THE INTESTINAL ANTIBODY RESPONSE TO BACTERIAL GASTROENTERITIS IN HUMANS

Justin LaBrooy M.B.B.S., M.R.C.P.(U.K.), F.R.A.C.P.

Department of Medicine,
The University of Adelaide,
Adelaide.
SOUTH AUSTRALIA.

A thesis submitted for the degree of Doctor of Medicine

July, 1979
ABSTRACT

THE INTESTINAL ANTIBODY RESPONSE TO BACTERIAL GASTROENTERITIS IN HUMANS

This thesis examines the antibody response to bacterial gastroenteritis in humans with particular reference to antibody levels in the intestine.

While understanding of the secretory antibody system has advanced greatly through animal experiments and studies with vaccines, the intestinal antibody response to gastroenteritis has been inadequately documented. This area is reviewed and the lack of appropriate antibody assays for measuring intestinal antibody is pinpointed as one of the fundamental reasons for this.

The effect of different storage temperatures on antibody activity in intestinal fluid was examined in preliminary studies.

The development and validation of a radio-immuno assay for measuring intestinal antibody is described.

Using this assay the antibody response in serum and gastrointestinal secretions was measured on two occasions in 16 adults and 9 children after Salmonella and Shigella gastroenteritis. Their responses were compared with controls who were either healthy or recovering from a minor attack of gastroenteritis with no defined bacterial agent responsible.

The results showed a consistent antibody response in the intestine which was mainly in the IgA class. Taking
the patients as a group, the serum response was of the same order of magnitude as the intestinal response but this was not so in individual patients. The response in the intestine was early and could conceivably play a part in recovery from the disease. The duration of the response will require further study. Antibody in saliva and in the IgA class in the serum did not appear to reflect the antibody response in the intestine.

The relevance of these results to immunity in bacterial gastroenteritis and further studies that need to be undertaken in relation to the possible development of vaccination programmes against these diseases are discussed.
STATEMENT

The material in this thesis has not been previously submitted for a degree in any University, and to the best of my knowledge and belief, it contains no material previously published or written by another person except where due reference is made in the text.

J.T. LaBrooy.
I acknowledge with gratitude the interest and guidance of Professor Derrick Rowley and Professor David Shearman in these studies, the unstinted technical assistance of Judith Remes and Diana Pyle, and the willing co-operation of Sister Dorothy Evans in the jejunal intubations. Sue Dixon and members of her department helped with notifying me of suitable subjects for study. Dr. Geoffrey Davidson and his colleagues at the Adelaide Children's Hospital provided samples from the children studied. My thanks are also due to all the patients who consented to be studied and then came back for more, and to their physicians for allowing me to carry out these studies. I would like to record a special word of thanks to those colleagues who volunteered as controls and are still my friends. I am grateful to Kim Lowen for the preparation of the figures, to William Nolan for the photography and to Joan Devaney and Beth Jaworskyj for the typing of the manuscript.

Finally I record my gratitude to my family who put up with many trials and tribulations including Salmonella gastroenteritis while this work was being done.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>TITLE</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>STATEMENT</td>
<td>iv</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>v</td>
</tr>
</tbody>
</table>

## CHAPTER 1. MAN AND INTESTINAL BACTERIA

- Control of the commensal flora ........................................... 1
- Intestinal infections caused by bacteria ......................... 1
  - A. Enterotoxin production ........................................ 4
  - B. Local invasion ............................................... 6
  - C. Enteric disease with systemic invasion .................... 8
- Immune response to bacterial enteritis in humans .............. 10
  - A. The immune response and gastroenteritis-recovery .......... 11
    - i. Correlation between recovery and increase in the immune mechanism 11
    - ii. Intestinal infection in immune deficiency states .......... 13
    - iii. Restoration of deficient immune function ............ 14
  - B. The immune response and gastroenteritis-protection ........ 14
    - i. Does bacterial gastroenteritis or vaccination prevent infection 15
    - ii. Do measures of immune function correlate with resistance to infection 21
- Conclusion ................................................................... 22

## CHAPTER 2. IMMUNITY AND THE GASTROINTESTINAL TRACT .......... 24

- Historical .................................................................. 24
- Current concepts of the intestinal immune system ............ 25
Interaction of immune responses manifest at secretory surfaces and in the rest of the body 32
Problems in studying intestinal antibody 35
Sampling and measurements of intestinal antibody 35
Dilutional effects 39
The effects of protein degradation 41
Assay techniques 45
Studies of the intestinal antibody response to bacterial gastroenteritis in humans 49
Aims of this thesis 60

CHAPTER 3. MATERIALS AND METHODS 63
Bacterial strains 63
Tetanus toxoid 63
Gel chromatography 64
Ion-exchange chromatography 64
Affinity chromatography 65
Protein estimations 66
  Extinction co-efficients 66
  Quantitative Folin assay 67
  Single radial immuno-diffusion 67
Qualitative determinations of protein purity 69
  Immuno-electrophoresis 70
  Ouchterlony analysis 70
Precipitin analysis of antisera 71
Immunoglobulin and light chain preparation 71
  Immunoglobulin A 71
  Immunoglobulin G 72
Light-chains 73
  Secretory IgA 74
<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Stability of antibody during storage</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>Background</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>Design of experiment</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>Results</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>Conclusions</td>
<td>85</td>
</tr>
<tr>
<td>5</td>
<td>Radio-immuno assay</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>Description of assay</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>Detailed steps in assay</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>Shape of immune curve</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>Experiments in development of the assay</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>Effects of washes</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>Effects of time on binding</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>Effects of temperature on binding</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>Effect of carrier protein</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>Volumes of antibody containing fluid</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>Excess of bacteria</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>Method of preparing bacteria for use in assays</td>
<td>92</td>
</tr>
</tbody>
</table>
Specificity of assay

Natural antibody or non-specific binding in non-immune sera

Radio-labelled secondary antibody

Maximum binding of secondary antibody

i. Determination of antibody activity of secondary antibody

ii. Binding of secondary antibody to polymerised immunoglobulin

Saturation of antigenic surface of primary antibody by secondary antibody

Background binding

End-point

Variation intra-assay and inter-assay

Standardisation of the assay

Quantitation of the assay

Inhibition of binding of secondary antibody by purified secretory IgA and IgG

The assay and intestinal antibody

CHAPTER 6. STUDY AND RESULTS

Subjects studied

Results on individual subjects

Comparison of antibody response in serum and in intestinal aspirate

Pattern of change in antibody response with time

Relationship between intestinal and serum antibody in individual patients

Immunoglobulin class of antibody in serum, aspirate and saliva

Correlations of intestinal antibody with salivary and serum IgA antibody

High responders

Persistence of bacteria
CHAPTER 7. DISCUSSION

The measurement of antibody in intestinal fluid 117
Temporal pattern of intestinal antibody response 119
Relationship of intestinal antibody to other measures of humoral immunity 120
Class of antibody 123
Persistence of bacteria in the presence of antibody 124
High antibody responders 125
Improvement in methodology - The assay 126
Improvement in methodology - Inhibitors of proteolysis 128
Improvement in methodology - Controls 128
Further studies arising out of this thesis 129

APPENDIX - Results from individual subjects 131

BIBLIOGRAPHY 168

FIGURES After Page

CHAPTER 2.

Figure 56

II-1 Antibody response to cholera

CHAPTER 3.

III-1 Analysis of IgA using immuno-electrophoresis 74
III-2 Analysis of IgG using immuno-electrophoresis 74
III-3 Analysis of light chains using immuno-electrophoresis 74
III-4 Analysis of secretory IgA using immuno-electrophoresis 74
III-5 Analysis of Anti-IgA and Anti-α serum using immuno-electrophoresis 77
III-6 Analysis of Anti-IgG and Anti-γ serum using immuno-electrophoresis 77
III-7 Analysis of Anti light-chain serum using immuno-electrophoresis 77
CHAPTER 4.

FIGURES

IV-1  Vibriocidal activity of serum off a Sephadex G200 column. 83

CHAPTER 5.

V-1  Representative binding-curve in the radio-immuno-assay. 89
V-2  Effect of washing bacteria prior to adding secondary antibody. 90
V-3  Time of incubation (Primary) 90
V-4  Time of incubation (Secondary) 90
V-5  Variation in binding of intestinal antibody with temperature. 90
V-6  Volumes of antibody containing fluid 91
V-7  Excess of bacteria used as an immuno-adsorbent. 92
V-8  Different preparations of bacteria and their function in the radio-immuno-assay. 93
V-9  Specificity of the R.I.A. in detecting antibody 94
V-10  " " " " " " " 94
V-11  Effect of absorptions on binding of serum from 'normal' controls. 95
V-12  Maximum binding of secondary antibody 98
V-13  Saturation of primary antibody by secondary antibody. 99
V-14  " " " " " " " 99
V-15  Varying amounts of secondary antibody and the end-point. 101
V-16  Increasing binding with increasing amounts of secondary antibody. 101
V-17  Selection of a suitable end-point for assay. 102
FIGURES

VI-1  Intestinal antibody responses (Adults-u/0.1ml)  116
VI-2  Serum antibody responses (Adults-u/0.1ml)  116
VI-3  Time course of intestinal antibody response  
      (Adults-u/0.1ml)  116
VI-4  Time course of serum antibody response  
      (Adults-u/0.1ml)  116
VI-5  Intestinal and serum antibody response  
      (Adults-u/mg.Ig)  116
VI-6  Time course of intestinal antibody response  
      (Adults-u/mg.Ig)  116
VI-7  Time course of serum antibody response  
      (Adults-u/mg.Ig)  116
VI-8  Intestinal antibody responses (Children-u/0.1ml)  116
VI-9  Serum antibody responses(Children-u/0.1ml)  116
VI-10 Time course of intestinal antibody response  
       (Children-u/0.1ml)  116
VI-11 Time course of serum antibody response  
       (Children-u/0.1ml)  116
VI-12 Intestinal and serum antibody response  
       (Children-u/mg.Ig)  116
VI-13 Time course of intestinal antibody response  
       (Children-u/mg.Ig)  116
VI-14 Time course of serum antibody response  
       (Children-u/mg.Ig)  116
VI-15 Dissociation of serum and intestinal antibody responses  116
VI-16 Relationship of intestinal and serum antibody  116
VI-17 Relationship of serum IgA antibody and intestinal  
      antibody  116
VI-18 Relationship of salivary antibody and intestinal  
      antibody  116
VI-19 Antibody response in the different immunoglobulin  
      classes (Adults)  116
VI-20 Immunoglobulin class of antibody in the serum of  
      children  116
### TABLES

