NaCl was used as the diluent for the antigen; sera diluted to 1 in 5 were used throughout. Doubling dilutions of the antigen was used; best results were obtained with 1 in 2 dilution of the antigen. Different combinations of the antigens were tried. The lines of precipitates obtained were marked as a, b, c, d, and e, and the common lines of precipitates obtained were given the same letter. The filtrates of N1, N2, N3, N4, N5, and N6 were studied in detail while the others were studued rather less. Table XXVI shows the results obtained,also illustrated by photograph 4.

# TABLE XXVI

## PRECIPITATION REACTION BETWEEN SERUM AND FILTRATE OF CLOSTRIDIUM DIFFICILE

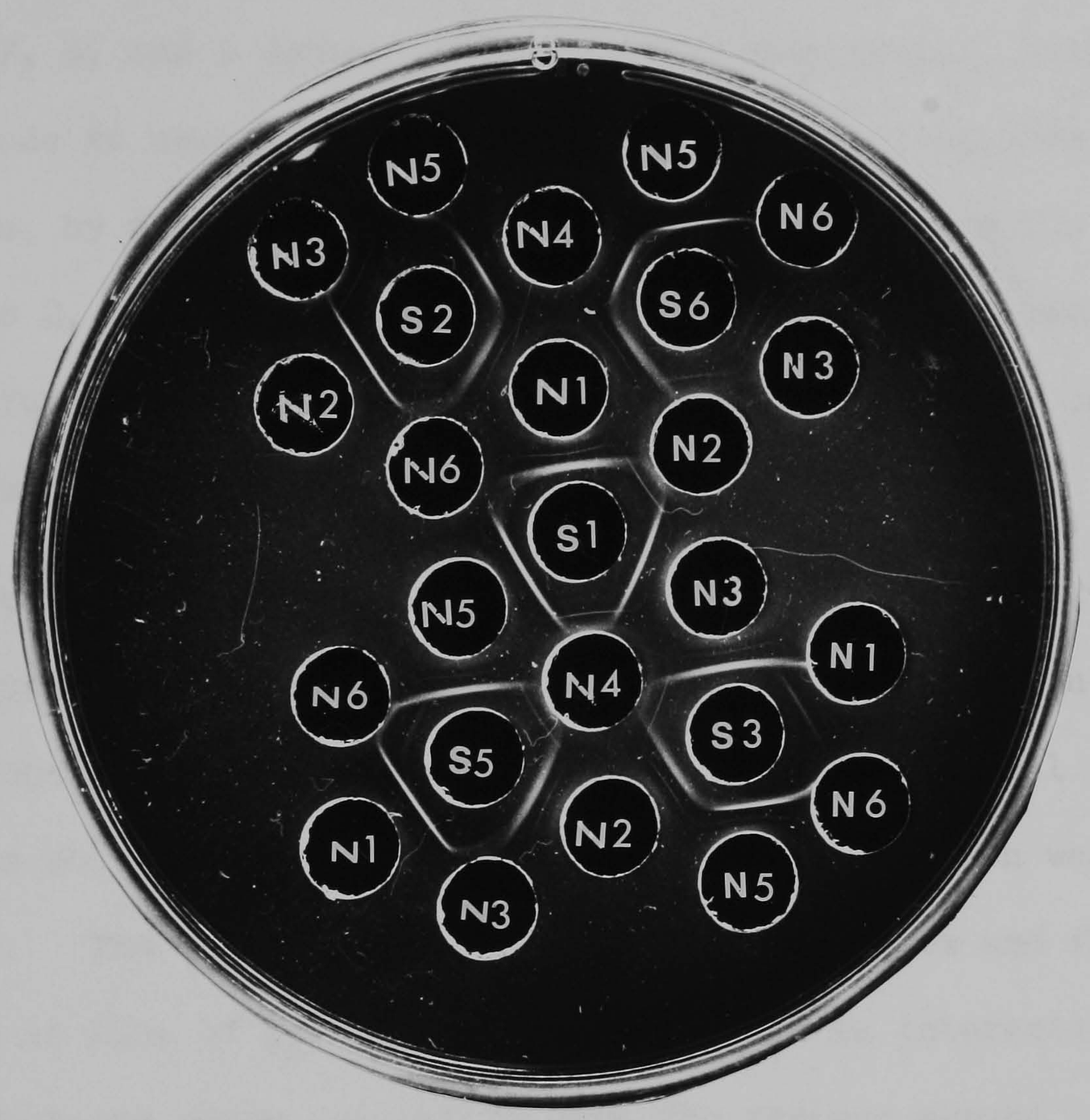
| Serum against strain | Result of diffusing serum against filtrate | | | | | |
|---|---|---|---|---|---|---|
| | N1 | N2 | N3 | N4 | N5 | N6 |
| N1 | abc | ab | ab | ab | ab | ab |
| N2 | ab | ab | a | ab | - | a |
| N3 | a | abc | abcd | acd | ab | a |
| N4 | a | a | ac | abc | a | a |
| N5 | a | ab | a | ac | abc | ac |
| N6 | ac | a | ac | ac | a | ac |

Each letter represents a line of precipitate of one type

PHOTOGRAPH 4

PRECIPITATION TEST BETWEEN SERUM AGAINST AND FILTRATES

OF STRAINS OF CLOSTRIDIUM DIFFICILE




S indicates serum against the strain

N indicates filtrate of the strain

Similarly precipitation was carried out with the antisera prepared against the different fractions of strain N4, the results obtained are given in table XXVII, and illustrated by photographs 5, 6, 7, 8, and 9 depicting some of the reactions. . Attempts were made to absorb some of the antiserum with filtrates of the strains, by mixing the antiserum with the antigen in the ratio of 1 to 2, and incubating the mixture at 37°C for 4 hours, centrifuging the mixture, and using the supernatant as serum diluting it to 1 in 5 and reacting it with the antigen.

From the tables XXVI and XXVII it is clear that different sera prepared against different strains and also differnt fractions respond to the precipitation reaction and all the strains showed at least one line of precipitate which was common. The control which contained normal serum and filtrates showed no line of precipitate. However it is interesting to note that the serum against N3 gave the maximum number of lines of precipitates; another important point to note is the fact that the specific serum always gave at least one line of precipitate more than the common lines of precipitate produced by other strains, with the exception of serum against N2 and N6. This indicates that all the strains are identical in some ways and at the same time possess some individuality also.

The analysis of the results in table XXVII, reveals that filtrates of strain N1, N2, N5 and N6 diffused against antiserum N4 have a single precipitation line in common, whilst N3 has two lines in common with the antiserum N4 which also has two individual lines. The antiserum against N4P produced .

TABLE XXVII

PRECIPITATION REACTIONS BETWEEN SERUM AGAINST DIFFERENT FRACTIONS OF STRAIN N4 AND THE STRAINS OF CL. DIFFICILE

| Filtrate of strain | Results of diffusing filtrate against serum produced against | | | | |
|---|---|---|---|---|---|
| | N4 | N4F | N4P | N4S | N4D |
| N1 | a | ac | ac | ab | ab |
| N2 | a | abc | ab | ab | ab |
| N3 | ac | ac | abc | abc | ab |
| N4 | abc | abcd | abcde | abcd | abc |
| N5 | a | a | ac | ab | a |
| N6 | a | a | a | ab | ab |
| N4I | abc | abcd | abcde | abcd | ac |
| T1 | a | ac | ab | a | a |
| T2 | - | a | - | a | - |
| T3 | a | ac | ab | abd | a |
| T4 | a | a | ab | abcd | a |
| T5 | - | - | - | a | - |
| T7 | a | ac | ab | a | - |
| T8 | - | - | a | a | - |
| T9 | a | a | ab | abd | ab |
| T10 | a | ad | ab | abcd | a |
| R1 | a | ac | a | a | - |
| R2 | - | a | ae | ac | - |
| R3 | a | ab | a | ab | a |

Each letter represents a line of precipitate of one type.

