THE AETIOLOGY OF ACUTE NON-BACTERIAL ENTERITIS

IN INFANTS AND YOUNG CHILDREN

by

GEOFFREY P. DAVIDSON, M.B., B.S. (Adel.), M.R.A.C.P.

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THE AETIOLOGY OF ACUTE NON-BACTERIAL ENTERITIS IN INFANTS AND YOUNG CHILDREN

SUMMARY

Acute enteritis is a disease of global importance and one of the commonest causes of childhood illness throughout the world. Most published surveys of acute sporadic enteritis in children conclude that no known aetiological agent can be identified in more than 20% of cases. The assumption has always been that the majority of these cases are due to an infectious agent, presumably a virus, but before this study was carried out no such agent had been identified.

In 1973 a light microscope study had revealed histological damage and disaccharidase depression in duodenal mucosal specimens of a majority of children with acute enteritis, in the absence of any recognizable bacterial or viral pathogen. Therefore initially I decided to study similar specimens using electron microscopy in addition to more routine techniques.

Electron microscopy of duodenal mucosa revealed morphologically similar virus particles of a type previously unrecognized in human disease in duodenal epithelial cells from 13 out of 20 children. No virus particles were observed in duodenal mucosa from normal children or mucosa obtained from the same patients after clinical recovery.
Indirect immunofluorescent techniques were used to help define localization of viral antigen. They also provided some evidence that virus particles from different patients appeared to share a common antigen.

The technique of differential centrifugation of faecal extracts was then developed to reveal the same virus particles in diarrhoeal faeces and the results obtained by electron microscopy of duodenal epithelial cells and faecal extracts were compared. This technique provided a relatively simple, sensitive and rapid method for locating the virus in large numbers of children and a year long survey to investigate the prevalence of this new virus in children with acute enteritis admitted to one hospital was undertaken. The survey conclusively showed this new virus to be the major aetiological agent of acute sporadic enteritis in infants and young children in Melbourne.

Immunological studies revealed an increased incidence of selective IgA deficiency in children admitted to hospital with acute enteritis compared with the normal population.

The study has clearly shown that this newly described virus (the "duovirus") was the major cause of acute enteritis in children in Melbourne.
STATEMENT OF AUTHENTICITY

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university, and to the best of my knowledge and belief, the thesis contains no material previously published, except when due reference is made in the text of the thesis.

GEOFFREY P. DAVIDSON
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The work reported in this thesis was carried out in the Department of Gastroenterology, Royal Children's Hospital, Melbourne over a two year period during the tenure of the Royal Children's Hospital Trainee Research Fellowship for 1973 and the Lady Latham Fellowship for 1974.

I am most grateful to Dr. Ruth Bishop who proposed this study and who performed the bacteriological studies. Without her assistance and advice the project would have been impossible.

It is a pleasure to thank Dr. R. R. W. Townley in whose Department this study was carried out. He provided much helpful criticism and guidance.

The author wishes to acknowledge the following collaborators for their part in the research.

Dr. I. H. Holmes, Department of Microbiology, University of Melbourne, who provided the electron microscope and virological expertise.

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Dr. C. S. Hosking, Department of Immunology, Royal Children's Hospital, Melbourne, for advice and suggestions regarding the immunological investigations.

Mr. I. Goller of the Department of Pathology, Royal Children's Hospital, for his valuable guidance with the immunofluorescent study.

I also wish to thank Mrs. R. Kay who prepared the histological sections and Mr. M. Murray who performed disaccharidase assays.

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I am finally most grateful to my wife Marion for her understanding during the preparation of this thesis and for typing the final manuscript.
CHAPTER I

AIM OF RESEARCH

OUTLINE OF THESIS

LITERATURE REVIEW
AIM OF RESEARCH:

From my review of the literature it was apparent that much time and effort had been expended looking for an aetiologica} agent in acute infectious non-bacterial gastroenteritis with little reward.

In temperate climates in particular a specific aetiologica} agent is often not identified but the epidemiology and clinical course of the disease strongly support infection as the cause.

Several earlier histopathological studies of the small intestine in children with acute enteritis had not been helpful in elucidating an aetiologica} agent. Toccalino et al (1972) demonstrated histological damage in the duodenum in 14 of 16 children with acute infectious non-bacterial gastroenteritis. More recently Barnes and Townley (1973) working at the Royal Children's Hospital in Melbourne confirmed that the duodenum was the major site of damage in 31 children with acute gastroenteritis. These studies suggested the disease should be more properly termed acute enteritis as the small intestine is the major site of damage.

With this background knowledge I decided to study prospectively duodenal tissue from infants and young children admitted to hospital with acute enteritis using the electron
microscope. This relatively new investigative tool had not previously been used to study acute enteritis in children. I also intended to study the biopsy material using accepted histological and biochemical methods in order to more fully evaluate this disease.

I also wanted to study several parameters of immune function in children with acute enteritis by measuring serum and secretory immunoglobulins. No previous studies of this nature have been performed in children with acute enteritis.

In order to make the assessment as complete as possible duodenal juice and faecal material were collected at the time of the biopsy and assessed bacteriologically and virologically. Portions of these samples were to be stored until a suitable method could be devised to allow for examination with the electron microscope.

I hoped that this study would provide further information on the aetiology of acute infectious non-bacterial enteritis in children and also some basic information on the immune status of children who acquire this disease.
OUTLINE OF THESIS:

Chapter I of this thesis reviews briefly the known bacterial pathogens in acute enteritis and then goes on to discuss in some detail the present situation regarding the aetiology of acute infectious non-bacterial enteritis.

Chapters II to V comprise the experimental work. Chapter II outlines my initial project in which I hoped to find an aetiological agent by using the electron microscope to study duodenal tissue at the height of the illness. Apart from the actual electron microscope examination of duodenal tissue, which was performed by Dr. I. Holmes and Mr. B. Ruck, the rest of the work was carried out by me.

The results obtained from this initial study led me to the preparation of a method for detecting the newly found virus in stools. This is the basis of Chapter III. Again Dr. Holmes performed the electron microscopy and Dr. Bishop carried out bacteriological examination of the duodenal juice. Having developed this much simpler method for detecting the new virus it was important to establish the place of the virus in relation to other known enteric pathogens. In order to do this, I studied prospectively all children with acute enteritis admitted to the Royal Children's Hospital, Melbourne, over a 12 month period and a suitable control group. I collected clinical information, stool
samples and acute and convalescent sera from these children. The results of this study are outlined in Chapter IV. The electron microscopy of faeces was performed by Mr. B. Ruck and Miss A. Veenstra.

In Chapter V, I have reported the immunological findings from the 20 children studied initially. Clinical material was obtained by me and Dr. C. S. Hosking performed the immunoglobulin analyses. The findings suggest that a child with selective IgA deficiency may develop a more severe illness when exposed to enteric pathogens.

These experiments have disclosed a new virus which proved to be the most important aetiological agent in Melbourne during the period of this study.

The name of this new virus is not yet decided. There have been several alternative names proposed since our original finding. These include reovirus-like agent, orbivirus and "rotavirus." We have proposed the name "duovirus" to describe the characteristic double-shelled capsid structure of the virus and to emphasize its involvement in enteric infection. To avoid confusion and wordiness this is the name that I have used throughout my thesis.
Throughout the world acute infectious non-bacterial enteritis is the commonest infectious disease in which an aetiological agent remains to be identified. Diarrhoeal diseases have a disproportionate effect on the countries that can afford them the least. Either directly or through interaction with malnutrition they are the largest single cause of death and loss of productivity. In developing nations a child has a 50% chance of dying before the age of seven years, primarily from diarrhoeal disease (Kretchmer, 1969). In India alone, 1.4 million children a year die from diarrhoeal diseases and this figure excludes deaths due to cholera (Leading article, 1975).

In developed countries acute enteritis causes significant morbidity and unnecessary mortality in infancy. Every year in Britain about 400 babies under the age of one, die with acute enteritis. Ten thousand require hospital admission and some 90,000 are treated at home (Kuzemko, 1969). In Melbourne during 1972, 539 children were admitted to the Royal Children's Hospital with acute enteritis. It was the primary cause of death in six children.

Many surveys of the aetiology of acute diarrhoeal disease in both developed and underdeveloped countries conclude that a specific pathogen cannot be identified in as many as 80% of patients (Sabin, 1963). In Melbourne during 1972, a recognized enteric pathogen was isolated in only 12% of patients.
The assumption has been made that viral agents are responsible for the majority of cases of acute enteritis in infancy and early childhood. However, proof of the association of viruses with acute enteritis has been difficult to establish.

I would briefly like to review the history of bacterial species known to cause acute enteritis and then to discuss in more detail the aetiology of acute non-bacterial enteritis.

**Bacterial Aetiology of Sporadic Acute Enteritis**

Since 1955 bacterial pathogens have not been recovered from more than 20% of infants under two years of age with diarrhoea in any study conducted in North America and Europe (Cramblett et al, 1971).

Bacteria which have been accepted as causing acute enteritis in children and adults include *Salmonella* sp., *Shigella* sp., enteropathogenic *E. coli*, *Vibrio cholerae*, *Clostridium perfringens* and *Staphylococcus* sp. (Grady and Keusch, 1971).

**Salmonella**

Since the isolation of the first member of the genus *Salmonella* from swine in 1885 by Salmon and Smith, more than 1,200 different serologic types have been identified (Prost and Riemann, 1967). Although there is a striking variation
in the pathogenicity of the various serotypes, almost all are capable of producing human disease. The most prevalent strain capable of producing acute enteritis in humans is *Salmonella typhimurium*.

**Shigella**

Dysentery was first linked with *Shigella sp.* in 1888 by Chantemesse and Widal but the more complete description of the organism by Shiga during a massive epidemic in Japan in 1898 caused his name to become firmly associated with this genus. *S. sonnei* is the species most frequently implicated in human disease (Grady and Keusch, 1971).

**Escherichia Coli**

*E. coli* had long been recognized as a normal bowel inhabitant being found in the faeces in 96% of normal people (Broido et al, 1972). The original concept that *E. coli* may cause disease came from studies by Jensen in 1892 of a natural diarrhoeal disorder ("scours") of newborn calves (Gordon et al, 1963). Smith and Orcutt (1925) associated the condition with special strains of colon bacilli identified bacteriologically and serologically. An aetiological relationship in man was first shown by Adam in 1923.
In England in 1945, Bray demonstrated "enteropathogenic" E. coli in 88% of patients from an epidemic of infantile diarrhoea and in 3% of controls. The development of serological classification at the same time by Kauffmann (1947) showed the outbreak described by Bray to be due to 0111:B4. In 1947, another serotype (055:B5) was responsible for an outbreak in infants in Scotland (Giles et al, 1949).

Well controlled challenge studies in adult volunteers using E. coli serotypes 0111:B4 and 055:B5 (Ferguson and June, 1952; June et al, 1952) and in an infant using the 0111:B4 serotype (Neter and Shumway, 1950) confirmed that this organism could cause diarrhoeal disease in humans. In 1967 Sakazaki and co-workers expanded the recognized serogroups to 22 and also demonstrated two distinct patterns of illness, one shigella-like and the other salmonella-like.

Acute enteritis due to pathogenic strains of E. coli is predominantly a disease of children under the age of two years (Ramsay, 1968) and has occurred mainly in nursery epidemics. However, the frequency of the asymptomatic carrier state of serotypically pathogenic E. coli makes it impossible to attach causal significance to the isolation of strains of this organism in sporadic episodes of diarrhoea (Solomon et al, 1961). Also, the more recent recognition of the poor correlation between serotypic enteropathogenicity and true enterotoxicity places even more doubt on attaching any significance to the isolation of an "enteropathogenic" serotype (Gorbach and Khurana, 1972).
Staphylococcus

Sterilized culture filtrates of a haemolytic Staphylococcus aureus were first shown in 1930 to be capable of causing a dose related enteritis in three volunteers (Dack et al, 1930). It has first been implicated as a cause of diarrhoea by Barber in 1914 (Grady and Keusch, 1971).

V. cholerae

After identification and culture of the vibrio during epidemics in Egypt and Calcutta in 1864, Robert Koch suggested that toxic material elaborated by this organism was the cause of the disease cholera. German bacteriologists, Pettenkofer and Emmerich doubted the association of V. cholerae with disease and they publicly drank a culture of Koch's bacillus. This nearly killed one and made the other quite uncomfortable (Topley and Wilson, 1964). Later Koch and Metchnikof, working independently were able to induce a similar diarrhoeal illness in the guinea pig and the suckling rabbit respectively (Banwell and Sherr, 1973). However, a further 75 years was to pass before it was demonstrated that cell free products from the vibrio would cause consistent fluid production in ligated rabbit ileal segments (De and Chatterjee, 1953) and severe diarrhoea when administered directly into the rabbit stomach (Dutta and Habbu, 1955).
**Clostridium perfringens**

This organism was first implicated as a cause of diarrhoea and cramps without fever or vomiting in the late 1890's. The reports remained controversial until Koch's postulates were fulfilled by the production of experimental disease in man (Hobbs et al, 1953). 

Cl.perfringens is important in food-borne diarrhoea (Centre for Disease Control, Georgia, 1970) and in "pig-bel" in the highlands of New Guinea (Murrell et al, 1966). It is not so far accepted as a cause of acute enteritis in infancy and childhood in Australia.

**Aetiology of Acute Infectious Non-Bacterial Enteritis**

**Historical Background**

The term 'acute infectious non-bacterial enteritis' was initially suggested in 1956 (Dingle et al, 1956) to denote a disease of uncertain cause, encompassing a number of syndromes. These included viral diarrhoea, epidemic diarrhoea and vomiting, winter vomiting disease, and epidemic nausea and vomiting.