#### CHAPTER 1.

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>After Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-1</td>
<td>Bacteria causing gastroenteritis and mechanisms implicated</td>
<td>4</td>
</tr>
<tr>
<td>I-2</td>
<td>Attack rates of Typhoid in various groups in the second outbreak in the same community</td>
<td>15</td>
</tr>
</tbody>
</table>

#### CHAPTER 4.

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>After Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV-1</td>
<td>Stability of IgM antibody in intestinal fluid at room temperature</td>
<td>84</td>
</tr>
<tr>
<td>IV-2</td>
<td>Stability of IgG antibody in intestinal fluid at room temperature</td>
<td>84</td>
</tr>
<tr>
<td>IV-3</td>
<td>Decay of IgG antibody in intestinal fluid at room temperature</td>
<td>84</td>
</tr>
</tbody>
</table>

#### CHAPTER 5.

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>After Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>V-1</td>
<td>Reduction of non-specific binding by using goat serum</td>
<td>90</td>
</tr>
<tr>
<td>V-2</td>
<td>Absorption of serum antibody as a test for specificity</td>
<td>94</td>
</tr>
<tr>
<td>V-3</td>
<td>'Kaufman-White' defined antigens of bacteria used in studies of specificity of the assay</td>
<td>94</td>
</tr>
<tr>
<td>V-4</td>
<td>Relationship of titre to end-point selected in assay</td>
<td>102</td>
</tr>
<tr>
<td>V-5</td>
<td>Quantitation of assay</td>
<td>104</td>
</tr>
</tbody>
</table>

#### CHAPTER 6.

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>After Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>VI-1</td>
<td>Comparison of antibody levels in controls with groups of patients</td>
<td>110</td>
</tr>
</tbody>
</table>
CHAPTER 1

MAN AND INTESTINAL BACTERIA

Nowhere is the spectrum of relationships that man has with bacteria as well seen as in the gastrointestinal tract. $10^{14}$ commensal bacteria at a conservative estimate are carried in the gut of a healthy adult (Gorbach and Levitan, 1970). This commensal flora can be further subdivided into 'resident' species and others that may be 'transient' visitors. The numbers of bacteria and the species predominating at various levels of the gastrointestinal tract remain fairly constant in health (Drasar and Hill, 1974).

This normally harmonious and symbiotic relationship between man and his fellow travellers is from time to time disrupted by the effects of 'pathogenic bacteria' which cause disease. That the term pathogen is appropriate when Salmonella typhi-murium causes gastroenteritis is undoubted; 'pathogens' may however be transiently present causing no symptoms, or be found for a variable time in the 'carrier state' after an attack of gastroenteritis. In these contexts the distinction between commensal and pathogen blurs. It may also be challenged by the ability of some normal 'commensal' species like Escherichia coli or Aeromonas to cause gastroenteritis in susceptible hosts.

Control of the Commensal Flora.

A wide variety of mechanisms have been demonstrated to play a part in maintaining the stability of the bacterial flora (Brandtzaeg, 1972); no doubt many others remain to be elucidated.
Many of these mechanisms are non-specific. To discuss one example: the most clearly demonstrated function of gastric acid is its role in the control of the gut flora. Hypochlorhydria greatly reduces the number of pathogenic organisms that need to be taken by mouth to cause gastro-enteritis. This has been well demonstrated for cholera (Cash et al., 1974 (a) ) and for Salmonella gastroenteritis (Giannella et al., 1971). It also allows a large increase in the numbers of commensals in the stomach and upper intestine (Drasar et al., 1969). Gastrointestinal motility, oxygen levels, the availability of iron and other nutrients within the bowel, the complex inter-relationships of the bacteria themselves and their products such as fatty acids and colicines are among the other non specific factors that may control the eco systems of the human gastrointestinal tract. This subject has been reviewed in depth by Drasar and Hill (Drasar and Hill, 1974).

Immunological mechanisms may provide a greater specificity in the control of the bacterial flora found in the intestine of the human host. Natural antibody is found in the serum and presumably the secretions of man and animals as they mature (Topley and Wilson, 1964). Mainly of the IgM class and of low affinity, natural antibodies may appear as part of the maturation process of the immune system without any antigenic stimulus whatever. On the other hand they may be stimulated by inapparent infections or contact with the specific antigens against which they are directed or by cross reacting antigens stimulating the plasma cells which produce them. They may well play a part in the natural resistance
to infection with pathogens. Even more specific control may be exerted by antibody that is stimulated by organisms that colonise the host. Animal studies where gnotobiotic animals were allowed to acquire a natural bacterial flora, suggest that the gut flora is a major stimulus to the production of serum immunoglobulins (Se1l and Fahey, 1964). There is clear evidence of a change in the small intestinal bacteria in pan-hypogammaglobulinaemia (Brown et al 1972). Progressive change in the antigenic structure of bacteria introduced into gnotobiotic mice apparently in response to antibody production against the bacterial antigens has also been demonstrated (Miller et al., 1972).

On the whole though, non specific mechanisms appear more significant than immunological ones in the control of commensal gut flora. The evidence for specific immunological mechanisms having an impact on the microbial ecology of the gut to the same extent as the lack of stomach acid, or interference with the normal flow of intestinal contents in 'stasis syndromes' do, is scant. It must be borne in mind however, that this lack of evidence for control of the gut flora by immune mechanisms may only reflect the difficulties in studying intestinal immunity (which will be discussed in the next chapter) and the enormous complexity of the commensal flora.

Intestinal Infections Caused by Bacteria

The importance of intestinal infection needs little emphasis. On a global scale it is probably the most common single cause of death. In underdeveloped countries it is intimately linked with malnutrition in a vicious circle of
cause and effect. Even in the most highly developed countries it is a major cause of paediatric morbidity and mortality. Though recent work has confirmed the long held suspicion that viruses are an important cause of gastroenteritis (Schreiber et al., 1977), there seems little doubt that in terms of severity of disease and net morbidity, bacterial pathogens cause more damage in the gut than their viral counterparts.

A wide variety of organisms can cause gastroenteritis (Table I-1). As mentioned earlier, the certainty with which any given attack of gastroenteritis can be ascribed to a species of bacteria found in the intestine of the patient, varies with the organism.

The patho-physiological mechanisms and host pathogen interactions vary considerably in different forms of bacterial gastroenteritis. This may be very relevant to the immunological response of the host and the impact of this response on the pathogen. The more important mechanisms, and their implications in relation to the generation of host-immunity and response of the pathogens to it will be discussed.

A. Enterotoxin Production

The classic example of a bacterium that multiplies within the gut and produces a toxin responsible for the manifestations of the disease is Vibrio cholerae. Studies in the late 1950s (Gangarosa et al., 1960) demonstrated that the intestinal mucosa of patients with cholera was intact and that the diarrhoea was a result of a biochemical disruption of epithelial cell function. An enterotoxin produced by Vibrio
TABLE I-1  Bacteria causing gastroenteritis and mechanisms implicated in each case.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Enterotoxin production</th>
<th>Invasion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus cereus</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Campylobacter</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Clostridium botulinum</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Clostridium difficile</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Clostridium perfringens</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Escherichia coli (some strains)</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Escherichia coli (some strains)</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Salmonella</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Shigella dysenteriae</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Shigella (other species)</td>
<td>?</td>
<td>+</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Vibrio cholerae</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Vibrio parahemolyticus</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Yersinia enterocolitica</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>* Aeromonas</td>
<td></td>
<td></td>
</tr>
<tr>
<td>* Pleisomonas shigelloides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>* Proteus mirabilis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>* Pseudomonas aeruginosa</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>* Streptococcus faecalis</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Indicates suggested (but unproven) causes of gastroenteritis.
Cholera was identified (Craig, 1965) and the link between this toxin and the choleraic diarrhoea is now generally accepted (Field, 1971). The enterotoxin attaches itself to the epithelial cells, activates adenyl cyclase to produce more cyclic AMP in these cells and this results in a nett flux of salt and water into the bowel lumen. Though there is rapid multiplication of the Vibrios in the bowel lumen during the disease, there is no evidence that Vibrios or their products need to enter the host tissues as an intrinsic part of the disease (Curlin, 1977). This means also that such antigen interaction as there is with host cells is almost entirely at the local level of the intestinal mucosa and the main antigenic stimulus is here. The corollary of this is that if the disease is affected by the immune process this must happen within the lumen of the gut. In animal systems the ability of these antibodies to agglutinate the Vibrios and to prevent them binding to the gut wall has been clearly shown (Freter, 1969; Steele et al., 1977). These are to date the only methods shown by which immunity could control the infection. It is also postulated as the method by which antibody protects against infection. There have been hints for a role played by antitoxin antibodies (Holmgren & Svennerholm, 1977) but this too would be for antibody acting at the epithelial surface or within the gut. The consensus of opinion is that the antibody demonstrated within the gut in human cholera is mainly secretory antibody and not transported serum antibody (Waldman et al., 1972), though the demarcations in this area are not clear. In a canine model of cholera the ability of serum antibody to protect against infection is good (Pierce and Reynolds, 1974). There is no
evidence that cells act within the lumen of the gut against bacteria, and so it seems unlikely that cell mediated immune mechanisms play any role in cure or resistance to the disease.

Escherichia coli species can cause gastroenteritis in a variety of ways (Du Pont, 1977). Enterotoxin production without invasion, causing disease closely analogous to cholera (Formal et al., 1971), is one of them.

B. Local Invasion

Gastroenteritis due to Salmonella enteritidis and Shigella species are characterised by local damage caused by bacteria multiplying in the intestinal mucosa. There is a variable degree of bacteraemia (Hook and Guerrant, 1977) in these infections but the predominant host-bacterial interaction is in the tissues and the lumen of the gut. In an animal model of Salmonella infections, penetration of the organisms into the subepithelial tissues is a feature of the disease (Takeuchi & Sprinz, 1967). The anatomical level of the intestine predominantly involved is uncertain (Drasar & Hill, 1974). The large intestine or the small intestine may be the main area of involvement. The mechanisms of the diarrhoea in Salmonella gastroenteritis are uncertain. No enterotoxins have been shown to be produced by Salmonellae up to now. The diarrhoea can be inhibited by Indomethacin and appears to be a prostaglandin driven secretory diarrhoea (Giannella et al, 1975).