- denotes absence of line of precipitation

PHOTOGRAPH 5

PRECIPITATION TEST BETWEEN SERUM AGAINST STRAIN N4 AND THE FILTRATES OF DIFFERENT STRAINS OF CLOSTRIDIUM DIFFICILE


S4 = serum against N4 strain

N = filtrate of strain (original )

T = filtrate of strain (isolated by me )

R = filtrate of strain (isolated by me )

PHOTOGRAPH 6

PRECIPITATION TEST BETWEEN SERUM AGAINST STRAIN N4 'FLOATING' PRECIPITATE AND THE FILTRATES OF DIFFERENT STRAINS OF CLOSTRIDIUM DIFFICILE


$S_4F$ = serum against floating precipitate ($N_4F$ )

N = filtrate of strain (original)

T = filtrate of strain (isolated by me)

R = filtrate of strain (isolated by me)

PHOTOGRAPH 7

PRECIPITATION TEST BETWEEN SERUM AGAINST STRAIN N4 'SINKING' PRECIPITATE AND THE FILTRATES OF DIFFERENT STRAINS OF CLOSTRIDIUM DIFFICILE


$S_4S$ = serum against sinking precipitate ($N_4P$)

N = filtrate of strain (original)

T = filtrate of strain (isolated by me)

R = filtrate of strain (isolated by me)

PHOTOGRAPH 8

PRECIPITATION TEST BETWEEN SERUM AGAINST $N^4$ 'SUPERNATANT'

AND THE FILTRATES OF DIFFERENT STRAINS OF

CLOSTRIDIUM DIFFICILE


$S_4S$ = serum against the supernatant of disintegrated cells of Cl. difficile ($N_4D$)

N = filtrate of strain (original )

T = filtrate of strain (isolated by me)

R = filtrate of strain (isolated by me)

PHOTOGRAPH 9

PRECIPITATION TEST BETWEEN SERUM AGAINST N4 DISINTEGRATED CELLS AND THE FILTRATES OF DIFFERENT STRAINS OF CLOSTRIDIUM DIFFICILE


$S_4D$ = serum against the disintegrated cells

N = filtrate of strain (original)

T = filtrate of strain (isolated by me))

R = filtrate of strain (isolated by me)

maximum number of lines against filtrate of strain N4 and N4I, and 1 to 3 lines of precipitate are common with the filtrate of other strains, the number of lines produced, by the filtrates of different strains of Clostridium difficile are nearly the same, It can be assumed that the cell-extract and the disintegrated cells (stroma) possess almost the same antigenic makeup with some minor differences, while the antigens produced by the organisms vary to some extent, and due to this factor the precipitin picture of the organisms varies.

## TOXIN NEUTRALIZATION TEST

Toxin and antitoxin mixtures have been widely used to determine the ability of the antitoxin produced in response to the toxin for neutralization of the toxins. Varying amounts of antitoxin are mixed with the lethal dose of the toxin, and in this way the amount of toxin is determined which would neutralize the toxin.

### Materials and Method

Strain N4 was used for toxin production, and the toxin tested for lethality.

The serum available (N4) was used as the standard serum and given an arbitrary value of 100 units per ml. As the LD50 was found to be 0.250 ml, 0.3 ml of the toxin was taken as the test dose, and to 1.2 ml of the toxin varying amounts of antiserum were added and the total volume of the mixture was made up to 2.0 ml by adding saline. The mixture was then mixed by rotamixer for a few seconds and left at room temperature for 30 minutes. Then from each mixture 0.5 ml was injected intravenously into each of two mice, which were observed for 7 days. The mixture that contained the minimum amount of antitoxin compatible with the survival of the mice given an injection of it was assumed to contain 5 units of antitoxin. This antiserum was tested against other toxins, and N4 toxin was used to test other sera.

The scheme for the toxin - antitoxin mixture is given on the next page.

Scheme for the Toxin and Antitoxin mixture

| Amount of toxin(ml) | Amount of serum(ml) | Amount of saline(ml) | Value of antiserum in units/0.5 ml of mixture |
|---|---|---|---|
| 1.2 | 0.8 | 0.0 | 20.0 |
| 1.2 | 0.6 | 0.2 | 15.0 |
| 1.2 | 0.5 | 0.3 | 12.5 |
| 1.2 | 0.4 | 0.4 | 10.0 |
| 1.2 | 0.3 | 0.5 | 7.5 |
| 1.2 | 0.2 | 0.6 | 5.0 |
| 1.2 | 0.15 | 0.65 | 3.75 |
| 1.2 | 0.10 | 0.70 | 2.5 |
| 1.2 | 0.05 | 0.75 | 1.25 |
| 1.2 | 0.04 | 0.76 | 1.0 |
| 1.2 | 0.0 | 0.80 | 0.0 |

The results obtained by injecting 0.5 ml of the mixture are recorded in table XXVIII. It can be concluded from the results obtained that 5.0 units of the serum are sufficient to protect the animal from the lethal effect of the toxin.

The experiment was repeated by varying the value of N4 antitoxin between 0.0 to 15 units and 0.3 ml of the filtrate of N1, N2, N3, N4, N5, N6 and 1.0 milligram of N4 precipitated and dried toxin was used, and it was found that one antitoxin (N4) was able to protect all the animals, the results are given in table XXIX.

In another set of experiments, 0.3 ml of the filtrate of N4 was used as the lethal toxin for mice, and the antiserum

## TABLE XXVIII

## TITRATION OF ANTISERUM N4 AGAINST THE LETHAL TOXIN OF STRAIN N4 OF CLOSTRIDIUM DIFFICILE IN MICE

| Filtrate of strain | Amount of filtrate (ml) | Units of antiserum | Results |
|---|---|---|---|
| N4 | 0.3 | 20.0 | Survived* |
| | 0.3 | 17.5 | Survived |
| | 0.3 | 15.0 | Survived |
| | 0.3 | 12.5 | Survived |
| | 0.3 | 10.0 | Survived |
| | 0.3 | 7.5 | Survived |
| | 0.3 | 5.0 | Survived |
| | 0.3 | 3.75 | Died in 96 hours |
| | 0.3 | 2.5 | Died in 72 hours |
| | 0.3 | 1.25 | Died in 72 hours |
| | 0.3 | 1.0 | Died in 72 hours |
| | 0.3 | 0.0 | Died in 72 hours |

* indicates that the mice survived till the end of the experiment (7 days )

TABLE XXIX

PROTECTIVE EFFECT OF N4 ANTISERUM AGAINST

THE TOXINS OF CLOSTRIDIUM DIFFICILE

| Filtrate of strain | Results of injecting into mice 0.5 ml of a mixture containing 1.2 ml of toxic filtrate and antitoxin units | | | | | | |
|---|---|---|---|---|---|---|---|
| | 15.0 | 12.5 | 10.0 | 7.5 | 5.0 | 3.75 | 0.0 |
| N1 | - | - | - | S | S | D | D |
| N2 | - | - | - | S | S | D | D |
| N3 | - | - | - | S | S | D | D |
| N4 | - | - | - | S | S | D | D |
| N5 | - | - | - | S | S | D | D |
| N6 | - | - | - | S | S | S | S |
| N4P | S | D | D | D | D | D | D |

- = Not used

S = Survived till the end of the experiment

D = Died

against N1, N2, N3, N4, N5, N6, N4S, N4D, N4P and N4F were used to see the protective effect of the various antisera, and the values for each antiserum was determined by adding varying amounts of antiserum, and the mixture made up to 0.5 ml and injected intravenously into the mice, the controls included mice injected with 0.3 ml of N4 filtrate with and without 5 units of antiserum. The values for the sera have been calculated against the standard N4 antisera, the results are given in table XXX.