The disease is self-limited, lasting 24-48 hours and consists of combinations of diarrhoea, nausea, vomiting, low grade fever, abdominal cramps, headaches and malaise.
The earliest report of an epidemic of diarrhoea in a nursery for newborn infants was that of Dick et al in 1928. Eighty-eight infants were affected and 27 died. The disease was characterized clinically by sudden onset of diarrhoea and weight loss. Regurgitation of feeding was frequent. Fever or subnormal temperature was usually recorded at some time during the course of the illness. Stools were watery without blood or mucus. No organisms recognized as being responsible for diarrhoea in infants was found until Morgan's bacillus (Proteus morgagnii) was recovered from stools of the last five fatal cases. It is doubtful that this was the aetiologic agent.

In 1931 McLean speculated on the viral origin of winter epidemics of vomiting and abdominal pain among children in the United States, but at this early stage no proof of viral aetiology was forthcoming.

Boardman in 1938 had noted that acute infectious gastroenteritis in California was occurring more frequently, both sporadically and in epidemics, and that in many cases no aetiological agent could be found. He very astutely suggested that there was an acute infectious gastroenteritis probably due to a filtrable virus, carried by the secretions of the upper respiratory tract. This was the first time acute gastroenteritis had been ascribed to a viral agent.
The development by Enders in 1949 of relatively simple and effective methods for isolation of viruses from faeces led to the isolation of a great number of viruses that are cytopathogenic in tissue culture. Many viruses, however, are harboured in healthy persons (Ramos-Alvarez and Sabin, 1956; Kalser et al, 1966) and this makes the significance of a number of the earlier poorly controlled studies very dubious.

The causal relation of a virus to infection must be proved by its increased incidence in patients during an epidemic as compared with its incidence in healthy persons at the time, but particularly by the demonstration of an increase in antibody titre to that virus during convalescence, or by reproduction of the disease in volunteers. The most convincing evidence of viral origin comes from the study of outbreaks or epidemics of acute diarrhoeal illness in which the majority of patients excreted virus and a majority of well children did not.

An important early study linking viruses aetiologically with acute enteritis was reported in 1958 from Cincinatti (Ramos-Alvarez and Sabin, 1958). Using cell culture techniques, Ramos-Alvarez and Sabin were able to demonstrate for the first time an eight-times greater viral excretion rate in children with summer diarrhoea than in control patients.
Since this study, much evidence has accumulated linking viruses with a significant number of cases of acute enteritis occurring either in epidemics or sporadically. The viruses most commonly isolated from stool culture are enteroviruses and adenoviruses.

**Enteroviruses**

Coxsackie viruses, ECHO viruses and polioviruses of the enterovirus group, have all been associated with diarrhoea. Studies of both epidemic and sporadic cases implicate more specifically the ECHO viruses. Sommerville (1958) noted a prevalence rate of 21% of enteroviruses among 223 children under five years of age suffering from diarrhoeal disease, while the comparable rate among 115 "control" children with respiratory disease was 15%. The rate of Coxsackie virus and poliovirus isolations from the two groups were essentially the same; ECHO virus isolations occurred more frequently among the cases of diarrhoeal disease as compared to those suffering with respiratory disease (5.4 percent and 2.5 percent respectively), but the numbers were too small to yield a statistically significant difference. In contrast to this, a large study of summer diarrhoea in white children in South Africa failed to reveal any aetiologic associations between enteric viruses and diarrhoeal disease (Koornhof et al, 1964). This was also the experience of Yow and her associates (1970) from a detailed study of diarrhoeal disease in Houston from 1964 to 1967. Viral agents were isolated from 27% of study patients and 19% of control subjects.
In several instances of small epidemics of diarrhoeal disease in young children and adults, aetiologic and epidemiologic investigations have incriminated one or more members of the ECHO virus group as the causative agents. The best documented was an epidemic of diarrhoea occurring in two nurseries for premature infants which was due to ECHO virus 18 (Eichenwald et al., 1958). In both nurseries, the agent was isolated only from sick infants and only those infants who had symptoms of diarrhoeal disease developed antibodies against ECHO virus 18 during the course of their illness.

ECHO virus 11 was isolated from rectal swabs from two of three cases of acute enteritis which occurred in rapid sequence among a group of closely associated laboratory workers (Klein et al., 1960). One of these patients showed a definite increase in the level of antibody against ECHO virus 11. An outbreak of diarrhoea in newborn infants and mothers has also been attributed to ECHO virus 11 (Berkovich and Kibrick, 1964).

ECHO virus 19 has also been reported to cause diarrhoeal illness after accidental ingestion, but has yet to be found as a cause of outbreaks or epidemics (Cramblett et al., 1962).
ECHO virus 14 was isolated from a nursery outbreak from three infants who had bloody diarrhoea and from 20 asymptomatic contacts (Lepine et al, 1960). Two of the three affected infants showed a significant increase in antibody titre against ECHO virus 14 during convalescence, but there was no increase in antibody titre in the 20 asymptomatic contacts.

Poliovirus type 2 (Giovanardi and Gergamini, 1963), Coxsackie B3 (Felici et al, 1962) and Coxsackie B5 (Farmer and Patten, 1968) have been implicated in the causation of small epidemics of diarrhoeal disease, both in children and adults.

Adenoviruses

The association of diarrhoea with adenovirus infections was first made through a study of pharyngoconjunctival fever, commonly caused by adenovirus types 3 or 7 (Kendall et al, 1957). In a study of acute enteritis in Mexico City, Ramos-Alvarez and Olarte (1964) found a 14-times higher incidence of adenovirus excretion in children with diarrhoea than in their controls. The commonest were types 1, 4 and 7. Similarly, in a study of 167 severely ill infants with gastroenteritis under two years of age in Chicago (Moffet et al, 1968) adenoviruses were isolated from 17% of affected infants compared with 5% of 95 normal infants.
Calculations of the overall incidence of virus isolations from patients and matched controls on a perennial basis vary greatly from study to study. Some of the factors responsible for this are the seasonal rise and fall in prevalence of enteroviruses, patient selection, specimen collection techniques, and the methods used in viral isolations. Cramblett et al (1971) in a survey of all perennial studies of acute enteritis that have been carried out in the United States and Western Europe since 1955, calculated that the mean isolation rate of viruses from stools of patients was 14%, whereas in matched controls the incidence was 10.5%. In some of the individual studies summarized, however, the isolation rates from sick patients exceeded that of controls in a ratio as high as 5:1. He also found from the survey that 65% of all cases remained unexplained bacteriologically and virologically. Cramblett summed up very clearly the views at the time when he stated that unless potentially new pathogens were discovered or remarkably new techniques became available, additional studies of the nature outlined in his survey were unlikely to be fruitful in elucidating the cause of the majority of cases of acute infectious non-bacterial enteritis.
Transmission Experiments

Despite the failure to isolate viral agents from many patients with acute infectious non-bacterial enteritis it was strongly suspected that unknown viruses were the cause of the infection in most patients. Evidence supporting this suspicion was indirect and came mainly from transmission experiments in man and animals.

Animal Studies

In the early 1940's, various groups of investigators attempted to produce diarrhoea in experimental animals by exposure to bacteria-free filtrates of stool suspensions from patients with diarrhoea. Light and Hodes (1943) reported the first apparently successful transmission of a filtrable virus from stools obtained from infants in four separate epidemics of diarrhoea of the newborn in Baltimore and Washington. Calves were injected intranasally with Seitz filtrates of the diarrhoea stools. A bloody mucoid diarrhoea followed two to five days later. Successive calf passage was readily accomplished with both filtered and unfiltered material. When convalescent serum from six babies from one epidemic was pre-incubated with stool filtrates complete or partial protection against intranasal infection of calves occurred. The demonstration of similar findings using Seitz filtered diarrhoeal stools from an epidemic of diarrhoea in the newborn in Michigan confirmed the work of Light and Hodes (Cummings, 1947).
A disease of infants and young children characterized by stomatitis and diarrhoea was described in 1944 (Buddingh and Dodd, 1944). A filtrable agent was isolated from the mouth washings and stools of sick infants that caused an inflammatory reaction when placed upon scarified cornea of rabbits. Serial passage with rabbit eyes was readily obtained. However, some control material caused the same type of reaction as that from sick infants. Also the agent failed to cause diarrhoea in animals. These facts cast some doubt upon the significance of this virus.

Hodes (1956) in an article on viral diarrhoea quoted work performed in 1948 by Verlinde in which he isolated virus from stools of young infants and showed that this agent was pathogenic for young ferrets. Whilst this work has not been confirmed, Hodes felt it was a convincing study.

**Human Volunteer Studies**

About the same time, human volunteer experiments with similar bacteria-free stool filtrates were carried out. However, results from these studies were inconsistent.

In 1945, a gastroenteritis attack rate of 53% was reported among volunteers who had inhaled filtrates of garglings or stools from diarrhoea patients, as compared with an incidence of 9% of naturally occurring gastro-enteritis in a matched control group (Reimann et al, 1945).
In another study conducted in New York (Gordon et al, 1947) an afebrile diarrhoeal disease could be transmitted to volunteers by oral administration of bacteria-free faecal filtrates or throat washings or ill patients. The infectious agent was called the Marcy strain. Seven serial human passages of this disease left little doubt that this agent multiplied in the human host and therefore was unlikely to be a filtrable toxin. Two Japanese studies reported the production of diarrhoeal disease in 50% of human volunteers by ingestion of stool filtrates from human cases (Yamamoto et al, 1948; Kojima et al, 1953). One of these workers was able to produce diarrhoeal disease in cats fed filtered suspension of stool from human cases (Yamamoto et al, 1948).

In 1953, Jordon transmitted a febrile gastrointestinal illness by the oral administration of bacteria-free faecal supernates obtained from an ill patient. He called this agent the FS strain. Transmission was not obtained with respiratory tract secretions. He also demonstrated absence of cross immunity between the FS strain and the previously described Marcy strain.

In summary then, these animal experiments and human volunteer studies indicated that agents of sub-bacterial size, presumably viruses, were most likely responsible for the many outbreaks of acute infectious non-bacterial enteritis. Extensive studies for recognized bacterial protozoal and helminthic pathogens had yielded negative
results. Before further advances could be made, better methods for identification and characterization of these viruses would need to be developed.

Recent Evidence for an Infectious Agent in Acute Non-Bacterial Enteritis

With the development of several newer techniques, a number of workers were encouraged to reopen investigation into the aetiology of acute infectious non-bacterial enteritis after a break of nearly 15 years. These new techniques included better methods for the detection of viruses using the electron microscope and newer immunological methods. Newer culture methods using foetal intestinal organ culture were also developed. A further advance had been the development in 1957 of the small intestinal biopsy capsule (Crosby and Kugler, 1957) which allowed closer histopathological study of the small intestine in acute enteritis.

In 1968 in Norwalk, Ohio, an outbreak of winter vomiting disease occurred (Adler and Zickl, 1969). The symptoms included nausea, vomiting, less frequently diarrhoea and a low grade fever. About 50% of students in an elementary school became ill. The secondary attack rate was 32% and an estimated incubation period of 48 hours was noted. The disease lasted 24 hours and remitted spontaneously.
Laboratory and volunteer studies were set up to study this disease which seemed to have an infectious aetiology. Stool specimens were negative for bacterial and viral agents. An inoculum was prepared from the diarrhoeal stool of an adult secondary case. Three serial passages in human volunteers were accomplished with this agent and the attack rate was about 67%. The same inoculum produced variable clinical symptoms in volunteers and it was felt that this represented the varied clinical manifestations of a single agent (Dolin et al, 1971).

The Norwalk agent did not replicate in tissue culture or foetal intestinal organ cultures (Blacklow et al, 1972). The agent was of extremely small size, less than 66 nm in diameter and probably less than 36 nm. This was determined by passage through filters of known pore size. Ether treatment of filtrates did not impair infectivity, suggesting lack of a lipid coat. It was relatively heat stable and resistant to acid treatment (Dolin et al, 1972). Short term homologous immunity was also demonstrated by repeated challenges to previously ill volunteers (Blacklow et al, 1972). Transient mal-absorption of D-xylose and lactose indicating transient enzyme deficiency and steatorrhoea were noted in some volunteers infected with the Norwalk agent. Some of these observations were also made in otherwise asymptomatic individuals (Agus et al, 1973).
By applying the recently developed technique of immune electron microscopy, Kapikian et al (1972) demonstrated aggregation of viral bodies from faecal material after incubation in convalescent serum from patients who had been infected with the Norwalk agent.

Summary

At the time of starting this study, it was apparent from the preceding literature review that very little was known about the aetiology of acute infectious non-bacterial enteritis in childhood.

In adults, a possible viral agent (the Norwalk agent) had been found in several epidemics of acute infectious non-bacterial enteritis. This agent had been shown to be of sub-bacterial size, to be capable of damaging the small intestinal mucosa and to be associated with typical signs and symptoms of acute infectious non-bacterial enteritis. The agent had also been visualized by immune electron microscopy in stools from infected patients. However, the extent of its involvement in adults is unknown and to date no studies have looked for this agent in infants or young children.
CHAPTER II

STUDY OF DUODENAL MUCOSA IN 20 CHILDREN

WITH ACUTE ENTERITIS.
INTRODUCTION:

I wanted to use the technique of duodenal biopsy to obtain duodenal tissue from children with acute enteritis and study this tissue by electron microscopy as well as more routine methods. The technique of duodenal biopsy used was developed in this department which now has an extensive experience with its safe use (Townley and Barnes, 1973).

Doubts have been expressed about the safety of this procedure in childhood (Partin and Schubert, 1966) but since the introduction of the present capsule (Watson paediatric intestinal biopsy capsule) into this department, over 2,000 duodenal biopsies have been performed with only one complication. An infant of 14 months developed malena which settled with conservative management.

Several parts of this chapter have already been published (Bishop et al, 1973 a, b; Davidson et al, 1975 c) and here I have outlined my contribution to this work. The electron microscopy was performed by Dr. I. Holmes.
CLINICAL MATERIAL

Patient Selection

The children studied here were selected on the following basis:

(i) They were suffering from acute enteritis which was defined as a febrile illness of less than 10 days duration, associated with diarrhoea and vomiting where there was no other cause for the symptoms.