In Shigella infections the pathogen invades and multiplies within the lining epithelial cells of the colon and this gives rise to widespread superficial ulceration. Enterotoxin production by Shigella dysenteriae that causes a secretory
diarrhoea has been recognised for some time (Keusch et al., 1970). Recent studies suggest that this same or a similar toxin may be produced by other Shigella species, possibly by all of them, and may be instrumental in fluid loss (Keusch and Jacewicz, 1977).

While some strains of E. coli cause gastroenteritis by enterotoxin production, others invade epithelial cells and produce a disease analogous to shigellosis in humans (Du Pont et al., 1971).

While the immunological stimulus to the host's immune system is clearly maximal in the gastro-intestinal tract in these infections, there is a distinct difference from the possible interactions outlined above with regard to cholera. The bacterial invasion of tissues implies that there is much greater likelihood of a barrage by bacteria of the extra-intestinal portion of the immune system. This systemic stimulus may vary in degree and duration between patients so that the immune response is probably correspondingly variable.

The effector limb of the immune response probably varies significantly in terms of its effects on invading bacteria, from its role in infections like cholera where the infection remains intra-luminal. Secretory antibody could clearly play the same role in the lumen of the gut and stop further entry of organisms. Serum antibody and leucocytes of all types, polymorphs, lymphocytes and macrophages, interact in the tissues against the invading bacteria. In addition, serum antibody probably diffuses into the lumen through the ulcerated mucosa to a greater extent than where there is no overt mucosal ulceration. Bacterial killing by
antibody of the IgG and IgM classes through complement mediated lysis, and phagocytosis and destruction of bacteria opsonised by these antibodies and complement are potential mechanisms whereby the tissues could be cleared of the invading organisms. The pathological appearance of the intestine under the microscope emphasises the cellular response (Robbins, 1967).

The position with regard to protection against the disease is less certain. Secretory antibody could, as in the enterotoxigenic type of disease, protect against pathogen colonisation of the gut by these bacteria and their penetration could be prevented. The possibility of serum antibody or cell mediated mechanisms playing a part in protection is unlikely. However, it must be borne in mind that disease in this group is synonymous with tissue invasion. It is conceivable that a secondary immune response involving cells and possibly serum antibody could be mounted in the incubation period of the disease and overcome the incipient tissue infestation.

C. Enteric Disease With Systemic Invasion

Typhoid is the classic example of a bacterial disease where entry is via the gastrointestinal tract, with bacterial multiplication and the disease process being both locally in the gut and in systemic sites throughout the body. Some of the Salmonella enteritidis group (e.g. Salmonella paratyphi) may in individual patients behave in the same way as Salmonella typhi.
The microscopic pathology in typhoid reveals the bacteria multiplying within cells of the reticuloendothelial system in the gut, spleen, liver and other tissues with predominantly a macrophage and lymphocyte response. (Robbins, 1967). Organisms can be isolated by blood culture in the first 7-14 days of the infection and may also be found in the urine and stools. The local immune response within the gut has not been well documented but Peyer's patches and the lamina propria of the small intestine are centrally involved in the characteristic pathology of the disease. Widal described the serum antibody response to this disease in the 1890s (Widal, 1896) and cellular immune mechanisms have been shown to be heightened (Kumar et al., 1974) not surprisingly.

There is clearly antigenic stimulation of immune mechanisms in the gut and systemically to a marked degree. The efferent limb of this arc could act at a variety of sites. The fact that typhoid bacilli appear to multiply within macrophages means that termination of infection is dependent on a heightened ability of these cells to kill the organisms as a prerequisite for cure. This does not rule out a role for serum antibody which may play a part in opsonising the bacteria. However, the fact that relapses of typhoid fever occur in the presence of high levels of serum antibody is strong argument against serum antibody being of major importance in cure or prevention of the disease (Hornick et al., 1970). Intestinal antibody may be of importance in the immunity of the carrier state in typhoid where virulent typhoid bacilli are poured out of the intestine with no invasion or disease of the host's tissues apart from the gall bladder, where the organisms multiply.
(Hornick et al., 1970) at least in some carriers.

The animal model approximating most closely to human typhoid is that of Salmonella typhi-murium or Salmonella enteritidis in mice. There is intracellular proliferation of the pathogen in macrophages and current concepts of immunity to intracellular bacterial parasites suggest that this depends largely on the ability of specifically immune T cells to activate macrophages (North 1973) and that while serum antibody may aid the process it is not central to immunity to Salmonella (Davies and Kotlarski, 1976).

It must be borne in mind that while many of our concepts in terms of immunity to gastrointestinal infections have originated or gained a more solid basis from animal models, the fact that these infections are not natural to the animal must add to the reservations with which the results are transposed to the human situation.

**Immune Response to Bacterial Enteritis in Humans**

As discussed above, the immune response to gut infections or vaccination against these infections may be manifest as increased levels of antibody and as heightened specific cellular activity against these bacteria. This heightened immune reactivity may be seen in the immunoglobulin and the cells of the general circulation and tissues. It may also be seen in the intestine and in other gut associated tissues. There may be dissociation in the immune response in these two areas and the next chapter will review current concepts of the local immune response in the intestine with particular reference to intestinal infection.
Though the work to be described in this thesis relates to infection with Salmonella enteritidis and Shigella, most of the data available pertaining to bacterial gut infections in man is from studies on cholera and typhoid.

Among the possible benefits of the immune response to bacterial infections are that it may be instrumental in (1) terminating the disease and (2) preventing another attack.

A. The Immune Response and Gastroenteritis - Recovery

In assessing whether a disease is terminated by specific immunological mechanisms, evidence normally derives from three sets of observations:

i. Consistent correlation between recovery and a measured increase in the activity of the immune mechanism thought to be responsible.

ii. An increase in frequency of the infection or failure of recovery from it in immune deficiency states affecting that particular immune mechanism.

iii. Recovery from the infection by subjects with the immune deficiency through restoring, by passive transfer of immunoglobulin or cells, the absent immune function.

i. Correlation Between Recovery and Increase in the Immune Mechanism.

In studies of patients with cholera, both Freter (Freter et al., 1965) and Waldman (Waldman et al., 1972) and their colleagues demonstrated the appearance of antibody in intestinal fluid consistently. In Freter's study of 20 patients, using a
Farr assay, an antibody response was present in all of them by the fourth day after admission which is in keeping with the hypothesis that intestinal antibody is responsible for terminating infection. A rise in serum vibriocidal antibody was seen in 64 out of 66 subjects who developed diarrhoea after oral inoculation with cholera in another study (Cash et al., 1974) with the maximum titre being at 2 weeks, the time of first sampling after disease. About 75% of subjects in Waldman's study also showed a serum antibody response that was well developed by 2 weeks after infection. As discussed above, the relevance of this serum antibody response appears much less to control of infection, than that of antibody in the intestine.

Similarly in a study of children with enteropathogenic E.coli (epEc) infection, McNeish (McNeish et al., 1975) claimed that an intestinal antibody response could be consistently demonstrated very early after the start of infection. No consistent serum antibody response was seen. Whether the E.coli infection involved enterotoxin production or invasion was not discussed. They mention, en passant, that in three patients with protracted E.coli enteritis, intestinal antibody levels were low. Certain reservations with regard to this particular study will be discussed in the next chapter but the results, if taken at their face value, lend further support to the concept that the appearance of intestinal antibody may play a role in the cessation of disease.

In typhoid the Widal test has been used from the last century for the diagnosis of acute typhoid by detecting a rise in serum antibody levels. Such a rise did not however imply that the infection would be terminated and as already
has been mentioned, clinical recovery from typhoid could be followed by relapse in the presence of high serum antibody levels. As mentioned earlier, current concepts of recovery from typhoid based mainly on animal studies, point to the importance of cellular rather than humoral mechanisms in recovery. A dissociation between cellular and humoral immune responses could therefore possibly explain the lack of a consistent relationship between serum antibodies and recovery from typhoid. This must remain speculative in the absence of sensitive measures of cell-mediated immunity and no good published data even with such measures (delayed type hypersensitivity and macrophage migration inhibition) as do exist.

Reed and Williams have published a study of the copro-antibody response to Shigellosis in which they demonstrate an antibody response compared to controls (Reed and Williams 1971). This study which is discussed in the next chapter is open to criticism with regard to methodology.

ii. Intestinal Infection in Immune Deficiency States.

Persistent diarrhoea is seen fairly commonly in immunodeficiency states. The association is most marked in subjects with T cell deficiency in whom the bacteria which cause gastroenteritis by invasion such as Salmonella, Shigella and Escherichia coli are responsible for the diarrhoea. This serves to underline the importance of the cellular arm of the immune response in terminating infections associated with tissue invasion. (Katz and Rosen, 1977). Giardia lamblia, a protozoan parasite, clearly causes diarrhoea frequently in
subjects with panhypogammaglobulinaemia (Parkin et al., 1972) but bacterial gastroenteritis caused by enterotoxin-producing non-invasive pathogens is distinctly uncommon in these subjects with a predominantly B-cell deficiency syndrome. A speculative suggestion for why the enterotoxin-producing non-invasive pathogens are not found more frequently as a cause of disease in subjects with gastro-enteritis is that this defect is lethal where these forms of gastroenteritis are common (Pierce and Rosen 1977).

iii. Restoration of Deficient Immune Function.

Thymic transplants when successful, result in cessation of the diarrhoea seen in T cell deficiency states, further suggesting that the deficiency of T cells is responsible for the persistence of pathogens.

In summary then, there is suggestive evidence that the recovery from at least some forms of gastroenteritis is a result of a specific immune response but as will be discussed later the evidence is too scanty to conclude this with certainty.

B. The Immune Response and Gastroenteritis - Protection.

There is more published data that can be considered in relation to the question "Does the immune response to bacteria that cause gastroenteritis prevent such an attack?" The complexity of this question can be simplified into the two parts:

(i) How well does one attack of bacterial gastroenteritis or vaccination protect against infection with the same organism?
(ii) How well do measures of immune function correlate with resistance to infection?

(i) Does Bacterial Gastroenteritis or Vaccination Prevent Infection?

The observation that a disease mainly afflicts children in areas where it is endemic, whereas in other countries epidemics of the same disease affect adults and children alike, is taken as evidence for immunity playing a role in prevention of the disease (Burnet, 1962). This so-called 'herd immunity' is seen in areas where cholera (Mosley 1969) and typhoid (Hornick 1970) are endemic. That this may be a relatively minor degree of immunity and apparent only with ingestion of low doses of the infecting organism must be borne in mind (Hornick et al., 1970).