It can be concluded from the tables XXIX and XXX that all filtrates can be neutralized by a single antiserum, and all the sera produced have the ability to protect the animal from the toxic effect of the filtrate of N4, the least effective antiserum is that against N6 which has a value of 40 units per ml, whilst the most effective is antiserum against the precipitated toxin of N4, having a value of 200 units, compared with our standard which was given an arbitrary value of 100 units per ml. Similarly it can be seen that the serum N4 protects the animal from the filtrates of different strains of Clostridium difficile requiring 5 or less than 5 units to protect the animals with the exception of the precipitated toxin of N4 strain (N4P) in which case 1 milligram of the precipitated and dried toxin required 15 units of the N4 serum for neutralization. The higher values of antisera N4P and N4F can be due to the route of injection.

These tests can fairly be criticised as not conforming exactly to the form of the "test dose" method, but the weakness

## TABLE XXX

## TITRATION OF ANTISERUM (N1 - N6) AGAINST THE FILTRATE OF STRAIN N4 OF CL. DIFFICILE

| Serum against strain | Amount of N4 filtrate (ml) | Amount of serum protecting the mice (ml) | Units of serum/ml |
|---|---|---|---|
| N1 | 0.3 | 0.1 | 50.00 |
| N2 | 0.3 | 0.1 | 50.00 |
| N3 | 0.3 | 0.1 | 50.00 |
| N4* | 0.3 | 0.05 | 100.00 |
| N5 | 0.3 | 0.075 | 66.66 |
| N6 | 0.3 | 0.125 | 40.00 |
| N4F | 0.3 | 0.0375 | 133.33 |
| N4P | 0.3 | 0.025 | 200.00 |
| N4S | 0.3 | 0.075 | 66.66 |
| N4D | 0.3 | 0.05 | 100.00 |

* Standard serum

These figures are very approximate; many more mice would have been necessary for more accurate results.

of the toxic filtrates made it impossible to work at a standard level. Though the values of the sera must be regarded as uncertain because of this and because of possible non-avidity (which could not be tested for), it is still clear that the lethal filtrates from all strains are antigenically the same.

## ANTIHYALURONIDASE

Antihyaluronidase are the antibodies produced against the hyaluronidase. The test is based on the same principle as that of hyaluronidase testing.

When testing for the antihyaluronidase, serum $N_4$ was chosen and given 100 arbitrary units. The test dose was determined for the filtrates of N1, N2, N3, N4, N5, and N6, by diluting them by 10 per cent difference, one unit of serum diluted in B.B.S. and mixed by inversion of the tubes, allowed to stand at room temperature for 30 minutes and 0.5 ml of synovial fluid diluted to contain 8 indicating doses was added and the test then proceeded as in the case of hyaluronidase test. The minimum amount of filtrate which, when mixed with one unit of antitoxin, produced a good spread in the ACRA test was taken as the test dose and was used to assay other sera in the usual way.

The results obtained are recorded in a tabular form given in table XXXI. The values obtained for the different sera against the filtrates of the strains of Clostridium difficile, are in agreement, and this indicates that the hyaluronidase produced is immunologically identical in all the strains of Clostridium difficile.

TABLE XXXI

VALUES OF SERA AGAINST STRAINS OF CLOSTRIDIUM DIFFICILE

FILTRATES IN HYALURONIDASE TEST

| Sera against strain or fraction | Value (units per ml ) of serum against filtrate | | | | | |
|---|---|---|---|---|---|---|
| | N1 | N2 | N3 | N4 | N5 | N6 |
| N1 | 50 | 50 | 50 | 50 | 50 | 48 |
| N2 | 60 | 60 | 64 | 60 | 66 | 60 |
| N3 | 50 | 50 | 50 | 50 | 50 | 55 |
| N4* | 100 | 100 | 100 | 100 | 100 | 100 |
| N5 | 80 | 80 | 80 | 80 | 78 | 80 |
| N6 | 40 | 40 | 40 | 40 | 48 | 50 |
| N4F | 120 | 120 | 120 | 116 | 132 | 120 |
| N4P | 200 | 200 | 200 | 200 | 200 | 200 |
| N4S | 60 | 60 | 66 | 60 | 60 | 66 |
| N4D | 100 | 100 | 100 | 100 | 100 | 100 |

* Standard serum

## ANTIHAEMOLYSIN

Antihaemolysin is the antibody against the haemolysin. In order to test the antihaemolysin activity of the antibody produced in response to the strains of Clostridium difficile. Antiserum N4 was taken as standard and given 100 arbitary units per ml. The test dose was determined for the filtrates of strain N1, N2, N3, N4, N5 and N6 by diluting them by 10 per cent difference, one unit of the serum diluted in 0.85 per cent NaCl solution was added to 1.0 ml of the diluted filtrate, mixed on a mixer for a short time, and allowed to stand at room temperature for 30 minutes and 1.0 ml of 2 per cent RBC added, mixed by gentle shaking and incubated in a water bath at 37°C for 2 hours. The minimum amount of filtrate mixed with one unit of serum and producing 50 per cent haemolysis was taken as the test dose of the toxin, and used to assay other sera in the usual way.

The values obtained for the different sera and filtrates have been recorded in a tabular form in table XXXII; it is quite obvious from the table that all antisera have the ability to neutralize the haemolysin produced by the strains of Clostridium difficile, but the value of the sera varies to some extent, indicating that probably more than one type of haemolysin is produced by the strains of Clostridium difficile.

TABLE XXXII

VALUE OF SERA AGAINST STRAINS OF CLOSTRIDIUM DIFFICILE

IN ANTIHAEMOLYSIN TESTS

| Sera against strain | Value (units per ml) of serum against filtrate | | | | | |
|---|---|---|---|---|---|---|
| | N1 | N2 | N3 | N4 | N5 | N6 |
| N1 | 200 | 200 | 100 | 100 | 100 | 200 |
| N2 | 200 | 200 | 160 | 150 | 200 | 200 |
| N3 | 100 | 160 | 160 | 160 | 160 | 130 |
| N4* | 100 | 100 | 100 | 100 | 100 | 100 |
| N5 | 100 | 200 | 100 | 150 | 200 | 200 |
| N6 | 130 | 130 | 120 | 120 | 130 | 200 |
| N4P | 60 | 100 | 100 | 200 | 100 | 100 |
| N4F | 50 | 50 | 50 | 50 | 50 | 50 |
| N4S | 50 | 50 | 50 | 80 | 50 | 50 |
| N4D | 60 | 60 | 50 | 100 | 100 | 60 |

* Standard serum

# CHAPTER VI

## ELECTRON MICROSCOPY

In spite of the fact that in the current era there have been vast advances in the field of microorganisms, the development of electron microscope technique has revolutionized our general outlook about microorganisms and the electron microscope has proved itself as an indispensable tool. Particularly in the field of cytology of bacteria it has revealed so much that sooner or later we will have to accept the facts and as suggested by Bisset that, because of their small size and the difficulty of observing the complexities of their structure by the methods usually employed they may be regarded as simple in form and phylogenetically primitive.