(ii) Parental consent had to be sought. To do this I spent a considerable amount of time with the parents of each child and explained in detail the intended investigations. In particular I pointed out that this was a research project with the aim of increasing knowledge of the cause of acute enteritis in the hope that it may aid therapy or prevention of the disease. I interviewed the parents of 21 children and in only one case was permission to conduct the investigation refused.

(iii) Only infants who had symptoms for less than five days at the time of study could be included.

(iv) Infants weighing less than 4 kgms. were excluded from the study as experience with duodenal biopsy in such small infants in this department was very limited.
PATIENTS

Twenty children aged from two to 33 months were examined by me. All were admitted to the Royal Children's Hospital, Melbourne, from April to September, 1973, with a diagnosis of acute enteritis.

The major clinical details of the patients are summarized in Table 1. Five children had a history of prior illness, mainly upper respiratory tract infections, one to three weeks before the onset of acute enteritis. One child had an upper respiratory illness at the time of admission. Fifteen infants required intravenous therapy for rehydration. All the patients recovered clinically over a period of four to nine days from onset of symptoms.

The symptoms of acute enteritis were present for varying periods of time prior to admission to hospital. This is shown in Table 2. In the majority of patients symptoms had been present for less than four days prior to admission to hospital.

The age distribution of the patients with acute enteritis is shown in Table 3. It can be seen that 17 of the infants were less than two years of age.
<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age (months)</th>
<th>Prior Illness</th>
<th>Major Symptoms</th>
<th>Temp. (°C)</th>
<th>Degree of Dehydration</th>
<th>IV Therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. (A.J.)* M</td>
<td>31</td>
<td>Upper respiratory tract illness for 2 days.</td>
<td>+</td>
<td>++</td>
<td>38</td>
<td>5%</td>
<td>No</td>
</tr>
<tr>
<td>2. (J.M.)* M</td>
<td>8</td>
<td>Otitis media cleared 1 week previously.</td>
<td>+</td>
<td>++</td>
<td>38.7</td>
<td>5%</td>
<td>Yes</td>
</tr>
<tr>
<td>3. (N.S.) M</td>
<td>4</td>
<td></td>
<td>+</td>
<td>++</td>
<td>38.7</td>
<td>5-10%</td>
<td>Yes</td>
</tr>
<tr>
<td>4. (C.K.) F</td>
<td>9</td>
<td>Upper respiratory tract illness for 1 week previously.</td>
<td>++</td>
<td>+</td>
<td>40</td>
<td>5-10%</td>
<td>Yes</td>
</tr>
<tr>
<td>5. (R.G.) M</td>
<td>5</td>
<td>Older sibling had enteritis 3 days previously.</td>
<td>+</td>
<td>++</td>
<td>38</td>
<td>5%</td>
<td>No</td>
</tr>
<tr>
<td>6. (G.M.) M</td>
<td>7</td>
<td>No</td>
<td>++</td>
<td>+</td>
<td>38</td>
<td>5</td>
<td>Yes</td>
</tr>
<tr>
<td>7. (T.A.) M</td>
<td>30</td>
<td>No</td>
<td>++</td>
<td>+++</td>
<td>38</td>
<td>5-10%</td>
<td>Yes</td>
</tr>
<tr>
<td>8. (D.G.)* M</td>
<td>14</td>
<td>No</td>
<td>0</td>
<td>+++</td>
<td>39</td>
<td>10%</td>
<td>Yes</td>
</tr>
<tr>
<td>9. (T.T.) M</td>
<td>19</td>
<td>No</td>
<td>++</td>
<td>+++</td>
<td>38</td>
<td>5-10%</td>
<td>Yes</td>
</tr>
<tr>
<td>10. (A.D.) M</td>
<td>13</td>
<td>No</td>
<td>+</td>
<td>++</td>
<td>36.4</td>
<td>5-10%</td>
<td>Yes</td>
</tr>
<tr>
<td>11. (A.H.) M</td>
<td>4</td>
<td>No</td>
<td>0</td>
<td>++</td>
<td>38.4</td>
<td>5-10%</td>
<td>Yes</td>
</tr>
<tr>
<td>12. (T.F.)* M</td>
<td>10</td>
<td>No</td>
<td>+</td>
<td>++</td>
<td>37</td>
<td>5-10%</td>
<td>Yes</td>
</tr>
<tr>
<td>13. (T.K.) F</td>
<td>6</td>
<td>Upper respiratory illness cleared 1 week previously.</td>
<td>+</td>
<td>+++</td>
<td>37.9</td>
<td>5-10%</td>
<td>Yes</td>
</tr>
<tr>
<td>14. (P.H.)* M</td>
<td>8</td>
<td>No</td>
<td>+</td>
<td>++</td>
<td>37.4</td>
<td>5-10%</td>
<td>Yes</td>
</tr>
<tr>
<td>15. (C.N.) F</td>
<td>2</td>
<td>No</td>
<td>0</td>
<td>++</td>
<td>38</td>
<td>5%</td>
<td>No</td>
</tr>
<tr>
<td>16. (A.P.)* M</td>
<td>33</td>
<td>No</td>
<td>+</td>
<td>+++</td>
<td>40.6</td>
<td>10%</td>
<td>Yes</td>
</tr>
<tr>
<td>17. (N.F.) F</td>
<td>11</td>
<td>Upper respiratory illness cleared 5 days previously.</td>
<td>+</td>
<td>++</td>
<td>38</td>
<td>5%</td>
<td>No</td>
</tr>
<tr>
<td>18. (K.B.) F</td>
<td>11</td>
<td>No</td>
<td>++</td>
<td>+++</td>
<td>37.9</td>
<td>5-10%</td>
<td>Yes</td>
</tr>
<tr>
<td>19. (M.S.) M</td>
<td>18</td>
<td>Otitis media cleared 2 weeks previously.</td>
<td>++</td>
<td>+++</td>
<td>37.9</td>
<td>5%</td>
<td>No</td>
</tr>
<tr>
<td>20. (L.S.) M</td>
<td>22</td>
<td>No</td>
<td>++</td>
<td>++</td>
<td>39</td>
<td>5-10%</td>
<td>Yes</td>
</tr>
</tbody>
</table>

* Patients in whom repeat biopsy performed.
TABLE 2

Length of Time Patients Symptomatic Prior to Admission to Hospital

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Number of Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>5</td>
</tr>
<tr>
<td>24 - 48</td>
<td>5</td>
</tr>
<tr>
<td>48 - 72</td>
<td>5</td>
</tr>
<tr>
<td>72 - 96</td>
<td>4</td>
</tr>
<tr>
<td>96</td>
<td>1</td>
</tr>
</tbody>
</table>
TABLE 3

**Ages of Patients with Acute Enteritis and of Control Infants**

<table>
<thead>
<tr>
<th>Patients</th>
<th>Age (months)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Under 6</td>
<td>6-12</td>
<td>12-24</td>
<td>Over 24</td>
</tr>
<tr>
<td>20 Patients with Acute Enteritis</td>
<td>4</td>
<td>8</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>4 Control Infants</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>
It was not desirable to subject normal children to duodenal biopsy to obtain duodenal mucosa for electron microscopy. For this purpose, parts of specimens from four infants ranging from six weeks to 30 months who required duodenal biopsy as part of the investigations for their chronic diarrhoea or failure to thrive, were used (Table 4). There was one child in each of the four age groups studied (Table 3).
**TABLE 4**

Diagnosis in Four Control Patients in whom Duodenal Biopsy was Performed

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. (A.W.)</td>
<td>6 weeks</td>
<td>Chronic diarrhoea for investigation.</td>
</tr>
<tr>
<td>2. (M.J.)</td>
<td>10 months</td>
<td>Chronic diarrhoea for investigation.</td>
</tr>
<tr>
<td>3. (A.M.)</td>
<td>15 months</td>
<td>Anaemia. Failure to thrive.</td>
</tr>
<tr>
<td>4. (R.D.)</td>
<td>30 months</td>
<td>Chronic diarrhoea for investigation.</td>
</tr>
</tbody>
</table>
SAMPLE COLLECTION

Nasopharyngeal Mucus

Samples of mucus were collected within 24 hours of admission to hospital, using a sterile nasopharyngeal mucus extractor (Sterimed). These were sent for routine viral culture within half an hour of collection.

Duodenal Specimens

These specimens were collected after rehydration and within 48 hours of admission to hospital. Five percent glucose solution was the only oral feeding given in hospital prior to sampling. Vitamin K (5 mg) was given by intramuscular injection the day before duodenal biopsy. Premedication with metoclopramide syrup (Maxalon) and quinalbarbitone (Seconal) was given 30 to 60 minutes before intubation and duodenal biopsy according to the following schedule -

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>Medication</th>
<th>Dosage</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; 6</td>
<td>No premedication</td>
<td></td>
</tr>
<tr>
<td>6-12</td>
<td>Quinalbarbitone</td>
<td>50-75 mg</td>
</tr>
<tr>
<td></td>
<td>Metoclopramide</td>
<td>2-3 mg</td>
</tr>
<tr>
<td>12-24</td>
<td>Quinalbarbitone</td>
<td>100 mg</td>
</tr>
<tr>
<td></td>
<td>Metoclopramide</td>
<td>3-5 mg</td>
</tr>
<tr>
<td>&lt; 24</td>
<td>Quinalbarbitone</td>
<td>150 mg</td>
</tr>
<tr>
<td></td>
<td>Metoclopramide</td>
<td>5-10 mg</td>
</tr>
</tbody>
</table>
Duodenal Juice:

A sterile radio-opaque red rubber catheter was manipulated into the fourth part of the duodenum and the position checked by fluoroscopy. The first 2 ml of duodenal aspirate were discarded. Samples of duodenal juice were then collected for bacterial and viral studies and secretory immunoglobulin A analysis.

Duodenal Mucosa:

Duodenal biopsy was performed 24 to 120 hours after onset of symptoms of acute enteritis (Table 5). Duodenal mucosa was obtained using a Watson paediatric biopsy capsule with a 2 mm port mounted on Kifa x-ray opaque catheter tubing. The capsule was passed into the fourth part of the duodenum within two or three cm of the ligament of Trietz using fluoroscopic control. The biopsy specimen was obtained from this site. The radiation dose was comparable to that required for a chest x-ray. The biopsy specimen was divided into four pieces which were to be used for histology, disaccharidase estimation, electron microscopy and immunofluorescence.
**TABLE 5**

Duration of Symptoms Prior to Duodenal Biopsy in 20 Patients with Acute Enteritis

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Number of Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-24</td>
<td>1</td>
</tr>
<tr>
<td>24-48</td>
<td>4</td>
</tr>
<tr>
<td>48-72</td>
<td>4</td>
</tr>
<tr>
<td>72-96</td>
<td>8</td>
</tr>
<tr>
<td>96-120</td>
<td>3</td>
</tr>
</tbody>
</table>
HISTOLOGY

Method

One portion of the duodenal biopsy specimen was fixed in Bouin's solution and stained with haematoxylin and eosin. Assessment of histological change was made blind by an independent observer (Dr. R. R. W. Townley) without knowledge of the clinical situation.

Specimens were graded as showing normal appearance, mild, moderate or severe change as described by Townley et al (1965) and as illustrated in Plate 1.

Results

The duodenal biopsy samples from all the 20 patients demonstrated evidence of histological damage. The abnormality was graded as mild, moderate or severe as depicted in Plate 1 (p. ). In two of these children the changes resembled those seen in coeliac disease (Plate 2). A summary of the light microscope findings is seen in Table 6.

The changes seen included villous flattening, replacement of normal columnar epithelium with cuboidal cells which occasionally showed marked vacuolation and infiltration of the lamina propria with inflammatory cells, the majority of which were plasma cells. Epithelial cell changes tended to be patchy in distribution, thus differing from the more consistent epithelial cell damage seen in coeliac disease.
a) Normal

b) Mild change: Broadening of villi, slight increase in mononuclear cell infiltration of lamina propria and early epithelial cell damage.
c) Moderate change: Considerable blunting of villi; obvious increase in inflammatory cells in the lamina propria and epithelial cell damage.

d) Severe change: Complete villous flattening, heavy cellular infiltration in lamina propria and gross disorganization of epithelial cells.
a) Coeliac disease with absence of villi, epithelial cell damage and marked infiltration of the lamina propria.

b) Severe damage in acute enteritis.
### TABLE 6

**Degree of Histological Change to Duodenum as Assessed by Light Microscopy**

<table>
<thead>
<tr>
<th>Degree of Change</th>
<th>Number of Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild</td>
<td>8</td>
</tr>
<tr>
<td>Moderate</td>
<td>10</td>
</tr>
<tr>
<td>Severe</td>
<td>2</td>
</tr>
</tbody>
</table>
On the basis of the histological findings, a repeat biopsy was considered necessary in the two children with severe change (T.A. and A.P.) to exclude the diagnosis of coeliac disease. Unfortunately T.A. was lost to follow-up but the repeat biopsy in A.P. showed complete resolution of the histological abnormalities (Plate 3).
a) Acute: showing severe damage

b) Convalescent: three weeks later showing complete resolution of damage.
DISACCHARIDASE ESTIMATION

Introduction

The major disaccharidase enzymes (sucrase, maltase, isomaltase and lactase) reside on the brush border lining the luminal surface of the small intestinal epithelial cells. Their function is to hydrolyse the disaccharide sugars in the diet (sucrose, maltose and lactose) to their component monosaccharides (glucose, fructose and galactose) which are then absorbed. Absence of these enzymes either congenitally or due to an acquired lesion (secondary to epithelial cell damage) leads to failure of hydrolysis and these unsplit sugars remain within the intestinal lumen and induce an osmotic diarrhoea. This condition is termed sugar intolerance and is seen in more then 50% of children under six months of age with acute enteritis (Barnes and Townley, 1973).

Method

A portion of the duodenal biopsy was stored in "Parafilm" at -20°C and used for disaccharidase assay within one to 10 days by a modification of the method of Dahlqvist (1964). The modification was made so that the LKB 7400 calculating Absorption Meter could be used for the determination of glucose in the final step. This required the use of Tris buffer and the disaccharidase reaction was then stopped by heating. Results were expressed as units/g wet weight (one unit of disaccharidase activity hydrolyses 1 μmole of disaccharide per minute at 37°C).
Normal values for this laboratory have been established (Barnes and Townley, 1973). The lower limits of normal were as follows; maltase 9.0, sucrase 3.5, lactase 1.0 units/g wet weight.