Marmion's publication (Marmion et al., 1953) on recurrences of typhoid in a Royal Air Force unit in Suez where there were two outbreaks of typhoid of different phage types in quick succession is the most widely quoted and possibly the only relevant observation on this aspect of the natural disease in the last 50 years. In his introduction Marmion reviews the literature up to that time and states "the general impression conveyed by the literature is that an attack of typhoid fever confers a considerable degree of immunity but that recurrences occasionally take place". As seen from the figures taken from this report (Table I-2) there was evidence of marginal protection against infection with attack rates of 20.4% compared to 31 - 38% in patients who had an attack of typhoid less than five months before compared to those who had not. The point is made that this
TABLE I - 2. Attack rates of typhoid in various groups in the second outbreak in the same community.

<table>
<thead>
<tr>
<th>Description of group</th>
<th>Number in group</th>
<th>Number contracting typhoid</th>
<th>Attack rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men exposed to infection in second outbreak (total).</td>
<td>688*</td>
<td>235</td>
<td>34.2</td>
</tr>
<tr>
<td>Men exposed but not infected in first outbreak, re-exposed in second.</td>
<td>384</td>
<td>146</td>
<td>38.0</td>
</tr>
<tr>
<td>Men infected in first outbreak, re-exposed in second.</td>
<td>54</td>
<td>11</td>
<td>20.4</td>
</tr>
<tr>
<td>Newcomers to station between the outbreaks, exposed to infection in second outbreak.</td>
<td>250</td>
<td>78</td>
<td>31.2</td>
</tr>
</tbody>
</table>

* Including 57 men who joined the station after the infected meal, only one of whom, a medical orderly employed in the sick quarters, contracted typhoid fever.

from Marmion et al., J. of Hygiene (Cambr.) 1953; 51: 260-267.
is the most favourable interpretation that can be given to the figures in support of the theory that previous infection protects, as many of the 54 who were re-exposed did not partake of an infected meal which triggered off and was the main cause of disease in the second outbreak. Another point of interest with regard to this outbreak is that all exposed individuals had received TAB vaccine, usually of the alcoholized variety. The conclusion reached by the authors is that "the specific immunity conferred by an attack of typhoid fever is of no more than moderate degree and in the event of re-exposure recurrences are not very unusual."

The only other data on second attacks of typhoid in the recent literature comes from the volunteer study carried out in the University of Maryland (Hornick et al., 1970 a and 1970 b). Few details are given of this aspect of the study. Volunteers who had previously had disease were rechallenged 2 - 12 months later with an identical dose of organisms as had caused disease on the first exposure. The fact that only 25% of these subjects became ill a second time was taken as evidence of "substantial immunity". It is difficult to accept this interpretation on the published evidence especially in view of the fact that the most commonly used dose in these studies, of \(10^5\) organisms, resulted in a disease rate of 28% on first exposure.

With regard to these studies it must be remembered that antibiotic treatment which possibly aborted the first attack before the full development of immunity and, in the case of Marmion's study, a change of phage type in the infecting organism may have predisposed to the second attack.
Typhoid vaccines have been widely used since the end of the 19th century. The first properly controlled field trial (Yugoslav Typhoid Commission, 1962) demonstrated that an alcohol-killed alcohol-preserved vaccine in use at that time, thought on theoretical grounds and on the results of animal studies to be most effective, was of low efficiency. Trials have demonstrated repeatedly, then and since, the effectiveness of vaccines in protecting against typhoid (Heijec, 1965; Polish Typhoid Committee, 1966; Ashcroft et al., 1964). The most impressive results are probably those of the field trial with an acetone killed vaccine in Guyana (Ashcroft et al., 1967) where 90% protection was shown over 7 years in a cohort of school children (5 - 15 years at the start of the study) with little diminution in protection until the fifth year after vaccination. This trial also suggested that one dose of the vaccine would probably suffice to achieve this protection. While the acid test of a vaccine is its effectiveness in the field, field trials too have their limitations. The degree of protection imparted by a vaccine may vary considerably in different age groups and in different areas reflecting differences in immune status prior to vaccination of the groups, and also the different patterns of exposure to the disease (Ashcroft 1967). The vaccines have been demonstrated to be effective in these studies compared to a 'placebo' control vaccine. In one trial (Yugoslav Typhoid Commission, 1964) the degree of protection against typhoid conferred by a 'placebo', Tetanus Toxoid, given to the control group, compared to individuals given no vaccine at all, was outstanding. This serves to underline the importance of other factors such as possible increased health awareness
arising from being part of such a study, that could unforeseeably influence results.

Volunteer studies by the Maryland group (Hornick et al., 1970 (a) and (b)) using this same acetone-killed vaccine shown to be the most effective in field trials, could demonstrate protection only with an ID$_{25}$ challenge dose of $10^5$ organisms. (ID$_{25}$ dose is the dose which results in disease in 25% of normal volunteers). The effectiveness at this dose level was 67% but no protection could be shown after prior immunisation, on challenge with an ID$_{50}$ dose of $10^7$ organisms. While part of the reason for this may be that the subjects in these studies had probably no previous exposure to the typhoid antigens in contrast to the subjects immunised in the field trials, it emphasizes the fact that the "immunity" may only be relative, apparent at one level of challenge but not at another.

In addition to these results with parenteral typhoid vaccines, protection against typhoid has been demonstrated after oral vaccination with attenuated typhoid organisms. While there are suggestions that these may confer a comparable or even greater degree of protection than the parenteral vaccines (Gilman et al., 1977), the duration of this effect is uncertain and there are no results of controlled field trials with these agents as yet.

The degree of immunity conferred by an attack of cholera to a second attack seems to be not very different to the position with regard to typhoid. Robert Koch noted in 1884 that acquired immunity to cholera "does not seem to persist for a long time because there is a sufficiency of examples to show that an individual who had been affected with cholera
during one epidemic fell ill with cholera a second time during another epidemic; but one hears but rarely that somebody had been attacked twice during the same epidemic". (Koch, 1884).

Reviewing the literature in the WHO monograph on cholera in 1959, Pollitzer echoed Koch's opinion. (Pollitzer 1959).

Woodward's study of cholera re-infection published in 1971 was the first study where both attacks of cholera were confirmed bacteriologically. (Woodward 1971). This study of 14 patients with two documented attacks of cholera suggested that the duration of immunity was short especially against re-infection with a heterologous organism. (8.8 months with a range of 1.5 to 29 months with heterologous infection; 27.3 months with a range of 11 to 60 months with the homologous organisms). The risk of re-infection appeared only slightly less than the risk of initial infection. The results in a volunteer study of resistance to a strain of cholera in the University of Maryland, on rechallenge after one attack, demonstrated immunity as judged by absence of diarrhoea, 2-12 months after the first attack, compared to a diarrhoea rate of 80% in controls with no previous exposure. When the rechallenge was with a heterologous organism, diarrhoea occurred in 4 out of the 6 subjects challenged suggesting little protection across the Ogawa-Inaba serotype difference in this group of subjects who did not live in an area where cholera was endemic.

In summary then, the evidence in 1978 is in keeping with Koch's comments in 1884, and Pollitzer's impression in 1959: cholera induces a moderate degree of short lived immunity against a second attack.
1885 saw the first attempt at vaccination against cholera by Ferran (Pollitzer, 1959). In 1927, A.J.H. Russell commented after a large scale trial of cholera vaccine "the case in favour of anticholera vaccine as a practical and cheap preventive measure is complete" (Russell, 1927). Yet Curlin in his review of cholera vaccination at the 1976 World Congress of Immunology (Curlin, 1977) agreed with Finkelstein's summary of 3 years before (Finkelstein, 1973) "some cholera vaccines offer a limited degree of protection for a limited period of time in some population groups". The most intensive and critical appraisal of cholera-vaccination was the series of field trials conducted under the auspices of the South East Asian Treaty Organisation in Bangla Desh. The results of these studies using whole cell cholera vaccines were published in the Bulletin of the WHO between 1964 and 1973. The initial reports were optimistic with the main conclusions reached as follows: the fall in case rate with age as well as with cholera vaccination correlated closely with the level of vibriocidal antibody titre; lower infection rates were seen in contacts with higher titres; the cholera infection rate fell about 50% with doubling of vibriocidal antibody titre. Though protection was around 80% in the season following 3 doses of a mixed Inaba-Ogawa vaccine, it dropped to 50% and 39% 1 and 2 years after vaccination respectively. In the later studies (Mosley and Aziz, 1972; Mosley et al., 1973) the claim that levels of vibriocidal antibody titre correlated with protection against infection was retracted and the cost effectiveness of cholera vaccines were found to be far less than that of the appropriate treatment for cholera. Trials with mono-
valent and toxoid vaccines have not suggested any of them as suitable for field use, though an Inaba vaccine gave 84% protection against Inaba cholera over 3 years follow up (Mosley et al., 1973). Field trials from the Phillipines in this period gave even less hopeful results with a moderate degree of very short lived immunity following parenteral vaccination (Azurin et al., 1967). No oral vaccination programmes can up-to-date, match even the meagre success of the parenteral vaccination schedules.

(ii). Do Measures of Immune Function Correlate with Resistance to Infection?

Benenson's review of the WHO sponsored field trials in British Guiana, Poland and Yugoslavia summarises the data that there was at the time with regard to serum antibody and protection from disease after vaccination against typhoid. No correlation with O and Vi antibodies were seen and the correlation with H antibodies did not, on other grounds appear to be a causal relationship (Benenson, 1964). Lack of any relationship between serum antibody and protection against challenge both after typhoid infection and anti-typhoid vaccination was found in the studies of the Maryland group (Hornick et al., 1970, b; Gilman et al., 1977).

In cholera, as mentioned before, initial reports held out hope that the pre-existing serum vibriocidal activity was a good index of protection against disease (Mosley, 1969). Further studies however proved that this was not the direct relationship initially hoped for (Mosley and Aziz, 1972; Mosley et al., 1973; Cash et al., 1974) particularly in individual patients. The original observation that serum
antibody levels of the "herd" are higher in endemic areas and there is some degree of "herd immunity" in these areas still is valid, though no more direct a relationship than this has been established.

It is clear then, as might have been predicted from the concepts outlined before of these and other enteric disease, that serum antibody plays little direct role in protection against them.