In order to have a closer look of Clostridium difficile, the strains were examined under the electron microscope, and some of the results obtained were quite interesting.

The organisms were seen in stained and unstained preparations and also sections of the organisms were cut.

### Materials and Methods

Strains of Clostridium difficile were grown in RCM, and centrifuged at 1,000 rpm for 30 minutes; the supernatant was decanted off and the cells were resuspended in saline and washed 3 times, resuspended in saline and fixed by adding 1 per cent formaldehyde solution and allowing them to stand for 10 minutes. They were then centrifuged and the supernatant was discarded; the fixed cells were resuspended in distilled water and again spun down and finally the cells were resuspended in distilled water so that a turbid suspension was formed.

This was mounted on the grid (with plastic film), and allowed to dry. When unstained preparations are to be examined the grid is mounted directly on the specimen holder and seen under the microscope; when stained preparations are to be examined, the grid containing the specimen is stained either by heavy metal or by negative staining.

In preparation for sectioning, the organisms are grown, fixed, dehydrated and embedded; the blocks are polymerised, and thin sections are cut, mounted and stained, and then observed under the electron microscope.

The organisms were grown in liquid medium, and the cultures were centrifuged and washed 3 times with phosphate buffer, and centrifuged to form a pellet. The pellet was transferred to a small glass - stoppered bottle 1.5 cm in diameter containing 1 - 3 ml glutaraldehyde fixative - (25 per cent glutaraldehyde in water with phosphate buffer to give a final concentration of 1.5 - 6.0 per cent glutaraldehyde in 0.05 - 0.1 M buffer at pH 6.8 - 7.6) and left at room temperature for 2 - 4 hours. Then the glutaraldehyde was removed as far as possible with Pasteur pipettes and the pellet was washed 3 times with phosphate buffer, and then post fixed with buffered osmium tetraoxide fixation, for 2 hours.

After post fixation the specimen was dehydrated by passing it through a series of alcohols at room temperature: 30 per cent for 15 minutes; 50 per cent for 15 minutes; 70 per cent (twice) for 15 minutes each time; 90 per cent for 30 minutes; and absolute alcohol (twice) for 60 minutes each time.

The specimens remained in the small glass-stoppered bottles

in which they were fixed; one solution was removed with a fine pipette and the next was poured in.

Embedding

The next step after dehydration is the embedding of the specimen. For this a recently devised "Low viscosity Epoxy Resin Embedding Medium" (Spurr, 1969) was used, and after some trials with others was found to be the most suitable medium, and was used for the study. The medium consists of ERL - 4206 (vinyl cyclo-hexene dioxide) 10.0 g; D.E.R. 736 (diglycidyl ether of polypropylene glycol) 6.0 g; NSA (nonenyl succinic anhydride) 26.0 g; and S-1 (dimethylamino-ethanol or DMAE), 0.4 g. The medium is easily and rapidly prepared by dispensing the components, in turn by weight, into a single flask, and mixed by shaking and swirling.

The infiltration of the medium was carried out by adding an equal quantity of the embedding medium to the amount of dehydrating fluid (absolute alcohol) retained in the vials at the last change. The mixture was swirled, and allowed to stand for 30 minutes. Then again a volume of the embedding medium equal to the total volume present in the bottle was added, and again swirled and allowed to stand for 30 minutes more. The mixture was drained from the bottle, and then a third amount of the medium was added, and swirled again and allowed to stand for 4 hours. The medium was then drained from the bottle, some more medium was added and the specimens were transferred to oven-dried capsules, which were then filled with the embedding medium. Polymerization was done by leaving

the filled capsules in an oven at 70°C for 12 - 18 hours. The castings were transparent and light yellow in colour.

The castings were removed from the capsules by cutting the capsules, and trimmed with a sharp razor blade, and phased for cutting on microtome. Small sections of the castings containing the specimen were cut with glass knives.

The sections were mounted on the grids and stained with heavy metallic stains, and some were examined without staining..

Observations

The following observations were made.

a) Whole cells; The whole cells were seen as elongated cells, with round ends and the size varied between 2.5 - 5.9 u by 0.3 - 1.5 u . It was interesting to note that when the cells were in dividing stage, they were fairly thick rods.

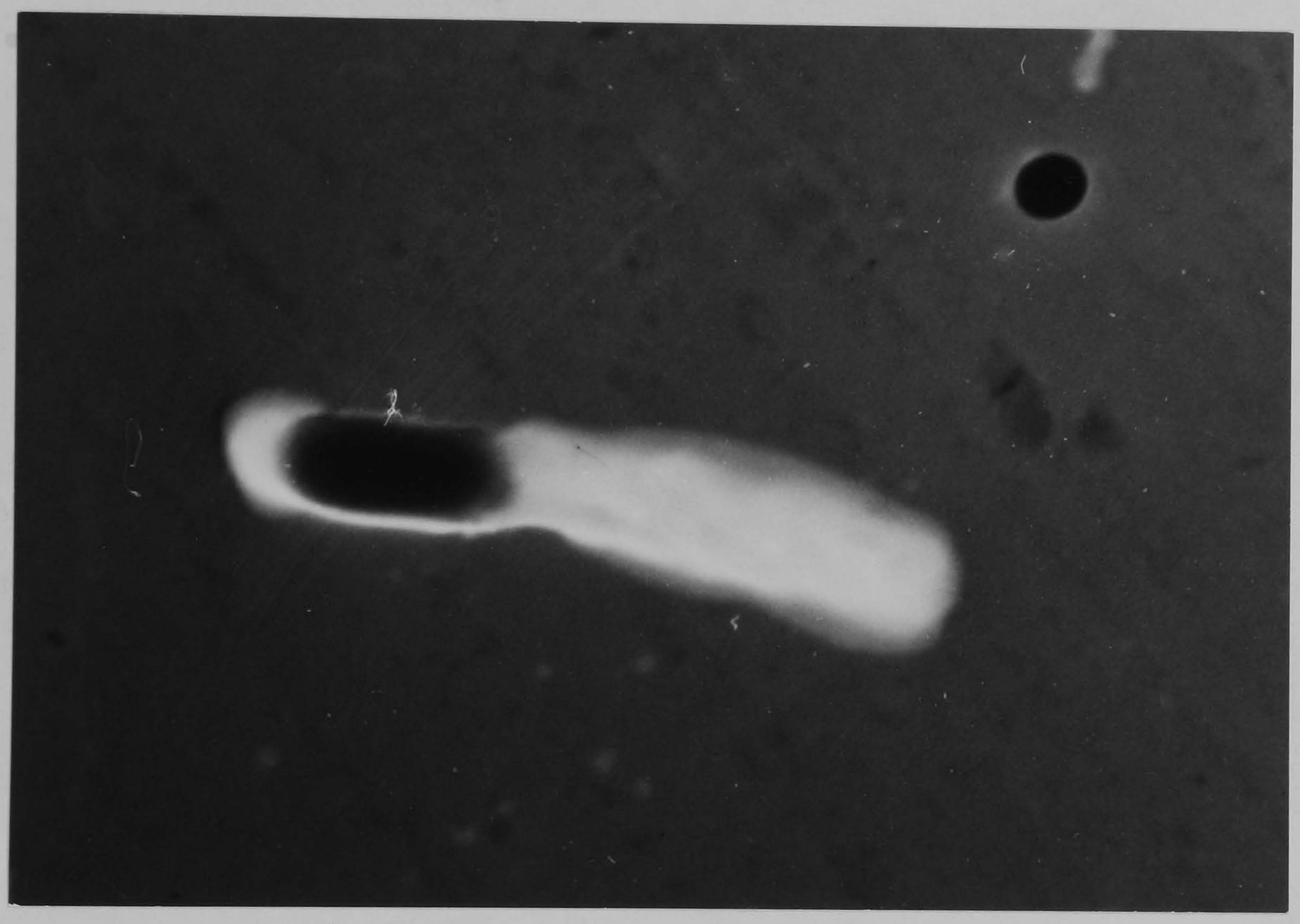
b) Dividing cell; The nucleus was seen dividing into four daughter nuclei, two of which migrated to the two ends, and the other two daughter nuclei migrated to a short distance, so that they werewere arranged in the cell at almost equal distance from one another. The septal walls then developed and the entire cell became like a beaded structure, finally the cell elongates and detach from the parent cells to form four daughter cells. the process is illustrated by electron micrograph photographs 10, 11, 12, 13, and 14.