Results

Depression of all disaccharidases measured was found in 13 of the 19 patients studied. Only two patients had normal levels of all disaccharidases (Table 7). Of the 13 specimens with generalized disaccharidase depression 11 showed moderate or severe histological changes. The two specimens with normal disaccharidases showed mild histological changes.

The results of disaccharidase levels in the six children who had repeat duodenal biopsies are shown in Table 8.

In three children with moderate histological change (A.J., D.G. and T.F.) the disaccharidase levels were extremely low initially, resembling the pattern seen in coeliac disease. To exclude this diagnosis I repeated the biopsy four to eight weeks later. Unfortunately, the specimen obtained from A.J. was too small to perform disaccharidase estimations but the duodenal histology had returned to normal. The other two children had normal histology and disaccharidase levels.

A further child (J.M.) continued to have diarrhoea after discharge from hospital and a repeat duodenal biopsy was indicated to exclude underlying disease. The histology was
<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (months)</th>
<th>Histological Change</th>
<th>Disaccharidases + Units/g wet wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Maltase</td>
</tr>
<tr>
<td>A.J.*</td>
<td>31</td>
<td>Moderate</td>
<td>2.0</td>
</tr>
<tr>
<td>J.M.*</td>
<td>8</td>
<td>Moderate</td>
<td>9.9</td>
</tr>
<tr>
<td>N.S.</td>
<td>4</td>
<td>Mild</td>
<td>6.0</td>
</tr>
<tr>
<td>G.K.</td>
<td>9</td>
<td>Moderate</td>
<td>2.2</td>
</tr>
<tr>
<td>C.G.</td>
<td>5</td>
<td>Moderate</td>
<td>7.7</td>
</tr>
<tr>
<td>T.A.</td>
<td>7</td>
<td>Moderate</td>
<td>3.8</td>
</tr>
<tr>
<td>T.G.</td>
<td>10</td>
<td>Moderate</td>
<td>2.0</td>
</tr>
<tr>
<td>T.T.</td>
<td>19</td>
<td>Moderate</td>
<td>6.8</td>
</tr>
<tr>
<td>A.D.</td>
<td>13</td>
<td>Moderate</td>
<td>5.6</td>
</tr>
<tr>
<td>A.H.</td>
<td>4</td>
<td>Moderate</td>
<td>10.6</td>
</tr>
<tr>
<td>T.P.*</td>
<td>10</td>
<td>Moderate</td>
<td>5.9</td>
</tr>
<tr>
<td>T.K.</td>
<td>6</td>
<td>Mild</td>
<td>13.3</td>
</tr>
<tr>
<td>P.M.*</td>
<td>8</td>
<td>Moderate</td>
<td>10.1</td>
</tr>
<tr>
<td>C.N.</td>
<td>2</td>
<td>Mild</td>
<td>8.1</td>
</tr>
<tr>
<td>A.P.*</td>
<td>33</td>
<td>Severe</td>
<td>2.4</td>
</tr>
<tr>
<td>N.F.</td>
<td>11</td>
<td>Mild</td>
<td>6.0</td>
</tr>
<tr>
<td>K.B.</td>
<td>11</td>
<td>Moderate</td>
<td>6.5</td>
</tr>
<tr>
<td>M.S.</td>
<td>18</td>
<td>Mild</td>
<td>13.1</td>
</tr>
<tr>
<td>L.S.</td>
<td>22</td>
<td>Mild</td>
<td>10.0</td>
</tr>
</tbody>
</table>

* Patients who had a repeat biopsy 3-8 weeks later.
+ The lower limits of normal for this laboratory are maltase 9.0, sucrase 3.5, lactase 1.0 units/g wet wt.
# Table 8

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (months)</th>
<th>Interval Between Biopsies (weeks)</th>
<th>Histological Change</th>
<th>Disaccharidase Levels (Units/g wet weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Initial</td>
<td>Repeat</td>
</tr>
<tr>
<td>1. A.J.</td>
<td>31</td>
<td>4</td>
<td>Moderate</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. J.M.</td>
<td>8</td>
<td>4</td>
<td>Moderate</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. D.G.</td>
<td>14</td>
<td>8</td>
<td>Moderate</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. T.F.</td>
<td>10</td>
<td>5</td>
<td>Moderate</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. P.M.</td>
<td>8</td>
<td>3</td>
<td>Moderate</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

M = Maltase, S = Sucrase, L = Lactase
normal but the disaccharidase levels suggested he was a heterozygote for sucrase-isomaltase deficiency (Kerry and Townley, 1965). His symptoms subsided when placed on a low sucrose diet.

Patient P.M. also continued to have diarrhoea after discharge from hospital. The disaccharidase levels in his initial biopsy suggested that he may have been a heterozygote for sucrase-isomaltase deficiency, but the pattern could also have resulted from the associated histological damage. The repeat biopsy showed a persistence of the disaccharidase abnormality with normal histology and he also improved when placed on a low sucrose diet.
ELECTRON MICROSCOPY

Method

A very small portion of duodenal biopsy tissue was divided and immediately fixed in 2% glutaraldehyde in Millonig buffer for two hours at 4°C. After a soak in buffer alone, the tissue was further fixed in 1% osmium tetroxide in Millonig buffer for two hours at 4°C, dehydrated in ethanol and embedded in 'Maraglas.' Thin sections were stained with uranyl acetate and lead citrate and examined by electron microscopy.

Results

Although routine viral cultures were generally negative, electron microscopy revealed the presence of virus-like particles in 13 of the 20 children (Table 9).

To enable a suitable comparison, Plate 4 is an electron micrograph of the apical portion of a normal duodenal epithelial cell showing its regular tall microvilli and normal cellular contents. When compared to the infected cell seen in Plate 5 the changes due to viral invasion become apparent. Apart from virus particles within the cytoplasm, the microvilli are quite irregular and the endoplasmic reticulum cisternae are quite distended. These changes were often patchy with apparently normal cells often adjacent to obviously infected cells.
TABLE 9

Electron Microscope Examination of Duodenal Mucosa in 20 Children with Acute Enteritis

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (months)</th>
<th>Duration of Symptoms before Biopsy (days)</th>
<th>Electron Microscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.J.</td>
<td>31</td>
<td>1 1/2</td>
<td>+++</td>
</tr>
<tr>
<td>J.M.</td>
<td>8</td>
<td>1</td>
<td>++</td>
</tr>
<tr>
<td>N.S.</td>
<td>4</td>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td>C.K.</td>
<td>9</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>R.G.</td>
<td>5</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>G.M.</td>
<td>7</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>T.A.</td>
<td>30</td>
<td>3</td>
<td>+++</td>
</tr>
<tr>
<td>D.G.</td>
<td>14</td>
<td>3 1/2</td>
<td>++</td>
</tr>
<tr>
<td>T.T.</td>
<td>19</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>A.D.</td>
<td>13</td>
<td>3</td>
<td>++</td>
</tr>
<tr>
<td>A.H.</td>
<td>4</td>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>T.F.</td>
<td>10</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>T.K.</td>
<td>6</td>
<td>4</td>
<td>++</td>
</tr>
<tr>
<td>P.M.</td>
<td>8</td>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td>C.N.</td>
<td>2</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>A.P.</td>
<td>33</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>N.F.</td>
<td>11</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>K.B.</td>
<td>11</td>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td>M.S.</td>
<td>18</td>
<td>3</td>
<td>+++</td>
</tr>
<tr>
<td>L.S.</td>
<td>22</td>
<td>1</td>
<td>+++</td>
</tr>
</tbody>
</table>

0 = negative for "duovirus"

+ = scanty numbers of "duovirus" particles

++ = moderate numbers of "duovirus" particles

+++ = profuse numbers of "duovirus" particles
PLATE 4. Apical portion of normal mature duodenal epithelial cell showing microvilli (MV), junctional complex (JC), and mitochondria (M). Saccules of smooth (SER) and rough (RER) endoplasmic reticulum are also seen. (x45,000)
PLATE 5. Apical portion of duodenal epithelial cell from a patient with acute enteritis. Microvilli (MV), a normal junction complex (JC) and mitochondria (M) are seen. Numerous virus particles (V) are aggregated within a greatly distended endoplasmic reticulum cisternae; arrows indicate enveloped particles. A reticulate inclusion (R, lower right) can be seen and some virus particles seem to be in continuity with this. (x45,000)
When virus particles were present they almost all had an electron-dense core (33 nm in diameter) enclosed by a moderately dense layer, interpreted as capsid with an outer diameter of about 67-70 nm. A variable proportion of particles (approximately 10%) within vesicles seemed to be enveloped, their total diameter was then 87-90 nm. These features are well shown in Plate 5.

The electron microscope appearance of some epithelial cells from 13 of the 20 patients with acute enteritis showed evidence of distortion of the cellular architecture with flattening of the microvilli and vacuolation of the cytoplasm. Virus-like particles were seen within cytoplasmic vesicles (Plate 5). These cytoplasmic vesicles containing virus particles were recognized on closer examination to be distended cisternae of the rough endoplasmic reticulum. In some sections, infected cells could be seen adjacent to normal looking epithelial cells. This is demonstrated in Plate 6 where a necrotic cell is discharging virus particles into the lumen of the small intestine. This serves to demonstrate, even at an ultrastructural level, the patchy involvement of the duodenum in acute enteritis. It also demonstrates the cytolytic effect of the virus.

There were particles within cisternae between the nuclear membranes, but there were none in the nuclei itself. Inclusions with a fine reticulate structure, which seemed to consist of masses of convoluted smooth membrane, were sometimes adjacent to cisternae containing particles. These
PLATE 6. Necrotic epithelial cell discharging contents into lumen of small intestine (L). Membrane bound groups of virus particles (V) are seen. Part of a normal epithelial cell (E) is seen on left of picture with normal microvilli (MV) (x45,000)
inclusions may be either a source of the membrane surrounding enveloped particles (R in Plate 5) or a by-product of budding. No virus particles were observed in any of the other types of cell in the lamina propria.

In six children who underwent repeat biopsy three to eight weeks after clinical recovery included four of the 13 children in whom virus particles were observed during the acute stage of the illness. A prolonged search (three to five hours) of duodenal epithelial cells using the electron microscope did not reveal virus particles in any of the six samples from convalescent patients. A similar search in duodenal biopsy specimens from the four control children failed to reveal virus particles.
IMMUNOFLUORESCENCE

Introduction

With the electron microscope, only a minute portion of the available duodenal tissue could be searched and it was difficult to be sure of the localization of infected epithelial cells along the villi. Also, although morphology of the virus particles from different patients was identical no information could be obtained about similarity of antigenic structure.

In order to study these parameters, I decided to use the technique of indirect immunofluorescence which had been used successfully to locate other viruses in tissue specimens (Hatch et al, 1961; McQuillan and Gardner, 1968).

The work described in this section has recently been published in detail (Davidson et al, 1975 c). I performed all the studies described here with the guidance and assistance of Mr. I. Goller.

Methods

Specimens were available from eight of the children I had subjected to duodenal biopsy one to five days after onset of symptoms of acute enteritis. The number was limited for several reasons. Firstly, my inexperience led to incorrect preservation of some of the initial
specimens and secondly, a number of the biopsy specimens were too small to allow me to carry out all the studies. Histologically normal duodenal mucosa was available from the four control children.

Sera were collected from seven children two to four days from onset of symptoms ("acute" sera) and 14-30 days later ("convalescent" sera).

All seven children had shown "duovirus" particles in duodenal mucosa or faecal extracts. Three control sera were obtained from a newborn infant, a child aged five months with no history of enteritis and a healthy adult. All sera from patients and controls were used at a 1 in 2 dilution. In addition, acute and convalescent sera from two patients were used in dilutions over a range 1 in 2 to 1 in 64.

Duodenal mucosa was processed by a modification of the technique described by Sainte-Marie (1962). After initial fixation in 95% ethanol at 4°C for two hours minimum (maximum overnight) the tissue was dehydrated at approximately 10 minute intervals in three successive changes of absolute alcohol at 4°C and two changes of xylene, the first at 4°C, the second at room temperature. Tissue was vacuum embedded for five minutes at 60°C, blocked in paraffin ('Paraplast plus,' melting point 56-57°C) and stored at 4°C until used. Sections 4-6μ thick were cut and were
floated on a 45°C water bath for a maximum period of two seconds. Deparaffinization of slides was carried out at 4°C in three changes of xylene, two of absolute ethanol and two of phosphate-buffered saline (P.B.S.) for 5-10 seconds in each bath.

Sections were incubated with serum for 30 minutes at 37°C and then washed twice in P.B.S. for 10 minutes. The sections were then incubated with fluorescein labelled antisera to human immunoglobulin G, A or M (Hyland laboratories) for 30 minutes at room temperature. Slides were washed twice with agitation in P.B.S. for 15 minutes and were mounted in 50/50 P.B.S./glycerol.

All slides were examined under blue light using a Leitz microscope, a dark-ground condenser, with BG38 and KP 490 excitation filters and an OG1 barrier filter. Slides were coded and read by two independent observers unaware of their source. The results were recorded with a Leitz Orthomat automatic camera.

Specificity of Immunofluorescence:

This was determined by incubating duodenal biopsy tissue containing "duovirus" particles with sera absorbed with "purified" virus particles. The "purified" "duovirus" particles were obtained by differential centrifugation of faeces from a child with acute enteritis. To absorb the sera,
faecal extract containing "duovirus" particles was diluted 1 in 2 with P.B.S., mixed with an equal volume of a 1 in 2 dilution of acute or convalescent serum, incubated for one hour at 37°C, overnight at 4°C and centrifuged at 2,000 g for 15 minutes. The supernatant was incubated with duodenal tissue. The deposit obtained after centrifugation was re-suspended in 1-2 drops of 0.002M Tris negatively stained with potassium phosphotungstate at pH 7 and examined by electron microscopy at an initial magnification of 30,000.