There are no published reliable estimates of other measures of immunity such as intestinal antibody or cell mediated immunity active in protection against these diseases, in humans. Hints of locally active protection come from the studies of the Maryland group (Hornick et al., 1970 a; Gilman et al., 1977) where prior oral vaccination decreases faecal excretion of Salmonella typhi on challenge compared to unvaccinated controls. That this cannot be the only means of protection possible is evident from the fact that parenteral vaccines effective in protection do not lessen faecal shedding of S.typhi.

Conclusion

Hetsch commented over 60 years ago, "The experience that the agglutinin and bacteriolysin content of the blood in man and animals is considerably higher after cholera vaccination than after spontaneous cholera attacks and that nevertheless even after the slightest spontaneous cholera attack the immunity is very considerably higher than after vaccination justifies the assumption that recovery from cholera produces a local immunity of the intestine which is not produced to such a degree in animal experiments and
through vaccination."

(Hetsch, 1912).

The observations reviewed in this chapter, namely that the gastroenteritides are self limiting (if not fatal in the short term), the modest degree of immunity to re-infection after recovery from cholera or typhoid, the increased resistance following vaccination in the short term against cholera, and in the longer term against typhoid, and the age associated immunity seen in areas where the disease is endemic, along with the poor correlation of serum antibody with immunity, underline the insight in these comments. The work done on local intestinal immunity to gastroenteritis and the reasons for the slowness of its progress will be reviewed in the next chapter.
CHAPTER 2

IMMUNITY AND THE GASTROINTESTINAL TRACT

HISTORICAL

The seeds of the idea that immunity in the intestine could be independent of measures of immunity in other parts of the body were sown about 60 years ago: Davies (Davies, 1922) observed the appearance of antibodies against Shigella in samples of stools with some evidence that these antibodies were not derived from the serum; Besredka (Besredka, 1927) at the Pasteur Institute in Paris, worked on local immunization as a means of preventing bacterial infections of the gut. The concept germinated with the clear-cut demonstration by Burrows and his group (Burrows et al., 1947) in the 1940s of the independence in time and in intensity of the antibody response in intestinal fluid from that of serum; they also showed that resistance to infection correlated better with antibody levels in intestinal fluid than in the serum. Burrow's work was done with a highly artificial model of cholera in guinea pigs that had originally been described by Koch (Koch, 1885). The relevance of this concept of local immunity to human disease had to wait for development in immunochemistry and cellular immunology around 1960. Heremans, as the first of his many contributions to the understanding of gut immunity, discovered a new class of globulin in the serum, \( \beta_2 \)-A globulin, later renamed IgA. Tomasi and co-workers (Tomasi and Ziegelbaum, 1963; Tomasi et al., 1965), described the predominance of IgA over IgG in the secretions of the gut and gut-related structures compared to their proportions in the serum. On the cellular side, Gowans' pioneering observations
on the path of circulation of large lymphocytes (Gowans and Knight, 1964) was followed by work which showed that lymphocytes antigenically stimulated in the gut after circulating through lymphatics and mesenteric lymph nodes to the thoracic duct returned to the intestine where many of them were apparent as IgA secreting plasma cells (Craig and Cebra, 1971; Hall et al., 1972; Guy-Grand et al., 1974). Evidence is also accumulating for a similar migratory pathway for T cells (Sprent 1976; Ogilvie and Parrott, 1977).

A variety of studies in the 1960s and 1970s have confirmed the validity of the concept of local immunity at secretory sites such as the intestine and respiratory tract, but has still left many unanswered questions. Rather than trace the development of these ideas over the past few years, current concepts of the secretory immune system will be reviewed with particular reference to their implication for studies described in this thesis.

Current Concepts of the Intestinal Immune System.

Several reviews of the intestinal immune system have been published in the last three years (Tomasi, 1976; Wright, 1976; Ferguson and MacSween, 1976).

Mammalian intestine appears to have specialised areas of mucosa which are adapted for trapping antigens. These are Peyer's patches and the appendix. The peculiarities of their anatomy that fit them for this task are their thin modified epithelium through which particulate antigens have been seen to be pinocytosed (Owen and Jones, 1974; Owen, 1977), and the aggregation of B and T lymphocytes in an orderly
manner just under this epithelium (Parrott, 1976 a and b). This would obviously facilitate contact of antigen with the precursors of cells specifically active in the immune response. It is currently thought that the primary stimulus of antigen is at these sites where antigen is processed and the migration of antibody producing cells start. (Cebra et al., 1977). It is not clear how important uptake through these specialised areas is compared to uptake through the vastly greater unmodified intestinal lining in providing the primary antigenic signal. B lymphocytes stimulated by the absorbed antigens have been demonstrated to migrate via the lymphatics to mesenteric lymph nodes and on through the thoracic duct into the general circulation. These cells then home mainly to the lamina propria of the intestine, where as mature plasma cells they secrete antibody. The B lymphocytes involved in this migration mature into plasma cells secreting IgA (Parrott, 1976). The proportion of lymphocytes bearing surface IgA relative to other lymphocytes increases progressively at various points along the migration pathway (Guy-Grand et al., 1974). In the lamina propria, the IgA secreting plasma cells form the predominant population of plasma cells. In man, the IgA they produce is dimeric and contains J-chain in contrast to the monomeric IgA produced by most plasma cells in the bone marrow. Prior to reaching the lumen of the intestine the dimeric IgA molecules pass through the epithelial cells of the gut and have secretory component attached to themselves in the process (Brandtzaeg and Baklien, 1977). Secretory IgA forms the predominant immunoglobulin in intestinal fluid (McClelland et al., 1972).
There is evidence to suggest that SIgA may bind to the mucus which overlies the epithelial cells of the intestine, possibly increasing its concentration at the cell surface where its potential effectiveness is maximal (Edwards, 1978).

Parotid saliva (Brandtzaeg, et al. 1970), pancreatic juice (Clemente et al., 1971) and bile (Dive and Heremans, 1974) like intestinal fluid contain IgA in a much higher proportion relative to albumin and IgG than does serum. Where these and other secretions of the gastro-intestinal tract have been studied, the IgA has been found to be mainly in the form of secretory IgA (Heremans, 1974). It seems likely that the IgA found in these fluids is produced by plasma cells found sub-epithelially. The lactating breast also appears to behave as an outpost of the gastro-intestinal tract at least as far as immunoglobulins and antibodies are concerned. (Allardyce et al., 1974; Goldblum et al., 1975). The evidence from these studies points strongly to antigenic stimulation in the intestine resulting in specific SIgA antibody production in the breast probably as a result of immunoblasts stimulated in the gut migrating to the breast.

Some evidence has been produced by Bienenstock's group suggesting that the antibody response at secretory sites may to some extent cross the barriers of organ systems. They have developed the concept of bronchus associated lymphoid tissue (BALT) and shown that an antigenic stimulus in the respiratory tract results in the appearance of antibody producing cells in the gastrointestinal tract, though the numbers are much less than in the respiratory tract (Rudzik et al., 1975).
There is no data regarding interaction between the intestine and the genito-urinary tract.

While IgA producing plasma cells are in general predominant at secretory surfaces in health, there are significant numbers of IgG and IgM producing plasma cells as well (Brandtzaeg and Baklien, 1976). There is no suggestion that the IgG cells are stimulated in the gut and home to secretory surfaces in the same way as the IgA cells do. The position with regard to IgM cells, which may replace the IgA cells in IgA deficiency, is less clear.

Further information regarding plasma cells and immunoglobulin production at secretory sites is being sought to expand understanding of this area. It is uncertain whether all IgA producing cells in the lamina propria of the intestine are derived from precursors that have migrated via the thoracic duct from gut associated lymphoid tissue. It is possible that some of the IgA plasma cell precursors may migrate directly across from specialised GALT structures to the lamina propria (Parrott, 1976b). Whether such a short circuit exists, and if it does, its relative importance has yet to be assessed.

A major unanswered question is what the signal is that enables the IgA plasma cells to home back to secretory sites. One suggestion is the presence of the antigen that initially triggered off the IgA secreting plasma cells. Ogra demonstrated in his experiments with polio vaccine instilled at various sites that the antibody response was maximal at the site of antigen replication (Ogra and Karzon, 1969a). This could result from preferential homing of IgA plasma cells to where the stimulating antigen is found or from antigen present at these sites driving the plasma cell population producing
antibodies specific to it, to expand. This would imply that antigen can cross the mucosa at sites that are not specialised GALT areas. Experiments in which homografts of sterile mouse intestine placed under the skin (Moore and Hall, 1972) and under the capsule of the kidney (Parrott and Fergusson, 1974) were seeded by IgA containing immunoblasts indicate that the presence of antigen cannot be the only signal to homing plasma cells. Another factor that may control the homing of IgA plasma cells is the presence of secretory component in epithelial cells. In their description of a 15 year old boy with secretory component deficiency, normal serum IgA but absence of secretory IgA and inability to synthesize IgA in the intestinal mucosa, Strober and his colleagues discuss this possibility (Strober et al., 1976). Tomasi had suggested it before (Tomasi and Yurchak, 1972).

The contribution of the intestine to serum IgA and to specific serum antibody is also an area of controversy. Animal work suggested that this was significant (Heremans, 1974). In humans the amount of dimeric IgA in the serum has been variously estimated at 10% (Heremans, 1974) and less than 2% (Radl et al., 1975). As the IgA produced in the lamina propria is mainly dimeric this would make the actual amount of serum IgA derived from the intestine much less than the estimates for rats and mice (Heremans, 1974). The presence in human bone marrow of plasma cells with the capability of secreting dimeric IgA (Radl et al., 1975) would imply that some of dimeric IgA in the serum came from the marrow. Though the plasma cells in the marrow secreting dimeric IgA may themselves be derived from GALT it means that
an even smaller proportion of serum IgA is secreted by cells in the intestine. While the relative proportion of dimeric to monomeric IgA in serum is small, it is possible that dimeric IgA may have a much shorter half life than monomeric IgA in the serum. The evidence that gives this hypothesis some weight is the discovery that the liver in rats clears dimeric IgA from the serum, and secretes it in significant amounts into the bile (Le Maitre Coelho et al., 1978) where it is found in the form of secretory IgA. There is no evidence as yet that this happens in man.