c) Spore ; The spores are seen as subterminal and slightly elongated, illustrated by elctron micrograph photograph 15.

d) Section of the cell; the section of the cell reveals a central mass of the DNA, illustrated by electron micrograph.(photograph 16 )

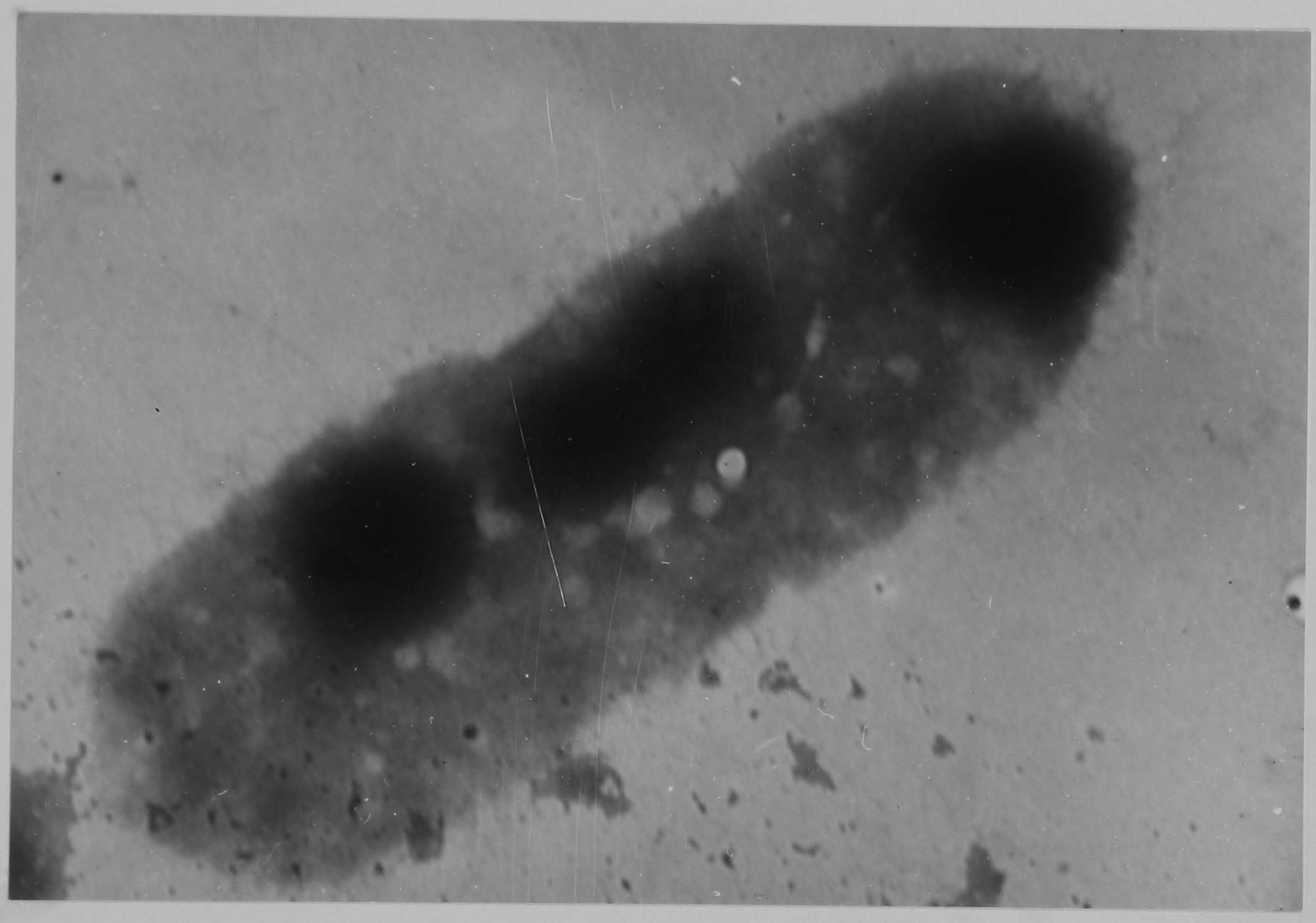
PHOTOGRAPH 10. ELECTRON MICROGRAPH OF SPORING CELL

OF CLOSTRIDIUM DIFFICILE x 15,000

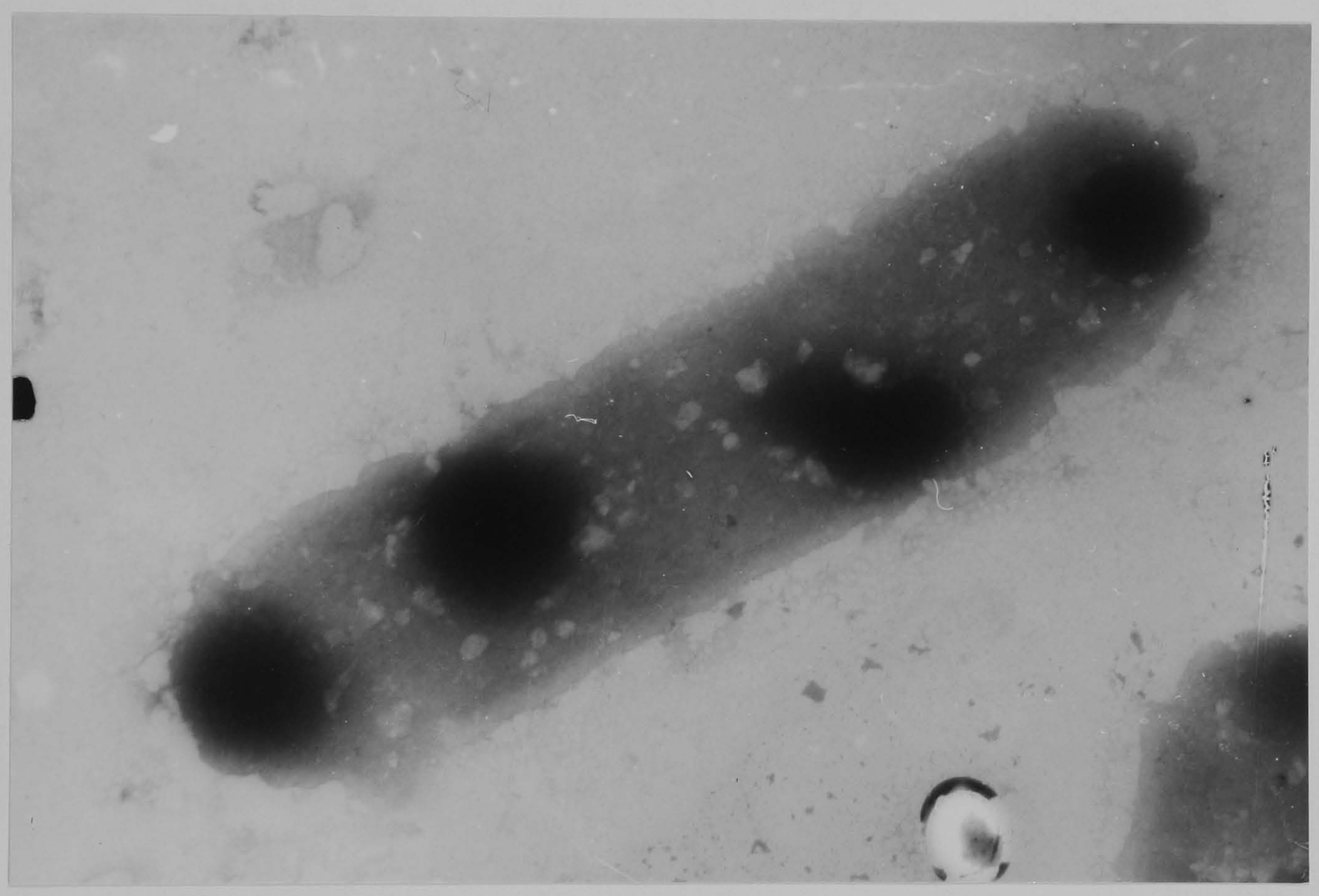


PHOTOGRAPH 11. ELECTRON MICROGRAPH OF WHOLE CELL OF CLOSTRIDIUM DIFFICILE x 25,000

PHOTOGRAPH 12. ELECTRON MICROGRAPH OF CELL DIVISION OF CLOSTRIDIUM DIFFICILE SHOWING THREE COMPARABLE REGIONS OF DARK STAINING AND THE SECOND IS IN THE PROCESS OF DIVISION x 24000