Reaction with Reovirus Antisera

Duodenal biopsy tissue containing "duovirus" particles within epithelial cells was incubated with rabbit antisera to reovirus type 1 (Long strain) type 2 (Jones strain) and type 3 (Abney strain). Fluoresceinated goat anti-rabbit gammaglobulin was used as the marker.
Results

Table 10 lists fluorescence observed in duodenal biopsy tissue incubated with a variety of sera at a 1 in 2 dilution. Fluorescence was seen within epithelial cells only when mucosa containing "duovirus" particles was reacted with acute or convalescent sera from patients known to have been infected with the "duovirus" (Plate 7a). This fluorescence occurred with fluorescein labelled antiserum to human IgM but not with similarly labelled antisera to IgG or IgA.

Convalescent sera absorbed by incubation with "purified" "duovirus" showed no fluorescence within epithelial cells in biopsy tissue containing "duovirus" (Plate 7b).

The centrifuged pellet after absorption with "duovirus" containing faecal extract was examined by electron microscopy and immune aggregates of "duovirus" particles were seen (Plate 8).

No fluorescence was seen in epithelial cells when control sera were incubated with duodenal mucosa containing virus particles, or when acute and convalescent sera were incubated with control mucosae containing no virus (Table 10).
**TABLE 10**

**Immunofluorescence in Biopsy Specimens of Duodenal Mucosa from Patients Infected with the "duovirus" and Controls**

<table>
<thead>
<tr>
<th>Sera</th>
<th>Infected with Virus</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Acute sera</td>
<td>28</td>
<td>10</td>
</tr>
<tr>
<td>Convalescent sera</td>
<td>26</td>
<td>11</td>
</tr>
<tr>
<td>Convalescent sera absorbed with</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>virus containing faecal extract</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control sera</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td>Reovirus antisera to types 1, 2</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>and 3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Positive = Particulate fluorescence in epithelial cell

2 Negative = No fluorescence in epithelial cell
a) Particulate fluorescence in epithelial cells of duodenal mucosa from a child with acute enteritis due to the "duovirus."
Original magnification x500

b) Abolition of particulate fluorescence in epithelial cells of duodenal mucosa after absorption of sera with "purified" "duovirus."
Original magnification x500

D = particulate fluorescence due to virus particles
E = epithelial cell layer
L = lamina propria
N = nucleus of epithelial cell
PLATE 8. Immune aggregate of negatively stained "duovirus" particles and antibody material (x100,000)
The distribution of fluorescence along one villus is shown in Plate 7a. Fluorescence attributed to the presence of the "duovirus" was particulate in nature and was located in a supra-nuclear position in the cytoplasm of duodenal epithelial cells. No fluorescence was seen in epithelial cell nuclei. Not all epithelial cells fluoresced in any one section. No specific fluorescence was seen in the cells of the lamina propria although diffuse non-specific fluorescence was observed in plasma cells in most sections. In each biopsy, distribution of epithelial cell fluorescence due to the presence of the "duovirus" was patchy; some sections reacted strongly while others showed little specific fluorescence. The number of fluorescing epithelial cells present was influenced by duration of symptoms. Biopsy tissue obtained more than four days after onset of symptoms of acute enteritis showed few areas of epithelial cell fluorescence.

The patchy distribution of the "duovirus" explains the inconsistent results with some sera. Of the total of 75 reactions between mucosa containing virus particles and sera from virus infection patients (Table 10), 54 showed positive fluorescence while 21 showed no fluorescence.

Mucosae containing "duovirus" particles showed fluorescence within epithelial cells when incubated with sera from other patients (Table 11). Only one homologous reaction (patient 5) was possible because most convalescent sera were collected after I ceased subjecting patients to duodenal biopsy, since it had become apparent that examina-
TABLE 11

Immunofluorescent Reactions Between Duodenal Mucosa Containing "duovirus" and Convalescent Sera from Children with "duovirus" Enteritis

<table>
<thead>
<tr>
<th>Tissue from Patient</th>
<th>Epithelial Cell Fluorescence with Convalescent Sera from Patient</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>3, 10, 16</td>
</tr>
<tr>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td>15</td>
<td>10, 11, 16</td>
</tr>
<tr>
<td>16</td>
<td>3, 8, 10, 16</td>
</tr>
<tr>
<td>17</td>
<td>11, 16</td>
</tr>
<tr>
<td>18</td>
<td>10, 11, 16</td>
</tr>
<tr>
<td>19</td>
<td>3, 10, 16</td>
</tr>
<tr>
<td>20</td>
<td>2, 8, 10, 11, 16</td>
</tr>
</tbody>
</table>

+ Numbers refer to patients as outline in Table 1.
tion of faecal extracts was a more sensitive method for detecting the virus.

Titres of paired acute and convalescent sera from two of the patients were estimated using consecutive sections from one biopsy specimen. Immunofluorescent titres were determined as the highest dilution of sera showing definite particulate fluorescence within epithelial cells. In both patients acute sera showed a titre of 1 in 4 for IgM antibodies, whereas paired convalescent sera showed a titre of 1 in 32.

No fluorescence was observed when reovirus antisera types 1, 2 and 3 were incubated with duodenal mucosa from two patients known to be infected with "duovirus."
DISCUSSION

Histology and Disaccharidase Levels

This study clearly confirms that severe histological change and marked depression of disaccharidase levels can occur in the small intestine in acute enteritis. This histological damage can be severe enough to be confused with the histological appearance seen in coeliac disease. Also generalized disaccharidase depression was more likely to occur in patients with moderate or severe histological changes.

Complete recovery of histological appearance and reversal of disaccharidase depression occurred as judged by repeat biopsy three to eight weeks after clinical recovery. An unexpected finding, but an important one from the patients viewpoint, was the diagnosis of an underlying disaccharidase abnormality in two of the 20 children. These children may have been predisposed by their underlying defect to a more severe illness.

Electron Microscopy

There is little reason to doubt that the virus particles observed in duodenal epithelial cells by electron microscopy were responsible for acute enteritis in 13 out of the 20 patients studied. The particles were present in duodenal epithelial cells during the
symptomatic stage of the illness but could not be detected in six of the same patients examined some weeks after clinical recovery. The presence of particles during the acute stage of the illness coincided with histological abnormalities and depressed disaccharidase levels in the duodenal mucosa. The location of virus particles in the cytoplasm of the epithelial cell was consistent with this damage.

It is unlikely that this virus particle is a common latent human virus. No previous electron-microscope studies on human duodenal epithelium in health and disease have described such a particle. Moreover, there were no virus particles found in the six convalescent biopsies or in the four control children who had biopsies to investigate failure to thrive or recurrent diarrhoea.

The morphology location and size of the particles and especially the occurrence of both enveloped and naked particles within cisternae, indicate that the particles observed closely resemble a virus belonging to the recently described orbivirus group (Borden et al, 1971; Murphy et al, 1971; Schnagl et al, 1971). This group is closely related to the reovirus group but is distinguished by certain details of its morphogenesis. The virus particles seen in these children with acute enteritis resembles very closely the viruses of epizootic diarrhoea of infant mice (E.D.I.M.) described initially by Adams and Kraft (1963) and calf scourds (Fernelius et al, 1972).
This would be similar to the age range of overt infection of mice by E.D.I.M. which is limited to the first three weeks of life. The infection of calves with the Nebraska calf scours virus follows a similar pattern. The age range of children vulnerable to this new virus may be similar to that for other childhood virus infections occurring in the first few years of life. For example, infection with respiratory syncytial virus causes bronchiolitis in the first year of life but by age seven nearly all children have developed neutralizing antibodies (Beem et al, 1964). This suggests that overt or subclinical infection has occurred early in childhood. It would appear, therefore, that as with other childhood viruses younger children seem to be at the greatest risk for acquiring the infection.

The timing of the duodenal biopsy in relation to the onset of illness was a very important factor in detecting the virus. Particles were present in greatest numbers in the duodenal epithelium in four of the five biopsies obtained earliest after onset of the illness. In six of the seven patients in whom no virus particles were observed in the duodenal epithelium despite histological and enzyme damage, biopsies were obtained more than four days after onset of symptoms (Table 12).
TABLE 12

Relationship Between Duration of Symptoms and Presence of "duovirus" Particles in the Duodenal Mucosa

<table>
<thead>
<tr>
<th>Duration of Symptoms Prior to Biopsy</th>
<th>Electron Microscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Virus Present</td>
</tr>
<tr>
<td>&gt;72 hours</td>
<td>8</td>
</tr>
<tr>
<td>&lt;72 hours</td>
<td>5</td>
</tr>
</tbody>
</table>
The patchy nature of the mucosal damage in acute enteritis in children as previously observed by Barnes and Townley (1973) might explain the difficulty in locating virus particles by electron microscopy in some of the patients.

**Immunofluorescence**

Indirect immunofluorescence tests used on duodenal mucosa already shown to contain the new virus confirmed that the location was only in the cytoplasm of epithelial cells of the lamina propria. Distribution of the "duovirus" in epithelial cells was patchy, both in adjacent villi from the same biopsy, and in adjacent epithelial cells on the same villus. The "duovirus" was difficult to detect in duodenal mucosa obtained more than four days after onset of clinical symptoms of acute enteritis. Previous studies in calves infected with a similar virus have shown that infected epithelial cells are shed rapidly and thus virus-containing cells may be difficult to find after the third or fourth day of the illness (Mebus et al, 1971 a).

Children with acute enteritis due to the "duovirus" developed IgM antibodies in sera as early as two days after onset of symptoms. The specific nature of the IgM response was shown by absence of fluorescence in epithelial cells after absorption of positive sera with "purified" virus obtained by differential centrifugation of faeces. In two children an eight fold rise in viral antibody titre occurred during convalescence.
The immunofluorescence results provided evidence that "duovirus" particles from different patients, as well as being morphologically similar, were also antigenically similar as "duovirus" particles fluoresced when incubated with convalescent sera from other "duovirus" positive patients.

Duodenal tissue containing "duovirus" particles did not fluoresce with specific antisera to reovirus types 1, 2 and 3. This emphasized that this virus is not a classical reovirus.
CHAPTER III

DETECTION OF THE "DUOVIRUS" BY ELECTRON MICROSCOPY
OF FAECAL EXTRACTS FROM 20 CHILDREN WITH ACUTE ENTERITIS.
INTRODUCTION

In the initial part of this study, virus particles were found by electron microscopy in epithelial cells of duodenal mucosae from children with acute enteritis. Liberation of these particles into the intestinal lumen was also observed (Plate 6 page 51). As a result of this it was logical to expect that these particles would be present in faeces. It was also apparent from earlier studies of acute enteritis in children that the use of routine viral culture methods for this new virus were unlikely to be successful (Cramblett et al, 1971).

This chapter describes my adaptation of a technique whereby the virus in diarrhoeic faeces is purified, concentrated and examined by electron microscopy. I adapted this technique from one that had been used by Turner et al (1973) for the detection of the Nebraska calf scours virus in stools.

The results outlined in this chapter have recently been published (Bishop et al, 1974a). Here I will describe my role in this part of the study which was to collect and correlate clinical data and specimens and prepare material for electron microscopy. Dr. Ruth Bishop carried out the bacterial cultures and Dr. I. Holmes performed the electron microscopy.
Clinical Material

Patients

Patients used in this part of the study have been described in detail in Chapter II of this thesis (page 25).

Controls

Control faecal specimens were obtained from nine children who had no gastrointestinal tract symptoms for two weeks before and two weeks after the day of collection. Only two of these nine children were in hospital - one for investigation of the urinary tract after previous infection, the other for investigation of mental retardation. The age range of the control group was similar to the group with acute enteritis.

Specimens

Faeces were obtained as early as possible after admission to hospital and no later than the day of duodenal biopsy in 18 of the 20 patients. A portion of each specimen was sent for routine bacterial and viral cultures. The remainder was stored at 4°C or -70°C until preparation for electron microscopy.
The method of collection of duodenal juice is described on page but the methods used for culture and the results will be described in the following section along with the faecal results.

Bacteriology

Methods

Specimens were plated immediately or after refrigeration for one hour. 0.05 ml of duodenal contents were placed on each of the following media: horse blood agar, McConkey agar, Rogosa agar, deoxycholate agar, Sabouraud agar incubated aerobically at 37°C; and on horse blood agar and Rogosa agar incubated anaerobically with the addition of 5-10% carbon dioxide. Faecal specimens were plated on the same media and were also incubated in selenite broth for 48 hours. Two drops of each specimen were dried at room temperature on a glass slide. A gram stain of this smear acted as a check on the adequacy of culture. A standard plating technique that allowed semiquantitative estimation of numbers present was used (Cregan and Hayward, 1953). Bacterial counts using the technique of Miles et al (1938) were compared with the results of plating, in order to assess numbers of organisms present. Yeast counts were made microscopically under high power in a haemocytometer. All bacteria isolated were subcultured and identified according to criteria described by Gibbs and Skinner (1966). E.coli strains were typed serologically using commercial polyvalent and monovalent antisera.
(BBL). The ability of strains to form enterotoxin was tested in ligated loops of rabbit intestine (Burrows and Musteikis, 1966). Yeasts were identified by biochemical tests and colonial morphology described by Martin et al (1937). *Candida albicans* was additionally identified by ability to form germ tubes (Taschdjian et al, 1960).

**Results**

**Duodenal Juice**

Duodenal juice from three of the 20 children was negative on culture.

In the remaining 17 children culture yielded varying amounts of bacteria regarded as normal flora of mouth, nose and throat, e.g. Streptococci of the viridans group, *Neisseria sp.*, *Staphylococcus albus*, *Veillonella sp.*, *Corynebacterium sp.*, and *Haemophilus sp.*

The results did not differ either qualitatively or quantitatively from those obtained from healthy children samples by intubation (Anderson and Langford, 1958).