The effect of continued or a secondary antigenic stimulus to the intestine is the subject of controversy. There is no evidence as to whether maximum effectiveness of a secondary stimulus is also at the GALT areas or in the rest of the intestine which has been seeded with plasma cells after the primary stimulus. The fact that there is pre-existing antibody after the primary stimulus, is likely to reduce antigen uptake and therefore the secondary stimulus (Walker et al., 1972). The very existence of an anamnestic or memory response in the secretory IgA system was consistently denied by Heremans and co-workers (Heremans, 1974). This probably reflects the difficulty of mounting an effective secondary local stimulus in the face of active antibody after the primary stimulus. More recent work in animals (Bloom, 1978) and in man (Mestecky et al., 1978) is in keeping with an accelerated and more intense secondary antibody response. Whether such a response does last longer than a primary response is uncertain. Further information on these points is of great importance in the development of
a better understanding of immunity to commonly met antigens and also to the development of better methods of vaccination against intestinal disease.

Most of the work on the local immune system done up to now has focussed on B cell stimulation, immunoglobulin production and their control. Evidence for T cell migration in a manner similar to that of IgA secreting plasma cell precursors comes from the work of Parrott and co-workers working with a model of Trichinella infection in mice (Ogilvie and Parrott, 1977). The existence of T cell sensitisation in Peyer's patches has been shown by Muller-Schoop and Good (Muller-Schoop and Good, 1975). The nett importance in the gut of T cell mechanisms in health and disease remains very uncertain. As was discussed in chapter 1, the possible importance of T cell mediated immune mechanisms in invasive infections and inflammatory bowel disease is easy to conceive of. What possible role cell mediated mechanisms could have in health is a more difficult question. No functioning cells have been found within the lumen of the adult intestine nor has the possibility of such a function been seriously suggested. Speculation on the role of the intra-epithelial lymphocytes (theliocytes) continues (Ferguson, 1977). They have been demonstrated to carry T cell markers and their prominence histologically suggests that they may be the key to unlocking the mysteries of T cell function in the healthy intestine.
Interaction of Immune Responses Manifest at Secretory Surfaces and in the Rest of the Body.

Recognition of secretory IgA as a marker of the local immune response and the migration patterns of SIgA producing plasma cells revived interest in the observations of Burrows (1947), that measures of immunity at secretory surfaces could be independent of the same measures within the blood stream, and that for diseases that entered or were mainly manifest at secretory sites resistance to infection might correlate best with measures of immunity at the local level. The independence of secretory and serum antibody from each other has been confirmed repeatedly in various models after oral vaccination, in relation to artificially stimulated immunity (Freter and Gangarosa, 1963; Girard and Kalbermatten, 1970; Mestecky et al., 1978). It has been more difficult to demonstrate this clearly in the natural state. There is probably a significant though possibly variable interplay between these two areas. Even in a condition such as cholera where it is postulated that bacteria remain completely within the lumen of the gut, a quite marked serum antibody response is seen. In the clearest study of the immune response to cholera, the serum response though slightly less, was of the same order of magnitude as the intestinal response (Waldman et al., 1972). This finding raises doubts on just how independent the local response is of the systemic one. Evidence on this point gleaned from different conditions using different assay techniques tends to be conflicting. The best dissociation of serum antibody from secretory immunity in humans has been demonstrated in
local vaccination with vaccines in Para influenza infection (Smith et al., 1966) and Rhino virus infection (Perkins et al., 1969) both viruses that multiply at the local site. Protection against natural disease correlated with secretory antibody rather than serum antibody, though serum antibody did follow local vaccination. Ogra's work done with polio virus (Ogra et al., 1968; Ogra and Karzon, 1969 (a) and (b)), a virus that multiplies locally as well as invades, showed that the capacity of virus to recolonise secretory sites depended on the pre-existing secretory antibody levels at those sites, but in most of his studies quite a marked serum antibody response was seen in addition to the secretory antibody response. In the few studies where a measurable anti-bacterial antibody response has been detected at the secretory surface but not in the serum, killed bacteria (Mestecky et al., 1978) or bacterial antigen (Girard and Kalbermatten, 1970) have been used as the stimulus.

The possibility of therapeutic gain in the form of effective immunisation against infections that either enter via or establish themselves at secretory sites, has spurred on interest in this area. Among these infections are dental caries, cholera and other infective enteritides, gonorrhoea and viral hepatitis. While local immunisation may appear more rational as a means of stimulating local defences, the feed-back inhibition of further antigen uptake (Walker et al., 1972) could act as a major impediment in this endeavour. The interdependence of the local and systemic immune responses generate the hope that combining local and systemic immunisation will facilitate the develop-
ment of local immunity more easily and possibly more effectively than by depending purely on local immunisation (Pierce and Gowans, 1975).

There seems then to be no doubt that there is a highly specialised and distinct compartment of the body's immunological system active at local secretory sites. Secretory IgA antibodies and antibody producing cells are at present the only practical estimate available of the activation of this local system, though there is good evidence that T cell mediated immune mechanisms may be stimulated at secretory sites distinct from cell mediated immunity in other tissues. In diseases active at these sites, where measurements of local immunity have been made, in many cases protection against disease has correlated better with local antibody than with serum antibody. A comparison of these two correlations needs to be made for other conditions affecting secretory sites. How well the secretory IgA antibody response represents other aspects of local immunity needs assessing.

Notwithstanding the distinction between immunity at local surfaces and systemically, different systems studied have shown varying degrees of overlap between these two compartments. On the present evidence it would appear that generalisations regarding the independence and the degree of importance of local and systemic immunity will not be possible, and each particular disease or condition will require individual consideration. Advances in the understanding of the pathophysiology of these conditions and in prophylaxis of disease at these sites require answers to these questions.
Problems in Studying Intestinal Antibody.

The major reason for the uncertainty about the role played by local intestinal immune mechanisms both in disease and health is the difficulty in obtaining information about its activity. There have been no published studies of local cell mediated immunity in the gut in humans and so discussion will be limited to local humoral immunity. The measurement of antibody activity within the intestine is beset by the problems of obtaining a satisfactory sample, the variation in antibody concentrations caused by the physiological processes of fluid secretion, digestion and absorption occurring within the lumen of the gut, and difficulty with designing an appropriate and effective assay for measuring antibodies as they exist in intestinal fluid.

A. Sampling and Measurements of Intestinal Antibody.

The only readily obtainable product of the human gastrointestinal tract is faeces. Though Davies (Davies, 1922) initiated interest in this area with his measurements of anti Shigella antibody in stools, the results obtained over the years on measuring antibody in stools have been very erratic and non-reproducible. Freter systematically looked at this question and his results (Freter, 1962) are the most definitive available. The assays he used were a "Farr" assay based on ammonium sulphate precipitation of radio-labelled antigen and bound antibody, a bacterial agglutination assay and a biological mouse protection test.
In essence he showed that there was continuing breakdown of antibody to a residue of less than 2% at 16 hours as measured in the Farr assay and in the mouse protection tests. There was also an inhibitor of agglutination present in stools. He demonstrated that markedly decreasing intestinal transit time by purgation with magnesium sulphate resulted in the preservation of 25% of ingested rabbit antibody (this was the mean of a series of results ranging from 4.5% to 84%). Demonstration of intestinal antibody in bacterial gastroenteritis of adults since this work by Freter has been mainly on liquid stools induced either by magnesium sulphate catharsis or by disease itself as in cholera. This would serve to reduce the length of time antibody produced in the small intestine lies around being degraded by gut or bacterial enzymes. In infants the studies of Ogra, Lodinova (Lodinova et al., 1973), and McNeish (McNeish et al., 1975), were carried out on stools obtained without purgation but the immature digestive power of the alimentary tract, and the faster intestinal transit time (Grybowski, 1977) in infants compared to adults may have helped preserve the antibody.

The only other practical way of getting intestinal fluid involves intestinal intubation. This demands considerable subject cooperation, skill on the part of the investigator and ready access to radiological facilities. As will be discussed below, gastric aspirate from normal subjects is unproductive as a source of secretory antibody.
This necessitates passing an intestinal tube into the duodenum or jejunum to obtain secretions with measurable amounts of antibody. The antibody in this fluid is clearly subjected to the digestive processes mentioned earlier and this will be discussed in more detail. It is still true to say however that the most meaningful work done up to the present in relation to gastrointestinal antibodies in adults has been carried out on fluid obtained by intestinal intubation.

Other fluids that have secretory IgA as their predominant immunoglobulin have been used in studies of local secretory immunity of the gastrointestinal tract. The assumption underlying these studies has been that the stimuli to the precursors of these cells probably occurred within the gut associated lymphoid tissues and that the antibody they produce reflects antibody production within the intestine. Colostrum, the peri-natal secretion of the breast, is very rich in immunoglobulin and though the concentration of immunoglobulin decreases when lactation is established there is still a comparable amount of immunoglobulin found in the breast secretions over 24 hours (Ogra and Ogra, 1978). The presence of specific antibody to Salmonella typhimurium in women infected three months before term but not in normal controls (Allardyce et al., 1974) emphasised the validity of this concept. The demonstration of cells in colostrum producing antibody against a specific E.coli fed to pregnant women shortly before term suggested that antibody produced in a distant site such as the breast against an ingested antigen was a consequence of seeding of immunoblasts stimulated in the
gut at that distant site (Goldblum et al., 1975). Saliva is another such fluid which is rich in secretory IgA; parotid saliva in particular is virtually free of any other immunoglobulin. Antibody activity against a variety of Streptococcus has been demonstrated in saliva after oral immunisation (Mestecky et al., 1978). The much greater ease with which saliva can be obtained than intestinal secretions makes it attractive as a fluid in which to determine the local immune response of the gastrointestinal tract. The correlation of secretory antibody levels in intestinal fluid with levels in saliva or colostrum remains as yet uncertain. This question was studied in the work described in this thesis. It must also be borne in mind that a variety of factors such as the site of primary and secondary antigenic stimuli may modify the output of antibody at various points in the alimentary tract. There may be no fixed correlation of antibody production at various points in the gastrointestinal tract to its total response to an antigenic stimulus, but a spectrum of relationships depending on the systems studied. Clearly the most relevant measurement of the local immune response in bacterial gastroenteritis is at the level of the intestine where the bacteria establish themselves and hence antibody in intestinal fluid must be the yardstick by which the significance of antibody levels elsewhere are measured.
**B. Dilutional Effects**

Unlike levels of circulating immunoglobulin which are not subject to major perturbations in the short term, the levels of immunoglobulin in fluids sampled in the gastrointestinal tract can vary widely. Even in the secretions of a single gland like the parotid or the pancreas, the fluid secreted per unit time can vary over a wide range (Brandtzaeg et al., 1970) with little change in the immunoglobulin secreted per unit time and therefore a great change in immunoglobulin concentration. Sampling fluid in the lumen of the jejunum is open to even greater variations as the fluid that is actually being sampled there arrives from a variety of sources—ingested fluid, gastric, pancreatic and biliary secretions, secretions of Brunner's glands and from the intestinal epithelium itself. It is assumed that most of the immunoglobulin in the gut is derived from the plasma cells of the lamina propria of the gut. But there are immunoglobulins in the other secretions and there is a wide variation in the rates at which fluids from these sources reach the intestine. Rough estimates of the contributions to fluid in the intestine over 24 hours are:

- **Saliva**: 1.5 l.
- **Gastric secretions**: 2.5 l
- **Bile**: 0.5 l
- **Pancreatic juice**: 0.7 l
- **Intestinal secretions**: 3.0 l

(Gamble, 1954).
Any fluid ingested is additional to these figures. Release of all these fluids are under nervous and humoral control and they are released at widely varying rates depending on food and nervous stimuli. In disease states the fluid reaching the intestine may increase greatly as in conditions of secretory diarrhoea seen in the gastroenteritides.