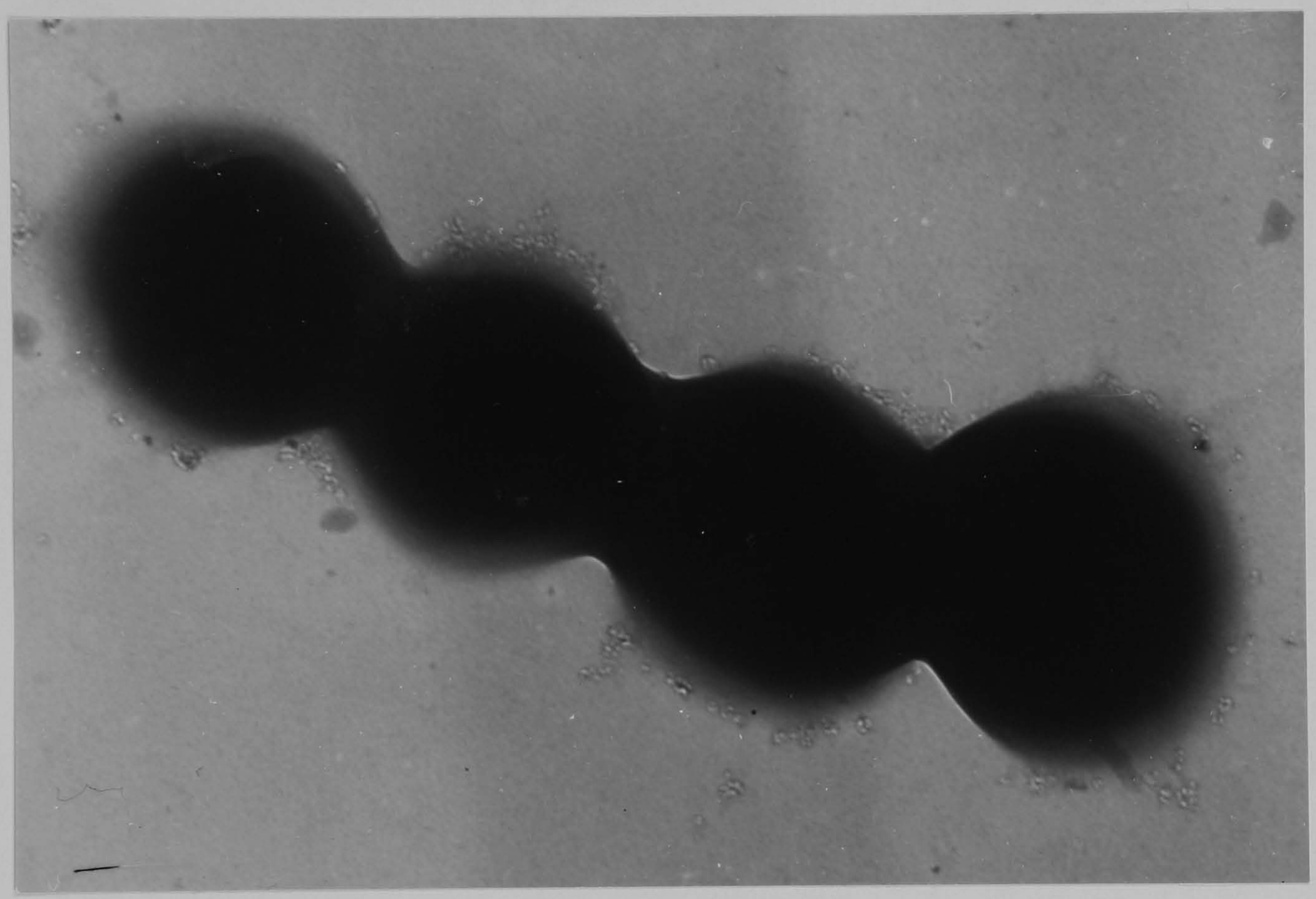


PHOTOGRAPH 13, ELECTRON MICROGRAPH OF THE CELL SHOWING FOUR COMPARABLE REGIONS OF DARK STAINING MATERIAL x 24,000

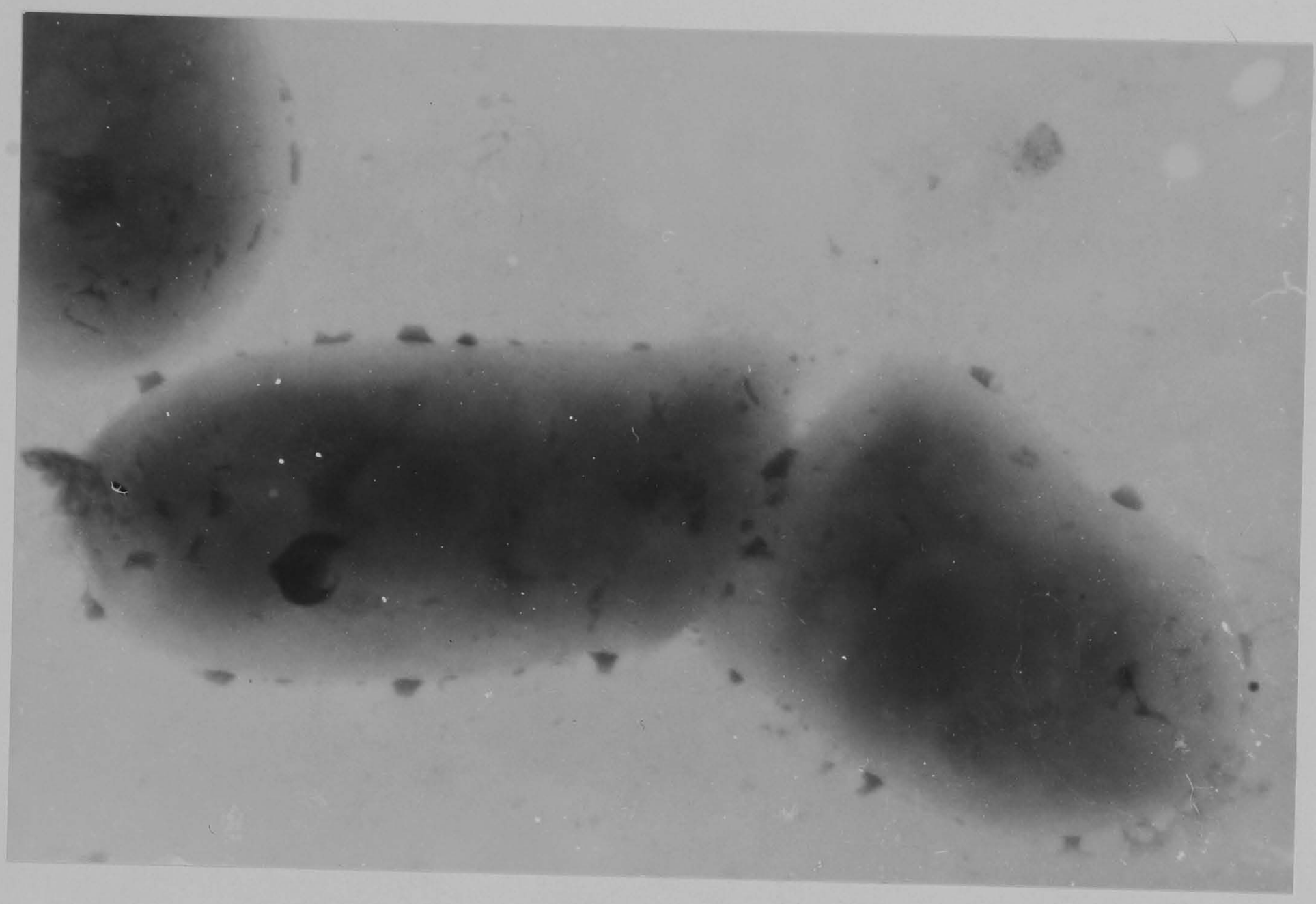


PHOTOGRAPH 14. ELECTRON MICROGRAPH OF THE CELL

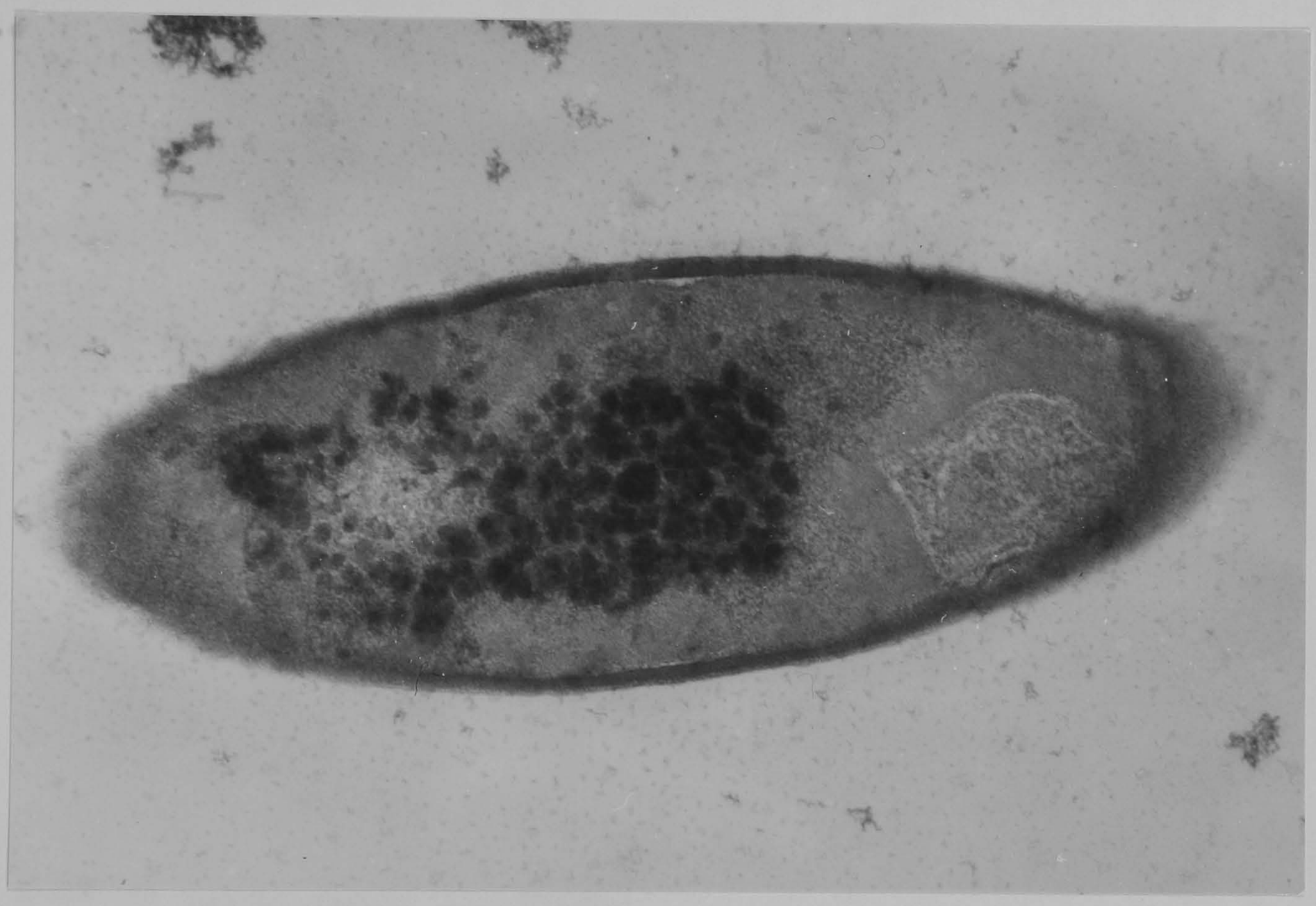
SHOWING CONSTRICTIONS INTO FOUR x 20,000



PHOTOGRAPH 15. ELECTRON MICROGRAPH OF THE DAUGHTER CELLS SEPARATING FROM THE PARENT CELL x 24,000



PHOTOGRAPH 16. ELECTRON MICROGRAPH OF THE SECTION OF CLOSTRIDIUM DIFFICILE SHOWING THE DNA x 32,000



# CHAPTER VII

## DISCUSSION

The work of Hall and O'Toole in 1935 was significant in two ways. Firstly, for the fact that to them must be attributed the first isolation, and therefore, the effective discovery of bacterium Clostridium difficile. Secondly however, and no less significantly, for the fact that these researchers, having applied a name to their discovery which implied and suggested the problems they had faced in isolating it, their reports of their work, do not disclose the methods of isolation used. We are left merely with the fact that they had discovered the organism in newborn children, and there the matter has rested for nigh unto 40 years.

It was believed that the organisms discovered was of a very slow growing nature, difficult to isolate, and inevitably overshadowed by other organisms present with it. It is perhaps for these reasons that little work has so far been done on Clostridium difficile.

In engaging myself upon discussion of the work and study course which I have conducted, I find it convenient to discuss my findings under three basic headings in the following terms :-

### The Isolation of Clostridium difficile

From work done by Professor S.D.Elsden and Dr. Meade, it had been suggested to Professor C.L.Oakley that the organisms with which I was working, purely by coincidence, and from a line of work entirely different from that upon which they were engaged, that Clostridium difficile produced paracresol.