*E. coli* was isolated in small numbers ($10^3$/ml) from four of the 17 children. None of the strains belonged to the serological types at present regarded as potentially enteropathogenic. Enterotoxin production was not detected in ligated loops of rabbit intestine.
Faeces

A recognised pathogen was isolated from only one infant of the 20 studied. This was *E. coli* 0125:B15 grown from the faeces of a 18 month old child. This child also had Coxsackie B5 grown from his faeces and particles of the new virus seen in duodenal mucosa and faeces. This organism failed to cause distension of ligated loops of rabbit intestine.

Virology

Methods

Mucus

Gel saline (2.5 ml) containing 80 ug/ml of gentamicin was added to mucus in the nasopharyngeal extractor. The contents were then agitated for 30 seconds and pipetted into a 5 ml sterile container to which had been added 0.25 ml mycostatin suspension (5,000 units/ml). Aliquots of 0.2 ml were then inoculated into human diploid fibroblast monolayer cultures, primary monkey kidney (cynomologous) and He La cells.

Duodenal Juice

Duodenal juice (0.2 ml) and an equal volume of foetal calf serum were added to 5 ml of Gel Saline containing 80 ug/ml of gentamicin. After mixing, 5 ml was pipetted into a sterile container to which had been added 0.5 ml of mycostatin suspension (5,000 units/ml). Aliquots of 0.2 ml were then inoculated into tissue culture media as for mucus.
Faeces

Solid faecal material was added to 5 ml of Gel saline containing gentamicin (80 μg/ml). The specimen was then centrifuged at 7,000 g in a Sorval refrigerated centrifuge. The supernatant was pipetted into a 5 ml sterile container, to which had been added 0.5 ml of mycostatin suspension (5,000 units/ml). Aliquots of 0.2 ml were then inoculated into tissue culture media as before.

All specimens were rolled on tissues for one hour. The cultures were given a medium change after 24 hours to avoid toxicity. Specimens were examined every two days until negative or the appearance of cytopathic change. Monkey kidney cells and human foetal fibroblasts were held at 36.5°C and the He La cells at 33°C. Cultures were terminated at 14 days.

Results

There were 15 virus isolations on culture of specimens from nine patients (Table 13). Only two enteroviruses were isolated. Coxsackie B5 was found in throat, duodenal juice and faeces of one child.

Poliovirus 1 and 3 were also found in another child who had received oral Sabin polio vaccine one week prior to onset of acute enteritis.
### TABLE 13

**Results of Positive Viral Cultures from Nine out of 20 Patients with Acute Enteritis**

<table>
<thead>
<tr>
<th>Patients</th>
<th>Nasopharynx</th>
<th>Culture Site</th>
<th>Duodenal Juice</th>
<th>Faeces</th>
</tr>
</thead>
<tbody>
<tr>
<td>N.S.</td>
<td>RSV</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>G.M.</td>
<td>rhinovirus</td>
<td>rhinovirus</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T.A.</td>
<td>CMV</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D.G.</td>
<td>-</td>
<td>rhinovirus</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T.T.</td>
<td>CMV</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A.H.</td>
<td>CMV</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T.K.</td>
<td>polio 1 &amp; 111</td>
<td>polio 1</td>
<td>polio 1 &amp; 111</td>
<td>-</td>
</tr>
<tr>
<td>K.B.</td>
<td>rhinovirus</td>
<td>rhinovirus</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M.S.</td>
<td>Coxsackie B5</td>
<td>Coxsackie B5</td>
<td>Coxsackie B5</td>
<td>-</td>
</tr>
</tbody>
</table>

CMV = Cytomegalovirus  
RSV = Respiratory Syncytial Virus
Electron Microscopy of Faeces

Methods

Faecal specimens were thawed and 1-5 g was thoroughly mixed by hand with 10 ml of phosphate buffered saline (P.B.S.) using a "Teflon" glass homogenizer. Trifluoro-trichloroethane ("Arkdrive," I.C.I.) 10 ml was then added and the sample was homogenized for a further 10 minutes. The homogenate was centrifuged at 10,000 g for 30 minutes at 4°C. The supernatant was removed, the volume measured and 8% polyethylene glycol 6,000 (Union Carbide) was added. The mixture was stored overnight at 4°C, then centrifuged at 4,000 g for 30 minutes and the supernatant discarded. The deposit was resuspended in 4 ml of P.B.S. This mixture was then layered on to 1 ml of 45% sucrose in 0.002M "tris" pH 7.0 and centrifuged at 100,000 g for 75 minutes. These conditions were selected to spin down virus particles of the size seen in duodenal epithelial cells but to leave enteroviruses and small agents in the supernatant.

The supernatant was discarded and the deposit was suspended in 2-5 drops of 01002M tris pH 7.0. This extract was then examined at an initial magnification of 30,000 in a Hitachi electron microscope after negative staining with potassium phosphotungstate (pH 7.0).
All faecal specimens were randomised and coded, so that the electron microscopists were not aware of the source of the extracts they were examining for particles.

Results

The new virus was observed in faecal extracts from 15 of the 18 specimens examined. Faeces were not obtained from two patients (Table 14). No children in the control group had virus particles in faecal extracts.

The particles of the new virus were recognizable in faecal extracts by their regularity, size and surface structure (Plate 9).

Bacteriophages of various morphological types were observed in faecal extracts both from controls and from children with acute enteritis. These were occasionally confusing at first glance, but the bacteriophage tails were easily recognized on closer examination. The appearance of the negatively stained virus particles was identical in all children and their morphology and morphogenesis were similar to the virus particles seen in ultra-thin sections.

Specimens contained predominantly double-shelled or single shelled particles or a mixture of both. Particles with a double-shelled capsid structure had a diameter of 70-75 nm and a characteristically sharp outline (Plate 10a),
**TABLE 14**

**Electron Microscope Examination of Faeces in 20 Children with Acute Enteritis**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (months)</th>
<th>Duration of Symptoms Prior to Examination (days)</th>
<th>Electron Microscopy of Faecal Extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.J.</td>
<td>31</td>
<td>1½</td>
<td>No faeces</td>
</tr>
<tr>
<td>J.M.</td>
<td>8</td>
<td>1</td>
<td>+++</td>
</tr>
<tr>
<td>N.S.</td>
<td>4</td>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td>C.K.</td>
<td>9</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>R.G.</td>
<td>5</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>G.M.</td>
<td>7</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>T.A.</td>
<td>30</td>
<td>3</td>
<td>+++</td>
</tr>
<tr>
<td>D.G.</td>
<td>14</td>
<td>3½</td>
<td>+</td>
</tr>
<tr>
<td>T.T.</td>
<td>19</td>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td>A.D.</td>
<td>13</td>
<td>3</td>
<td>No faeces</td>
</tr>
<tr>
<td>A.H.</td>
<td>4</td>
<td>3</td>
<td>+++</td>
</tr>
<tr>
<td>T.F.</td>
<td>10</td>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td>T.K.</td>
<td>6</td>
<td>4</td>
<td>++</td>
</tr>
<tr>
<td>P.M.</td>
<td>8</td>
<td>5</td>
<td>+++</td>
</tr>
<tr>
<td>C.N.</td>
<td>2</td>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td>A.P.</td>
<td>33</td>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td>N.F.</td>
<td>11</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>K.B.</td>
<td>11</td>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td>M.S.</td>
<td>18</td>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>L.S.</td>
<td>22</td>
<td>1</td>
<td>+++</td>
</tr>
</tbody>
</table>

0 = negative for "duovirus"
+ = scanty numbers of "duovirus" particles
++ = moderate numbers of "duovirus" particles
+++ = profuse numbers of "duovirus" particles
PLATE 9. Negatively stained "duovirus" particles (arrowheads) and assorted debris (B = bacterial flagellum) in a typical electron microscope field of a faecal extract (x45,000)
PLATE 10. "DUOVIRUS" PARTICLES

a) "Double shell" or complete particles with a characteristically sharp outline. x180,000

b) "Single shell" particles including one empty penetrated by stain. x180,000
corresponding to the particles with capsid diameter of 67 nm observed in ultra-thin sections of duodenal epithelium. Plate 10b shows single-shelled particles (60 nm in diameter) which exhibited a much more obvious capsomer structure than cores of reovirus particles; they closely resembled typical orbiviruses. In our preparations, we observed single-shelled particles far more frequently than double-shelled ones. This may have been a consequence of the method of processing of faecal samples but conditions in the gut and the time faecal specimens remained at room temperature prior to being frozen may also have influenced this finding.
DISCUSSION

Microbiology

Bacteria:

There is no evidence to suggest that bacteria played a role in the aetiology of acute enteritis affecting patients in this study. A possible pathogen, *E. coli* 0125:B15, was found in the faeces of one patient. This organism did not produce distension of rabbit gut loop and therefore was probably not an enterotoxin producer.

The bacteriological findings strongly support evidence from the literature that bacteria have little to do with the aetiology of most cases of sporadic acute enteritis.

Viruses:

Only two enteroviruses were isolated using routine viral culture methods. In one child, Coxsackie B5 was isolated from nasopharyngeal mucus, duodenal juice and faeces. This child also had the "duovirus" observed in duodenal mucosa and faeces. The other child had polio types 1 and 2 cultured from all three sites but had received oral Sabin polio vaccine two weeks prior to onset of acute enteritis.
The significance of the isolation of Coxsackie B5 in relation to aetiology of the acute enteritis is very dubious. Ramos Alvarez and Sabin (1958), who studied 153 children with acute enteritis and 100 matched controls, recovered enteroviruses from nearly 50% of those with diarrhoea. However, both groups showed the same incidence of Coxsackie and poliovirus isolations.

Electron Microscopy of Faeces

The results of electron microscopy of duodenal mucosa and faecal extracts from 20 children with acute enteritis are summarized in Table 15. The new virus was seen in duodenal epithelial cells from 13 out of 20 patients with acute enteritis and in no control child. Electron microscopy of negatively stained faecal extracts revealed virus particles in 15 out of 18 children with acute enteritis and in no control child. Thus, combining the results of duodenal mucosa and faeces, the new virus was observed by electron microscopy in 17 out of 20 children with sporadic acute enteritis in Melbourne.
TABLE 15

Frequency of the "duovirus" by Electron Microscopy of Duodenal Mucosa and Faecal Extracts

<table>
<thead>
<tr>
<th>&quot;duovirus&quot; present</th>
<th>Location</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;duovirus&quot; present</td>
<td>duodenum</td>
<td>13/20</td>
</tr>
<tr>
<td>&quot;duovirus&quot; present</td>
<td>faeces</td>
<td>15/18</td>
</tr>
<tr>
<td>&quot;duovirus&quot; present</td>
<td>duodenum and/or faeces</td>
<td>17/20</td>
</tr>
</tbody>
</table>
In four children electron microscopy of duodenal mucosa was negative, but examination of negatively stained faecal extracts revealed virus particles. The converse of this did not apply. Therefore, it seemed that electron microscopy of negatively stained faecal extracts was a more sensitive method for detection of "duovirus". It was also a much simpler, safer and more rapid method than electron microscopy of duodenal mucosa.

The "duovirus" clearly differs from the parvovirus-like particles recently detected by immune-electron microscopy in faeces from adults with gastroenteritis acquired naturally (Paver et al, 1973) or by ingestion of stool filtrates containing Norwalk agent (Kapikian et al, 1972). Electron microscopy of jejunal mucosa in the latter group did not show any virus particles (Agua et al, 1973).

The successful development and application of this relatively simple, sensitive and rapid technique now made the study of larger numbers of children possible. My next step was to use this technique to study the importance of the "duovirus" in relation to other recognized enteric pathogens in sporadic acute enteritis in infants and children.
CHAPTER IV

AETIOLOGICAL SURVEY OF ACUTE ENTERITIS IN
INFANTS AND CHILDREN IN MELBOURNE.
INTRODUCTION

The technique of faecal extraction provided a safe, rapid and relatively sensitive method of detecting the "duovirus." I now wanted to use this method to study the relative importance of this new virus in my hospital community.

There were a number of questions regarding the "duovirus" that I hoped would be answered by surveying one community over a twelve month period.

The questions I set out to answer were the following:

1. The prevalence of the "duovirus in any one community.

2. Seasonal incidence of the "duovirus."

3. Relative importance of the "duovirus" compared to other known enteric pathogens.

4. Age group of children affected by the "duovirus" and if possible the age of those susceptible to infection.

5. Duration of "duovirus" excretion.

6. Mode of spread of the "duovirus."
In order to carry out this survey, I studied prospectively all children with acute enteritis admitted to the gastroenteritis ward of the Royal Children's Hospital, Melbourne over a 12 month period and a suitable control group. I have recently reported this work (Davidson et al., 1975a,b). Again I have to thank Dr. Ruth Bishop for doing the bacteriology and Mr. B. Ruck and Miss A Veenstra who carried out the electron microscope examination of stool specimens.

Clinical Material

Patients

From November 1, 1973 to October 31, 1974, I obtained faeces from all children with acute enteritis admitted to the gastroenteritis ward of the Royal Children's Hospital, Melbourne. The term "acute enteritis" was previously defined (page 24).