In health the bulk of the fluid secreted into the intestine is reabsorbed so that about 1.0 litre reaches the colon and 0.2 litres is excreted in stools. There is no evidence to suggest that intact immunoglobulin is reabsorbed and what scanty data there is suggest that an increase in concentration of immunoglobulin occurs in the lower reaches of the small intestine. Tending to reduce the concentration of immunoglobulin is proteolytic breakdown of immunoglobulin both by the enzymes produced by the body and by bacteria. The interaction of a variety of factors would thus result in variation of immunoglobulin concentrations at any position in the intestine at different times. It would also result in variations at different levels of the gut.

These variations were limited as far as possible in the studies described in this thesis. Intestinal fluid was aspirated from the same fixed point, just beyond the duodeno-jejunal flexure. The subjects were fasted for eight hours before the study; as food is the most potent stimulus to gastrointestinal secretion, this would lessen dilutional effects. The expression of antibody activity
relative to immunoglobulin concentration is a further means of circumventing the variation caused by dilutional factors as presumably the secretion of non-specific immunoglobulin and specific antibody are affected to the same extent by these factors. Antibody levels in all body fluids sampled in these studies, were expressed both in terms of volume and relative to immunoglobulin concentration.

The Effects of Protein Degradation

Immunoglobulins being proteins are subject to enzymatic digestion continuously in the lumen of the intestine. In the upper part of the alimentary tract, these enzymes are mainly produced by the body while in the colon and lower ileum, bacterial enzyme degradation may be more important.

In spite of the presence of a plasma cell population in the gastric mucosa there is no detectable immunoglobulin in acid secretions from normal individuals (Odgers and Wangel, 1968). That this is a result of digestion by pepsin which only acts at a low pH was elegantly shown (McClelland et al., 1971) in a study which demonstrated the presence of large amounts of immunoglobulin in secretions from the stomach maintained at a neutral pH by perfusion of sodium bicarbonate through it. In further studies using purified preparations of secretory IgA, IgG and IgM, and pepsin obtained commercially, the rapid degradation of these molecules by pepsin so that they could no longer be detected in quantitative radial immunodiffusion or by immunoelectrophoresis was shown (Samson et al., 1973).
The major proteolytic enzymes active in the intestinal lumen at a near neutral pH are trypsin and chymotrypsin which are secreted by the pancreas. (Gray, 1973). They are endopeptidase enzymes that cleave amino-acid chains at CO:NH linkages internally in contrast to exopeptidases which break off amino-acids singly from the ends of the chain. In experiments using purified secretory IgA, serum IgA and serum IgG and subjecting these proteins to the actions of trypsin, chymotrypsin and duodenal fluid, Brown and co-workers (Brown et al., 1970) demonstrated the striking resistance of secretory IgA to the actions of these enzymes and to duodenal fluid. Monomeric IgA and IgG were degraded but the destruction was limited to the heavy chain portion of the molecule. The antigen binding Fab units remained intact and in experiments using monomeric IgG with antibody activity against diphtheria toxin they were seen to retain their binding ability completely after trypsin digestion. These results were confirmed by Samson and colleagues (Samson et al., 1973). Exopeptidases, in both pancreatic secretions and in the unstirred layer where they are secreted by the intestinal epithelial cells also play a part in the protein breakdown. They are probably less important in breaking down larger molecules than the endopeptidase enzymes discussed (Gray, 1973) but there are no published data relating to their individual effects on immune globulin structure and function (Brown et al., 1970). In view of the evidence that one, if not the major way that antibodies within the intestine act against pathogenic bacteria is by binding to them and interfering with their
ability to adhere to the lining of the gastrointestinal tract (Freter, 1969; Gibbons, 1974; Steele et al., 1977), the finding that Fab fragments remain after digestion of immunoglobulins by enzymic action in the gut is of great interest. In an animal model of cholera, fragments of immunoglobulin digestion that retained their binding capacity were found to have a protective activity comparable to the intact molecules (Steele et al., 1975). In view of these findings any meaningful assay of intestinal antibody should have the ability to measure binding fragments which have lost their class specific heavy-chain moieties.

Bacteria too may degrade immunoglobulin. Plaut and co-workers have described the production of microbial protease (Plaut, 1978) by strains of bacteria as diverse as Streptococcus sanguis, which is of major importance in the aetiology of dental caries, and Neisseria gonorrhoeae, another important pathogen at a secretory site. Production of this enzyme by intestinal pathogens has not been reported as yet. This protease only cleaves the Fc portion in molecules of the IgA₁ sub-class and leaves the antigen binding capacity of the fragments intact. The chances of other enzymes with the ability to break down immunoglobulin, being produced by others of the myriad strains of bacteria colonising the human intestine must be high. Even in the stools of patients with cholera (Northrup et al., 1970), IgG and IgM and much of the IgA was found, on sucrose density gradient centrifugation, to be small molecular weight fragments, though there was some apparently intact
secretory IgA. The results obtained by Reed and Williams (Reed and Williams, 1971) are probably compatible with the results of Northrup et al though the methods of determining protein size in this study are not given in detail.

It is clear then that from the time immunoglobulin enters the intestinal lumen it is subject to the action of proteolytic enzymes. The extent to which any degradation that results, interferes with its activity in the system under study can only be surmised in the absence of sufficient clear data on the degree of degradation and on the mechanisms by which antibody acts within the gut in that particular system.

Various methods have been used by workers to reduce enzyme damage to immunoglobulin. These include heating to 56°C for one hour to denature enzymes (Plaut and Keonil, 1969) and the addition of trypsin inhibitors, such as soya bean trypsin inhibitor (Waldman et al., 1971) and trasylol (Samson et al., 1973). Clearly these techniques could not prevent enzyme damage to immunoglobulin which could be extensive before the samples are actually retrieved (McClelland et al., 1971) but it was hoped that they would reduce damage prior to and during storage of the samples and in the assay itself. While this is conceptually sound, studies undertaken to demonstrate the effectiveness of these steps in preserving immunoglobulins in an antigenically recognizable form and in preserving antibody activity have failed to show any benefit compared to samples that have been quickly frozen and used in assays immediately after thawing (Samson et al., 1973; Horsfall et al., 1978).
Work done on storage of immunoglobulin shows that even at -20° there is continued breakdown of IgG and IgM in intestinal fluid presumably due to continued enzyme activity though this was not prevented by trasylol (Samson et al., 1973). Secretory IgA appeared stable under the conditions of storage even in the absence of the trypsin inhibitor. The question of whether this degradation during storage could be reduced or prevented by even lower temperatures has been the subject of experiments to be described in this thesis.

C. Assay Techniques.

Over and above the variety of factors that decrease the amount of antibody in intestinal fluid and introduce variability into the results, the difficulty of designing a sensitive but robust assay to measure antibody in intestinal fluid has been the major impediment to experimentally dissecting the function of locally produced antibody in the intestine.

Historically many of the most sensitive assays commonly used in measuring serum antibody have depended on complement fixation. This whole group of serological reactions has been irrelevant to the measurement of intestinal antibody for a variety of reasons. Neither antibody fragments with degraded Fc portions but intact binding Fab portions, nor antibodies of the IgA class bind complement (Heremans, 1974). Furthermore intestinal fluid has a high level of anti-complementary activity. In addition, complement-mediated antibody reactions are highly biased towards magnifying IgM against all other classes.
Agglutination reactions using the relevant bacteria or red blood cells sensitised with bacterial antigens have been the most widely used methods of studying intestinal antibody. In the introduction to his article on the detection of copro-antibody (Freter, 1962), Freter quotes many other studies where results using agglutination assays had been marked by irreproducibility. He goes on to say of his study "It will be shown that the irregular results obtained by earlier workers most likely reflected shortcomings in the technics available at that time and thus were not indicative of a basic irregularity in the copro-antibody response of man". In his discussion he outlined a number of factors such as an agglutinin inhibitor in faeces and the tendency to spontaneous agglutination that made agglutination a far less reliable test than the Farr assay he was using. These results were with 'copro-antibody' in stools obtained by magnesium sulphate purgation. Girard and de Kalbermatten in their report on "Antibody activity in human duodenal fluid" found bacterial agglutination far less sensitive than haemagglutination (Girard and de Kalbermatten, 1970), while Reed and Williams (Reed and Williams, 1971) found indirect bacterial haemagglutination unusable in their hands because of inability to obtain consistently negative controls, on attempting to measure intestinal antibodies to Shigella in stool fluid with this technique. In Waldman's study (Waldman et al., 1972) of the antibody response to cholera, a variety of antibody estimates were tried. While antibody
was found in the faecal samples of most of the patients studied and in the intestinal fluid of all of them, no antibody was found in either type of sample by tube agglutination or micro-agglutination technique. It may be that agglutination using bacteria or sensitised cells is easier to perform with intestinal fluid obtained from infants than from adults. Lodinova (Lodinova et al., 1973) and McNeish (McNeish et al., 1975) have published data based on the use of these techniques in studies on infants in the last six years. On balance though, these assays seem unsuitable for measurement of intestinal antibody for many reasons. They are insensitive when compared to more recently described methods such as radio-immunoassay and enzyme linked immuno assay. They do not measure monovalent binding fragments that may have meaningful biological actions in the intestine. Non-specific agglutination is a problem when these assays are used with adult intestinal fluid.