From these tentative thoughts, I was prompted to investigate the degree to which Clostridium difficile would resist paracresol. Finding by experiments that this microorganism resisted paracresol up to a concentration of 0.4 per cent in liquid media, I felt it appropriate to investigate in parallel the degree of resistance to ortho-cresol, meta-cresol, and indeod phenol; in each case, I found an identical degree of tolerance. I thus found myself in a position similar to that of a gardener faced with a seed which he had been told would be difficult to grow, who instead of trying varying degrees and mixture of compost and vegetable matter, had attempted to grow his seed in gravel and found it to flourish in this inhospitable medium. This unorthodox line of approach has demonstrated quite clearly that Clostridium difficile is not as its name suggests difficult to isolate.

The essential problem in growing the culture in normal media rests on the fact that Clostridium difficile is an exceptionally slow-growing organism and in such media is normally rapidly outgrown by other organisms so that it perishes. It is therefore only by growing it in the effectively sterile conditions indicated by the use of cresols or phenol, that Clostridium difficile can be isolated and can flourish albeit slowly, in conditions free from the contamination of other organisms.

The Growth Pattern of Clostridium difficile

The typical growth pattern of the organisms in liquid medium (RCM), is of a highly unusual character, indeed, there is no

recorded evidence of any other organism growing in a similar pattern. The peculiar characteristics of this growth are clearly shown in the photographic illustrations to my thesis. The pattern of supernatant formation, and the characteristics of that supernatant seem to demonstrate that, as Clostridium difficile grows, it produces in its growth, a substance which, of itself, inhibits the growth of other organisms. I am strongly inclined to the view that, that substance is indeed paracresol as suggested from Elsden and Meade's work in a different field.

At this point, I would mention one further controversial issue left to us by way of dispute between Hall and O'Toole on the one hand, and Snyder on the other, relative to the ability of the organisms to liquefy gelatin, Hall and O'Toole maintained that Clostridium difficile did 'NOT' liquefy gelatin, whilst Snyder was of the opinion that after an incubation period of 3 weeks, gelatin would and could be liquefied. Of these two contrary views, my work demonstrates that Snyder's view was the correct one, and the organisms do liquefy gelatin. My own test was done by Frazier's method, but I found that the process of gelatin liquefaction could be sharply accelerated by incubating the organisms in an atmosphere of hydrogen plus 5 per cent carbondioxide.

The Exotoxins

In experiments with toxins, I have found that filtrates of Clostridium difficile injected intracutaneously in the guinea - pig caused the death of the animal. This notwithstanding the

fact that the reactions produced in the skin of the guinea-pigs did not of itself suggest adequate reasons for the death.

My experiments on this subject have confirmed the earlier findings of Oakley.

Snyder's findings, to the effect that the toxins are neutralized by a single antiserum have also been confirmed. The strains resemble each other to a marked extent in their morphological and other reactions. The organisms of Clostridium difficile produce hyaluronidase and the serum against one strain neutralizes the hyaluronidase activity of the others in proportion to its antihyaluronidase content.

There was little written work on the subject of toxins, to be found, apart from a reference to the fact that the organisms produce exotoxins, my experiments have demonstrated that the organisms also have the ability to haemagglutinate and haemolyse R.B.C. and both these activities are separable by dialysis.

The toxin, when concentrated by ammonium sulphate produced two distinct precipitates, and the degree of lethality varied. One precipitate was lethal to mice, whilst the other proved non-lethal. Both precipitates produced antibodies when injected into rabbits, and the antibodies thus produced, neutralized the toxins, whilst at the same time destroying their agglutinating ability. Obviously ammonium sulphate precipitation concentrates toxins considerably and eliminates a good deal of non-toxic material.

It is unusual for an antitoxin to be produced as a result

of intravenously injecting a toxin, and moreover, the sera produced was found to possess almost all the properties of antibody.

In experiments on pregnant rabbits, it was found that intravenous injections of the toxin, about 7 days before birth produced an abortion, and similar treatment 3 days before the expected date of birth, led to stillbirth. My experiments along these lines have been inadequate to produce positive conclusions along these lines, but there seems to be the clear indications that the toxins have a part to play in creating either abortion or stillbirth, which in turn suggests some relationship to the presence of organisms in newborn babies, and may indeed be a contributory factor in some cases of premature or stillborn birth. This however is obviously a matter for lengthy and much more complex investigations.

Electron microscopy has revealed some interesting facts in support of suggestions by Bisset. Bisset's work was related to the cytological structure of a group of Escherichia coli and his theory as to the pattern of cell division were illustrated by him in diagramatic form.

During the course of my work on Clostridium difficile, I have been able to obtain actual micrographs which clearly show the pattern of bacterial cell division, and demonstrate beyond doubt that, as Bisset had suggested, the organisms divides into 4 daughter cell by the process of nuclear division and transference of the nuclei.

## SUMMARY AND CONCLUSION

I received three strains of Clostridium difficile from the department and three strains were received from ATCC. The organisms were labelled as strain N1, N2, N3, N4, N5, and N6 and most of the study was centred upon these six strain.

26 strains were isolated by me, 8 from the faeces of 25 newborn babies (32 per cent cases) under the age of 10 days, 1 from a rabbit which died from the injection of one of the strains of Clostridium difficile, 1 from an abscess of the thumb, 6 from soil and sand, 6 from animal excreta, and 4 from hay. All these strains were studied to some extent and have formed the basis for a revised description of this organism.

Clostridium difficile is widely distributed in nature, is Gram positive, heavily bodied, measuring about 2.5 - 5.9 μ by 0.3 - 1.5 μ , strictly anaerobic spore forming rods; the spores are subterminal; the cell wall is round at both ends; the organism is feebly motile and ferments glucose, mannose, xylose, fructose, mannitol, salicin, with the production of acid and gas; and sucrose, maltose, and soluble starch with the production of acid but no gas. Clostridium difficile will liquefy gelatin in 3 weeks and reduce nitrates, but casein is not affected by it. The organism is toxic to mice, guinea-pigs and rabbits, and produces haemolysin and hyaluronidase and haemagglutinate R.B.C.; it does not produce deoxyribonuclease. The organisms are resistant to cresols and phenols, and can be grown in the presence of 0.15 - 0.2 per cent of these substances. Strains that were isolated without growth in phenol or cresols

are resistant to these substances.

The organisms growth can be compared to that of a corn field in liquid medium. The growth commences as a 'tear drop' projecting and gradually extending downwards as if the stems of growth are held up by air bubbles.

Effective antisera can be produced against the toxin and the organisms. The antiserum produced against one strain gives protection against the toxins of other strains. Also the sera of the strain have the ability to neutralize haemolysin and hyaluronidase produced by other strains. The haemolytic activity of the toxin varies from strain to strain but it is usually within the limits of 8 and 32. The antihaemolysin produced has value that varies with the filtrate against which it is tested, suggesting that more than one type of haemolysin i is present. The organisms seem to be quite consistent in most of their characters.

The toxin production is at its peak when the culture is 72 hours old; and is detectable thereafter for up to 7 days of incubation. The toxin can be concentrated by ammonium sulphate precipitation.

Electron microscopy suggests that the organisms divide by cell division and 4 daughter cells are formed from one parent cell.

It is however my essential hope that the value of my work will be found to lie in what I hope to have demonstrated as being a foolproof method of isolating and growing the organisms of Clostridium difficile, and because of the unusual media in

in which this organism can be cultured, I would hope that this method may open up many avenues of experimentation in an era in which "difficile" has now become "facile".

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