A total of 378 children (209 male and 169 female) aged from 10 days to 12½ years were examined (Table 16). Most of the children had presented to the Casualty Department of the hospital with acute enteritis. However, 25 children had been transferred to the gastroenteritis ward from other wards in the hospital, when symptoms of acute enteritis developed two or more days after admission for various illnesses.
# TABLE 16

**Age Range of Patients with Acute Enteritis and of Control Infants**

<table>
<thead>
<tr>
<th></th>
<th>Under 6 Months</th>
<th>6-12 Months</th>
<th>12-24 Months</th>
<th>2-5 Years</th>
<th>Over 5 Years</th>
</tr>
</thead>
<tbody>
<tr>
<td>378 Acute Enteritis Patients</td>
<td>98 (26%)</td>
<td>77 (20%)</td>
<td>115 (30%)</td>
<td>79 (20%)</td>
<td>9 (4%)</td>
</tr>
<tr>
<td>116 Control Infants</td>
<td>46 (39%)</td>
<td>22 (19%)</td>
<td>22 (19%)</td>
<td>22 (19%)</td>
<td>4 (4%)</td>
</tr>
</tbody>
</table>
Controls

Faeces were also obtained by me from a control group of 116 children (69 male and 49 female) aged from 10 days to six years (Table 16), admitted to one ward of the hospital during the same year. This control group included 16 healthy children admitted to hospital for social reasons, 51 children admitted with respiratory illnesses who showed no symptoms referable to the gastrointestinal tract and 49 children with gastrointestinal problems not due to acute enteritis (chronic diarrhoea, failure to thrive, feeding problems). Table 17 shows the diagnoses in these 100 children.
TABLE 17

Diagnoses in 100 Control Children

a) Symptoms Outside Gastrointestinal Tract

1. Respiratory Illness

<table>
<thead>
<tr>
<th>Condition</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upper respiratory tract infections</td>
<td>14</td>
</tr>
<tr>
<td>Bronchiolitis</td>
<td>13</td>
</tr>
<tr>
<td>Croup</td>
<td>5</td>
</tr>
<tr>
<td>Pneumonia</td>
<td>5</td>
</tr>
<tr>
<td>Epiglottitis</td>
<td>3</td>
</tr>
<tr>
<td>Whooping cough</td>
<td>2</td>
</tr>
<tr>
<td>Asthma</td>
<td>2</td>
</tr>
</tbody>
</table>

2. Others

<table>
<thead>
<tr>
<th>Condition</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Congenital heart disease</td>
<td>3</td>
</tr>
<tr>
<td>Urinary tract infection</td>
<td>3</td>
</tr>
<tr>
<td>Viral pneumonia</td>
<td>1</td>
</tr>
</tbody>
</table>

b) Symptoms Referable to Gastrointestinal Tract

<table>
<thead>
<tr>
<th>Condition</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic diarrhoea</td>
<td>23</td>
</tr>
<tr>
<td>Failure to thrive</td>
<td>10</td>
</tr>
<tr>
<td>Feeding problems</td>
<td>6</td>
</tr>
<tr>
<td>Gastrooesophageal reflux</td>
<td>3</td>
</tr>
<tr>
<td>Pancreatic achylia</td>
<td>2</td>
</tr>
<tr>
<td>Pyloric stenosis</td>
<td>2</td>
</tr>
<tr>
<td>Acute appendicitis</td>
<td>1</td>
</tr>
<tr>
<td>Ulcerative colitis</td>
<td>1</td>
</tr>
<tr>
<td>Short gut syndrome</td>
<td>1</td>
</tr>
</tbody>
</table>
Methods

Sample Collection

Specimens of faeces were collected from all patients within 24 hours of admission to hospital. In 16 patients, I collected specimens over a number of days whilst they remained in hospital in order to determine the period of virus shedding in stool.

Bacteriology

Specimens of faeces were cultured as described on page 72. \textit{Ecoli} isolated from each patient were tested against antisera to the recognized enteropathogenic types. All isolates were tested in ligated loops of rabbit intestine for enterotoxin production.

Virology

Specimens of faeces were cultured for viral pathogens as outlined on page 74.

Electron Microscopy of Faeces

Portions of all faeces samples were stored at 4°C or -70°C and then extracted by the method of differential centrifugation as described earlier on page 77.

All faecal specimens were randomly coded so that the electron microscopist was unaware of the source of the extracts being examined for particles.
Results

Bacterial Cultures

Recognized enteric pathogens were cultured from faeces in 52 of the 378 patients with acute enteritis and in four of the 116 control infants (Table 18). The "enteropathogenic" E. coli strains isolated were 0111:B4, 0142:K86, 0125:B15, 055:B5 and 026:B6. None of these isolates caused distension of ligated loops of rabbit intestine.

Viral Cultures

Results of routine viral cultures of faeces are listed in Table 19. Adenoviruses were cultured from 17 patients with acute enteritis and two control infants. Enteroviruses were cultured from six patients and nine controls. 5.5% of the patients with acute enteritis had either adenovirus or enterovirus cultured from their faeces.

Electron Microscopy of Faeces

The newly discovered "duovirus" was found in 52% of patients with acute enteritis and in no control infant (Table 20).

Adenoviruses were seen in approximately similar percentages in both groups. In four patients and two control infants, adenoviruses were seen by electron microscopy and also cultured (Table 21). Adenoviruses are quite readily distinguished from the "duovirus" because of their different morphological features (Plate 11).
TABLE 18

Incidence of Bacterial Pathogens in Faeces from Children with Acute Enteritis Compared with a Control Group

<table>
<thead>
<tr>
<th>Enteric Pathogen</th>
<th>378 Children with Acute Enteritis</th>
<th>116 Control Children</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmonella sp.</td>
<td>40 (11%)</td>
<td>1 (1%)</td>
</tr>
<tr>
<td>&quot;Enteropathogenic&quot; E.coli</td>
<td>7 (2%)</td>
<td>3 (3%)</td>
</tr>
<tr>
<td>Shigella sp.</td>
<td>5 (1%)</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>52 (14%)</strong></td>
<td><strong>4 (4%)</strong></td>
</tr>
</tbody>
</table>
TABLE 19

Viral Cultures of Faeces from 378 Children with Acute Enteritis and 116 Controls

<table>
<thead>
<tr>
<th>Virus</th>
<th>Acute Enteritis</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus type 1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>type 2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>type 3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>type 5</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>untyped</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>17 (4%)</td>
<td>2 (1.7%)</td>
</tr>
</tbody>
</table>

| Enteroviruses          |                 |         |
| poliovirus 3           | 1               | 1       |
| ECHO virus 22          | 0               | 4       |
| 30                     | 1               | 1       |
| Coxsackie A9           | 1               | 3       |
| untyped                | 3               | 3       |
|                        | 6 (1.5%)        | 9 (7.7%)|
# Electron Microscopy of Faeces from Patients with Acute Enteritis and from the Control Group

<table>
<thead>
<tr>
<th>Enteric Pathogen Present</th>
<th>Acute Enteritis</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;duovirus&quot;</td>
<td>197 (52%)</td>
<td>0</td>
</tr>
<tr>
<td>adenovirus*</td>
<td>16 (4%)</td>
<td>8 (6.8%)</td>
</tr>
</tbody>
</table>

* In four "duovirus" patients and two controls adenoviruses were also cultured.
### TABLE 21

**Adenovirus Isolations made by Electron Microscopy and Culture**

<table>
<thead>
<tr>
<th></th>
<th>Acute Enteritis</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seen by Electron Microscopy alone</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td>Seen by Electron Microscopy and Cultured</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Cultured only</td>
<td>11</td>
<td>1</td>
</tr>
</tbody>
</table>

**Total**

27

9
a) Adenovirus particles (x90,000)

b) "Duovirus" particles (x90,000)
Summary of Viral Findings

The results from both electron microscopy and routine viral cultures are shown in Table 22. The "duovirus" was present in 52% of cases of acute enteritis and in no control child. This compares with adenoviruses and enteroviruses which were isolated with equal or greater frequency in the control group as in affected patients. These latter groups of viruses were found also in only a very small percentage of patients compared to the "duovirus."

Seasonal Incidence of "duovirus"

Acute enteritis was most common during the winter months (Fig. 1) when the incidence of "duovirus" infection was also at its peak (Fig. 2). The "duovirus" was the most common enteric pathogen during all but the two hottest months of the year (February and March). The high incidence of "duovirus" infection in November may be due to the very small number of patients seen during that month.
TABLE 22

Incidence of Viral Pathogens seen by Electron Microscopy or Cultured from Faeces from Patients with Acute Enteritis and the Control Group

<table>
<thead>
<tr>
<th>Enteric Pathogen</th>
<th>Acute Enteritis</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;Duovirus&quot;</td>
<td>197 (52%)</td>
<td>0</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>27 (7%)</td>
<td>9 (8%)</td>
</tr>
<tr>
<td>Enterovirus</td>
<td>6 (2%)</td>
<td>9 (8%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>230 (61%)</strong></td>
<td><strong>18 (16%)</strong></td>
</tr>
</tbody>
</table>
Figure 1: AETIOLOGIC AGENTS OF ACUTE ENTERITIS IN CHILDREN IN MELBOURNE Nov.1973 – Oct.1974
Figure 2: MONTHLY PERCENTAGE OF PATIENTS INFECTED WITH DUOVIRUS SALMONELLA sp OR ADENOVIRUS
Relation of the "duovirus" to other known Enteric Pathogens

The monthly percentage of patients infected with "duovirus," *Salmonella* species or adenovirus is shown in Fig. 2. Disregarding the November percentage which was possibly misleading owing to the small total number of patients, infection with the "duovirus" showed a peak incidence of 73% in patients admitted with acute enteritis during early winter (June). The peak frequency of infection with *Salmonella* species (33%) occurred during late summer (March). Adenoviruses were most common during the months December, January and February, but as stated previously, the frequency did not differ from that of the control group.

**Age Group Affected by the "duovirus"**

Figure 3 shows that infection with the "duovirus" was a common cause of acute enteritis in each age group studied up to five years of age. The highest percentage of infections (66%) occurred in the six to 12 month age group. The youngest child affected was 10 days old and the eldest 12½ years.
Figure 3: RELATION OF DUOVIRUS EXCRETION TO AGE OF PATIENT

( ) total number of patients in each group
Duration of "duovirus" Excretion

Excretion of "duovirus" in 16 patients was followed during their stay in hospital and this is charted in Fig. 4. No virus particles were seen in faeces obtained from any patient after eight days from onset of symptoms. Maximum excretion of virus particles in stool seemed to occur on the third or fourth day from onset of symptoms. The numbers of virus particles in each stool specimen could only be estimated approximately with the electron microscope. This would tend to make the estimation of maximal excretion relatively accurate but the estimate of duration of excretion may be quite inaccurate due to lack of sensitivity. It is probable that the electron microscope would not detect virus particles if they were present in a concentration less than $10^5$ virus particles per millilitre of faeces.

During this study, one child, admitted twice with acute enteritis (aged six months and 14 months), showed "duovirus" particles in faeces on both occasions. Eight other children, readmitted for acute enteritis, showed no "duovirus" particles on second admission.

Mortality Due to the "duovirus"

There were four fatal cases of acute enteritis during the period of this study giving a mortality rate of 1%. The "duovirus" was located in faeces obtained just after death in one of three of these children. No faeces were obtained from
Figure 4: DURATION AND DEGREE OF DUOVIRUS EXCRETION IN FAECES FROM 16 CHILDREN WITH ACUTE ENTERITIS
the fourth child, but the "duovirus" was recovered from faeces of a sibling with acute enteritis examined at the same time.

Hospital-Acquired Infection

Twenty-five patients developed symptoms of acute enteritis whilst in hospital for another illness. The "duovirus" was found in faecal extracts from 22 of these patients. Four of these 22 children had been admitted to the gastroenteritis ward with chronic diarrhoea two to eight days before onset of acute enteritis with fever. Electron microscopy of faecal extract on the day of admission was negative. Twelve of the 22 patients had been admitted to one ward two to seven days before onset of acute enteritis. This ward is close to the gastroenteritis ward and shares some staff. The remaining six patients in whom acute enteritis developed were scattered in wards remote from the gastroenteritis ward.
DISCUSSION

This survey has shown that "duovirus" infection is the most common cause of sporadic acute enteritis in children less than five years old, requiring admission to hospital in Melbourne. For most of the year, approximately 50% of children admitted were infected with this virus, with a peak frequency of 73% during the winter. The frequency of infection declined to 23% during the hottest month of the year. The frequency of infection with this new virus might have been higher than determined in this survey. Excretion of virus ceased rapidly in some patients, and faeces obtained after four days from onset of symptoms might not have contained virus particles in sufficient numbers to be detected by the relatively insensitive method of electron microscopy.

It was not possible to assess the frequency of the "duovirus" in milder childhood enteritis not requiring admission to hospital, but it will obviously be an important area for future study.

The incubation period of "duovirus" infection is probably less than 48 hours, as judged from the group of patients acquiring infection in hospital. Infection with the virus seems to be brief, since no child was observed to excrete this virus after eight days from onset of symptoms. Excretion may persist longer but the present method of detection is not sufficiently sensitive to
enable a fuller assessment. Maximal excretion of virus particles seemed to occur on the third to fourth day from onset of symptoms of acute enteritis. This fact must be taken into account when planning similar surveys in other communities so that every effort is made to study patients as early as possible after onset of symptoms.

The disease can be fatal. Infection with the "duovirus" is the most common cause of enteritis developing in hospital, particularly in children less than 12 months of age, admitted to wards close to the gastroenteritis ward. The mode of spread, however, remains to be elucidated. Both hand to mouth and airborne spread are likely. Airborne spread of a morphologically similar virus causing epizootic diarrhoea of infant mice (EDIM) has been described (Kraft, 1957).

This survey also reveals other recognized enteric pathogens as much less important causes of acute sporadic childhood enteritis. Infection with Salmonella sp. is common only during the summer months. It is not clear whether adenovirus infection causes enteritis in some children. The role of E. coli in acute sporadic enteritis in children in developed countries needs to be re-evaluated in the light of the current findings. Serological types of E. coli regarded as "enteropathogenic" were seldom isolated in this survey and those that were failed to demonstrate enterotoxicity in a validated test system. However, tests looking specifically
for elaborated enterotixins were not performed. A previous survey of acute enteritis in children in Melbourne also failed to show evidence of colonization of the upper small intestine by enterotoxin producing strains of *E. coli* (Bishop et al, 1974b). These findings are in accord with a number of other surveys of acute sporadic diarrhoeal disease in children in which "enteropathogenic" serotypes of *E. coli* have been commonly found in asymptomatic as well as symptomatic individuals (Ramos-Alvarez and Sabin, 1958; Solomon et al, 1961).

No recognised enteric pathogens were identified in 20-30% of children admitted during most months of the year. Undetected infection with the new virus could explain the aetiology in some of these children. It is also possible that other undetected aetiological agents were present. For example, the parvovirus-like particle (Norwalk agent) which has been shown to cause enteritis in older children and adults (Kapikian et al, 1972) would not be revealed by techniques used in this study.

This new virus belongs to the family *Reoviridae* but differs in morphology, serology (Kapikian et al, 1974) and polypeptide composition (Rodger et al, 1975) from both of the recognized genera (orbivirus or reovirus). The virus is related morphologically and serologically to the viruses of epizootic diarrhoea of infant mice (EDIM) and Nebraska calf scours (Kapikian et al, 1974). In view of this it is
suggested that these three viruses causing enteritis in man, mice and calves, should be placed in a new genus called "duovirus" to describe their characteristic double-shelled capsid structure and to emphasize their involvement in enteric infection (Davidson et al, 1975b).
CHAPTER V

EVALUATION OF SERUM AND SECRETORY IMMUNOGLOBULINS

IN 20 CHILDREN WITH ACUTE ENTERITIS.
INTRODUCTION

Estimation of serum immunoglobulin (Ig) levels and secretory immunoglobulin A (SIgA) levels in duodenal juice have not been previously reported in children with acute enteritis. This study provided a good opportunity to investigate these parameters with the aim of determining whether there was any deficiency of humoral immunity which may predispose this group of children to acute enteritis. I collected clinical data and specimens and Dr. C. S. Hosking did the laboratory analyses.

Clinical Material

Serum immunoglobulin estimations were performed on 19 of the 20 children described in detail in Chapter II of this study.

Duodenal juice was assayed for SIgA levels in 17 of the 20 children described earlier.

Methods

Secretory Immunoglobulin A

Duodenal juice was collected as outlined previously (page 32). The juice was stored at -70°C for a maximum of six weeks prior to testing. Secretory immunoglobulin A (SIgA) levels in duodenal juice were measured by radial
immunodiffusion (Mancini et al, 1965). The standard used was pooled colostrum. Full strength was designated 100 u/ml. Standards were dilutions of full strength colostrum.

Serum Immunoglobulins

Blood was taken soon after admission at the time other blood tests were being performed. The serum was stored at -70°C until analysed. Immunoglobulin levels were measured by automated immuno-precipitation (Shelton et al, 1974 a). Levels were compared to percentiles for each age group as described by Shelton et al (1974 b).

Results

Table 23 shows the results of serum IgA and IgM levels and SIgA levels in duodenal juice in 19 children with acute enteritis. Serum IgA levels were within the normal range in all 19 children and are not included in the table.23.

Eight of the 19 children had serum IgM levels at or above the 95th percentile for age (Shelton et al, 1974 b). There was no apparent relation between the degree of elevation of IgM and the length of illness prior to testing. In six of these eight children the "duovirus" was found in duodenal mucosa or faeces.
TABLE 23

Serum Immunoglobulin M and A and Secretory Immunoglobulin A Levels in 19 Patients with Acute Enteritis

<table>
<thead>
<tr>
<th>Patient</th>
<th>Duration of Symptoms Before Test (days)</th>
<th>Age (months)</th>
<th>New Virus in Duodenum or Faeces</th>
<th>Serum IgM (%ile)</th>
<th>Serum IgA (%ile)</th>
<th>SIgA in Duodenal Juice (mg/100ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.J.</td>
<td>1½</td>
<td>31</td>
<td>Yes</td>
<td>81</td>
<td>91</td>
<td>Not tested</td>
</tr>
<tr>
<td>J.M.</td>
<td>1</td>
<td>8</td>
<td>Yes</td>
<td>40</td>
<td>80</td>
<td>12</td>
</tr>
<tr>
<td>N.S.</td>
<td>4</td>
<td>4</td>
<td>Yes</td>
<td>&gt;95</td>
<td>64</td>
<td>Not tested</td>
</tr>
<tr>
<td>C.K.</td>
<td>5</td>
<td>9</td>
<td>No</td>
<td>95</td>
<td>68</td>
<td>11.8</td>
</tr>
<tr>
<td>R.G.</td>
<td>4</td>
<td>5</td>
<td>No</td>
<td>11</td>
<td>94</td>
<td>7.2</td>
</tr>
<tr>
<td>G.M.</td>
<td>2</td>
<td>7</td>
<td>No</td>
<td>36</td>
<td>88</td>
<td>7.2</td>
</tr>
<tr>
<td>T.A.</td>
<td>3½</td>
<td>30</td>
<td>Yes</td>
<td>69</td>
<td>10</td>
<td>Absent</td>
</tr>
<tr>
<td>D.G.</td>
<td>3½</td>
<td>14</td>
<td>Yes</td>
<td>17</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>T.T.</td>
<td>4</td>
<td>19</td>
<td>Yes</td>
<td>37</td>
<td>67</td>
<td>18.0</td>
</tr>
<tr>
<td>A.D.</td>
<td>3</td>
<td>13</td>
<td>Yes</td>
<td>&gt;95</td>
<td>50</td>
<td>8.0</td>
</tr>
<tr>
<td>A.H.</td>
<td>3</td>
<td>4</td>
<td>Yes</td>
<td>63</td>
<td>95</td>
<td>Absent</td>
</tr>
<tr>
<td>T.F.</td>
<td>5</td>
<td>10</td>
<td>No</td>
<td>&gt;95</td>
<td>Trace</td>
<td>Absent</td>
</tr>
<tr>
<td>T.K.</td>
<td>4</td>
<td>6</td>
<td>Yes</td>
<td>95</td>
<td>Trace</td>
<td>Absent</td>
</tr>
<tr>
<td>P.M.</td>
<td>5</td>
<td>8</td>
<td>Yes</td>
<td>&gt;95</td>
<td>40</td>
<td>1.2</td>
</tr>
<tr>
<td>C.N.</td>
<td>4</td>
<td>2</td>
<td>Yes</td>
<td>80</td>
<td>49</td>
<td>10.0</td>
</tr>
<tr>
<td>A.P.</td>
<td>4</td>
<td>33</td>
<td>Yes</td>
<td>56</td>
<td>88</td>
<td>7.2</td>
</tr>
<tr>
<td>N.F.</td>
<td>1</td>
<td>11</td>
<td>Yes</td>
<td>95</td>
<td>74</td>
<td>Absent</td>
</tr>
<tr>
<td>K.B.</td>
<td>4</td>
<td>11</td>
<td>Yes</td>
<td>60</td>
<td>50</td>
<td>Absent</td>
</tr>
<tr>
<td>M.S.</td>
<td>3</td>
<td>18</td>
<td>Yes</td>
<td>95</td>
<td>67</td>
<td>Absent</td>
</tr>
</tbody>
</table>
In eight children SIgA was absent in duodenal juice. There is no normal range available with which to compare the results in the other children. Salivary IgA levels were not tested. Three of these eight children also had absent serum IgA. There did not appear to be any correlation between absence of secretory IgA and the degree of duodenal mucosal damage. Seven of the 13 children in whom the "duovirus" was present had absent secretory IgA (54%) compared to one of four children in whom the "duovirus" was not detected (25%).
DISCUSSION

Immunofluorescent studies have previously shown that the predominant antibody formed was of the IgM class (page 57). It has been well established that IgM is formed as a primary response immunoglobulin (Humphrey and White, 1971) and serum levels rise rapidly in the first year of life (Buckley et al, 1968). Savilahti (1972) in a recent study of children aged three months to 16 years showed that, although at all ages IgA plasma cells in the gut and IgA levels in the intestinal juice were higher than for other immunoglobulins, IgM plasma cells and intestinal juice levels were relatively higher in the younger groups. Thus, our finding that the major antibody response was of the IgM type was not unexpected.

Selective IgA deficiency has been defined by Ammann and Hong (1971) as -

(i) Serum levels of IgA ≤ 5 mg/100 ml
(ii) No deficiency of other immunoglobulins
(iii) Normal antibody mediated immunity
(iv) Normal cell mediated immunity

The incidence of selective IgA deficiency in the normal population has been variously estimated at between 1 in 500 (0.2%) and 1 in 700 (0.14%) making it the commonest immunodeficiency state.
In this study, three of the 20 children had absent secretory and serum IgA. This absence could not be explained on the age of the children. Specific studies of cellular immunity were not done, but a normal growth pattern and lymphocyte count rules out a major defect of cell mediated immunity. The incidence then of selective IgA deficiency in this group of children with acute enteritis is about 100 times that found in the normal population.

IgA appears to be the predominant immunoglobulin in intestinal secretions (Plaut and Keonil, 1969) and a number of studies suggest that locally produced antibody may play a major role in protection against gastrointestinal viral infections (Scott et al, 1972; Ogra, 1971). Thus, the finding here could imply that children with selective IgA deficiency are more prone to viral enteritis than normal children.

The finding in five children with acute enteritis, absent SIgA but normal serum IgA, although a theoretical possibility has not been previously described (Ammann and Hong, 1973). The method used to measure secretory IgA would not detect levels below 0.8 mg/100 ml (Mancini et al, 1965). However, it is unlikely that lack of sensitivity is the reason for the findings as the majority of children in whom secretory IgA was present had high levels. This would be in accordance with the findings of Agus et al (1974) who
demonstrated in vitro a rise in secretory IgA synthesis in jejunal mucosal biopsy specimens 48-72 hours after experimentally induced viral enteritis using the Norwalk agent.

It is known that loss of SIgA activity of the order of 10-20% may occur during storage at -70°C for a few months (Tomasi and Grey, 1972). In an attempt to prevent this loss all specimens were stored for a maximum of six weeks prior to testing. However, some loss of activity could have occurred during this time.

A further possible reason for the absence of duodenal secretory IgA in the presence of normal serum levels may be the blocking of secretion into the gut lumen due to epithelial cell damage. Brandtzaeg (1974) has proposed that secretory IgA is secreted by pinocytosis and viral damage to the epithelial cell may interfere with this mechanism. However, there was no clear correlation between the degree of histological damage and SIgA levels.

It will obviously be important to follow up these initial studies in order to determine their significance. However, it does appear at present that children with selective IgA deficiency are at a greater risk for needing admission to hospital with viral enteritis.
CONCLUSION

The main aim of the project was to apply the technique of electron microscopy to studying the aetiology of acute infectious non-bacterial enteritis in infants and young children. I hoped this would reveal an aetiological agent for the disease.

Electron microscopy of duodenal mucosa led to the recognition of virus particles of a new type, not hitherto associated with enteritis in humans, but a well defined cause of enteritis in calves and mice. The virus was found only in damaged duodenal epithelial cells from affected children. Virus particles were not seen in any other cell of the lamina propria. The morphology of the virus was the same in each of the 13 children in whom it was found.

Immunofluorescent studies confirmed the epithelial cell location of the virus and also indicated that there was antigenic similarity between virus particles from different patients.

A technique for detection of the virus in faeces was developed which provided a simpler, quicker and more sensitive method for locating the virus. Combining the results of electron microscopy from duodenal mucosa and faeces, this new virus was observed in 17 out of the 20 patients with acute enteritis. The virus was not found
in the duodenum or faeces from control children in and out of hospital. The technique of faecal extraction provided the means for locating the virus in a large series of children and a year long survey to investigate prevalence of the new virus in children with acute enteritis admitted to the Royal Children's Hospital, Melbourne was undertaken. The findings of the survey have conclusively shown the new virus to be the major aetiological agent of acute sporadic enteritis in infants and children in Melbourne. Recovery of the virus reached a peak of 73% during winter. All age groups up to five years of age were susceptible to infection, which was occasionally fatal. Duration of infection was brief with a probable incubation time of less than two days.

Serum immunoglobulin studies have shown the main response immunoglobulin to be of the IgM class. A markedly increased incidence of selective IgA deficiency was noted in the small group of 20 children studied initially. Also a possible new condition of isolated secretory IgA deficiency was uncovered. These findings will need to be confirmed by other studies but do suggest that children requiring admission to hospital with acute enteritis may be more susceptible to enteric infection.
The evidence that the "duovirus" is associated with acute enteritis is now unequivocal. Virus particles have been seen in the epithelial cells of duodenal mucosa from children with acute non-bacterial enteritis and in faecal extracts from the same children examined by electron microscopy. It infects epithelial cells only during the symptomatic stage of the illness when it is associated with histological abnormalities and depressed disaccharidase levels in duodenal mucosa. Seroconversion has been demonstrated in some children. During the 12 month survey, "duovirus" was not isolated from any of the controls studied.

Other known enteric pathogens were of minor importance in this survey which was conducted in a developed community. At the present time there has not been a complete survey looking for the "duovirus" in a developing country and thus the relative importance of this new agent in these areas is not known.

Further clinical, ultrastructural and biochemical characterization of this virus will depend on cell culture adaptation of the virus. This has already been achieved for the Nebraska calf scours virus (Mebus et al, 1971 b) a morphologically and antigenically related virus (Flewett et al, 1974).
Acute infectious non-bacterial enteritis is a major cause of death in childhood in developing countries. Until now, an aetiological agent could be found in only a small minority. The study I have described here clearly shows the "duovirus" to be the major cause of acute enteritis in children in Melbourne. If similar studies in both developed and developing countries confirm this finding then an aetiological agent for this major disease has at last been found. Vaccination against this virus would then be an important and realistic goal for future control of this serious childhood illness.

ADDENDUM

During the progress of this study it became apparent that our findings were very important. In order to obtain confirmation by others and also make people aware of this new virus various aspects of the study were published as the study proceeded.

The first confirmation of our work came from a group in Birmingham, England (Flewett et al, 1973). In 1974, workers in many different parts of the world, including Canada (Middleton et al, 1974), Rhodesia (Cruickshank et al, 1974), U.S.A. (Kapikian et al, 1974), India (Holmes et al, 1974), Norway (Orstavik, et al, 1974) and Japan (Konno et al, 1975), described finding similar particles in faecal specimens from more than 50% of children with acute enteritis. We also confirmed the presence of the virus in Singapore (Tan et al,
1974), Australian aboriginal communities in Central and Western Australia (Sexton et al, 1974) and in New Guinea (Bishop, R. F., personal communication).

A recent leading article in the Lancet (Leading article, 1975) provides an excellent summary of current thoughts on this new virus which was first found in Melbourne and has now been abundantly confirmed by others.
BIBLIOGRAPHY