In the 1960s, Freter used a Farr assay to demonstrate that copro-antibody could be measured with greater reproducibility than had hitherto been possible. (Freter, 1962). The titres of antibody he measured using this technique were still low and in animal experiments in our laboratory this technique proved less sensitive than other forms of radio-immune assay. (Horsfall and Rowley, 1979). For this reason, the assay developed for use in the studies described in this thesis was based on measurement of antibody or antibody fragments that bound to bacteria using radio-labelled anti-antibody. It was possible to detect all
binding fragments using Anti light-chain antibody, IgA antibody using anti alpha antibody and IgG antibody using anti gamma antibody.

Various biological assays have been used for measuring antibodies in specific disease states. Among these are the baby mouse or infant rabbit protection test (Chaicumpa and Rowley, 1972; Jenkin and Rowley 1960) used for measuring antibodies in cholera, and the ligated loop (DuPont, 1978) for measuring toxin and by inhibition, antitoxin, in enterotoxigenic E.coli diarrhoea. They suffer from the limitations of being specific for one particular disease state, of being time consuming and in many cases fairly finicky.

The assay that has probably been of greatest use in studying antibody production in the gut in animal studies has been the Jerne plaque assay (Dresser and Greaves, 1973) which quantitates the number of plasma cells producing antibody against specific antigen. By using suitable enhancing antisera data can also be gained about the Ig class of the antibody being secreted by the cells. This involves biopsying tissue and in humans, this would raise the problems of ethics and patient acceptability; even more important, harvesting lymphocytes and plasma cells from gut biopsies in sufficient numbers to obtain meaningful numbers of plaque forming cells is an exercise that has yet to be achieved.
Studies of the Intestinal Antibody Response to Bacterial Gastroenteritis in Humans

Much of the information on which our concepts of secretory immunity are based come from animal studies. The aim of inducing local immunity to E.coli gastroenteritis in pigs and cattle has been achieved (Porter et al., 1977). On the whole though, much of the animal work relating to local immunity to gastroenteritis is based on artificial models of human disease and this must place major reservations on the results obtained.

In spite of all the difficulties outlined, some attempts to study the gastrointestinal antibody response in human to organisms in the gut have succeeded to a greater or lesser degree in achieving their objective. Important among them are observations in humans after vaccination with viruses and bacteria such as those of Ogra and his colleagues working with polio virus (Ogra et al., 1968; Ogra and Karzon, 1969 a & b), Hanson and his group (Jodal et al., 1977) and Mestecky (Mestecky et al., 1978). These studies and the general concepts which they have given rise to have been touched on earlier in this chapter. They will not be discussed in more depth as the major object of this thesis is to determine whether the concepts generated in models of "bacterial infection" in humans and animals do hold in the context of actual disease. The rest of this chapter will concentrate on reviewing what has actually been demonstrated with regard to the local immune response to bacterial gastroenteritis in humans.
The earliest relevant observations were those of Davies (Davies, 1922). The material he studied was the fluid portion of dysentery stool or saline extracts of stools, and also serum from patients with dysentery. The antibodies were measured by an agglutination assay. What Davies claimed to show was the presence of agglutinins in the stools of 18 out of 24 patients with dysentery as shown in the figures below:

<table>
<thead>
<tr>
<th>No. of Cases</th>
<th>A.histolytica +ve</th>
<th>B.dysenteriae +ve</th>
<th>Agglutination to B.dysenteriae</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>0</td>
<td>15</td>
<td>18</td>
</tr>
</tbody>
</table>

As seen from this table his only controls were two patients with amoebiasis, though in the course of his discussion Davies mentions that the intestinal exudates did not agglutinate cholera vibrios and Bacillus proteus X19. Davies advanced evidence that the agglutinins he demonstrated were not serum derived because the agglutinin titres were actually higher in the gut derived fluid than in the serum. Further evidence for this was based on the presence of agglutinins in the serum consequent on TAB vaccination; their concentration in the intestinal fluid was used as a marker of the contribution of serum antibody to the intestinal fluid. While Davies' interest focussed mainly on the possibilities local gut antibodies presented in
diagnosis, he did present the earliest suggestion that the intestinal antibody response was not a reflection of serum antibody, especially in gut infections.

From the time of Davies' study up to the 1960s there were sporadic attempts made to measure intestinal antibodies. Freter in his review of this work (Freter, 1962) emphasised the irregularity and irreproducibility of the results obtained in these studies. After developing and using a Farr assay in experiments based on immunisation (Freter, 1962), he used this assay to study the copro-antibody and serum antibody response in cholera patients (Freter et al., 1965). Aspiration from the duodenum or jejunum was used for obtaining the intestinal fluid. This was continued for several days and the fluid obtained was stored on ice and titrated on the same day. Serum was obtained on the first day of the study. Copro-antibody was demonstrated by the fourth day in all of the patients studied, supporting Freter's claim that intestinal antibody production was a regular occurrence. On the basis of the relative levels of serum antibody in patients and the time of production of copro-antibody, Freter suggested that there was no correlation between serum and copro-antibody. The conclusions reached in the study seem valid as far as they go. There is however no discussion of absolute levels of antibody taken as significant in the intestinal fluid nor of the quantitative pattern of rise either in serum or intestinal aspirate. A control group was used for comparison only of serum antibody with
strikingly little difference between the groups. The lack of a control group of normal subjects or patients with non-choleraic diarrhoea from the same area for comparison of intestinal antibody is perhaps the major deficiency in this study.

Five years after Freter's publication, Northrup and Hossain (Northrup and Hossain, 1970) published the results of work in which they attempted to define the immunoglobulin class of antibodies found in intestinal secretions in cholera, and to quantitate them. They obtained intestinal aspirate by intubation, collected it on ice and froze it immediately. After storage at -20°C, the samples of fluid were heated at 56°C for 30 minutes in an attempt to eliminate enzyme activity. (No evidence was published to confirm that this was achieved). After being centrifuged, the jejunal fluid was lyophilised and re-dissolved in 1/10 its original volume. The assay used for measuring antibody was based on the technique used by Ogra to measure anti-viral antibody. It depends on the binding and precipitation of antibody by antigen in an agar-gel. Serial dilutions of the antibody containing fluid (serum or reconstituted jejunal fluid), were placed in wells round a central well containing anti IgA, anti IgG or anti IgM antisera. Precipitin lines formed and non precipitated antiserum and immunoglobulin were washed off. Radio-labelled cholera antigen was introduced into the central well, diffused out to react with any precipitated anti-cholera antibody, and after washing out of non precipitated iodine-labelled
antigen, gave lines on photographic paper. The results of this study do not make any readily understood pattern. Intestinal antibody was demonstrated in all nine patients whose aspirates were studied, with surprisingly more subjects showing IgG antibody (8/9) than IgA (6/9) or IgM (5/9). Some attempt at studying the time course of the antibody response had been made but the impression given by the publication is that the response was irregular. No quantitation or even titres of immunoglobulin or antibody in the intestinal fluid are given except for one patient. A case is made for the serum IgA antibody being gut derived in that it tended to rise later in the course of the study after bacteria disappeared from the gut. On the data presented, the rise in serum IgA antibody could well have been proportionate to the rise in serum IgG antibody, most of the estimates of serum IgG antibody later on in the illness being greater than 1/128, the highest titre measured. On balance, this study added little to what was known before. It confirmed, probably, an antibody response in the intestine in patients with cholera, though the lack of controls, irregularity of the response and the uncertainty of the titres measured in intestinal fluid cloud this issue. In a previous publication on this same intestinal fluid, Northrup and other workers (Northrup et al., 1970) produced evidence that IgM and IgG in the secretions were partially degraded. In view of this, the predominantly IgG class response they claim to demonstrate is difficult to understand and raises questions on the specificity of the anti sera used (no information was published relating to this). Taking these results at their
face value, they would suggest a ratio of over 200:1 of antibody in serum compared to intestinal fluid.

The best study of the intestinal antibody response to a local bacterial infection in humans is the one carried out by R. Waldman and his colleagues and published in two parts in the Journal of Infectious Diseases, the first part (Waldman et al., 1971) dealing with immunoglobulin levels in serum, intestinal fluid and faeces, and the second part (Waldman et al., 1972) focussing on serum and intestinal antibody. This was a prospective study done on patients with naturally occurring cholera. The results obtained were compared with samples from patients with non-choleraic diarrhoea admitted to the same hospital in India, and with samples from 'normal' Indians and 'normal' Americans. Samples in the patients with diarrhoea were taken in the acute stage of the illness, at 2 weeks and again at 4 weeks after presentation. The intestinal fluid obtained by jejunal intubation, was collected on ice with sodium azide to prevent bacterial multiplication and soya bean trypsin inhibitor to slow down tryptic digestion. The fluid was then frozen and lyophilised. It was resuspended in 10% of its original volume, insoluble material spun out and the supernatant stored at -10°C till assayed. The faecal samples obtained were the diarrhoeal fluid in the acute stage and fluid obtained by magnesium sulphate purging at the later stages. It was collected on ice with azide and a trypsin inhibitor, filtered through gauze and centrifuged. Protein was precipitated using 50% ammonium sulphate. This was resuspended in 5%
of its original volume, and treated in the same way as the intestinal fluid from then on. Analysis of both the intestinal fluid and faecal samples collected and stored in this way, on Sephadex G200 did not reveal any evidence of breakdown of even IgG or IgM. On the estimates made of immunoglobulin in the stool in the acute stage, of the order of 5 grams of immunoglobulin was poured into the intestine every 24 hours of which 75% was IgA. In the second paper, comparisons of various techniques of measuring intestinal antibody were made. Some of the results obtained were discussed earlier in this chapter. In addition to finding agglutination techniques useless for measuring intestinal antibody, vibriocidal antibody was found in less than 30% of faecal samples and in less than 20% of intestinal fluid samples. Two assays based on antibody binding to antigen were found the most useful in these experiments. A radioactive radial immunodiffusion assay in which antibody diffuses out of a central well radially and binds with cholera LPS in the agar and then is demonstrated autoradiographically after washing, by flooding the plate with radio-labelled anti Ig serum which binds to the bound antibody, revealed antibody in virtually all samples from patients with cholera and in virtually none of the patients with non choleraic diarrhoea. The most complete results were obtained using an assay very similar to the one used in the studies described in this thesis. The basis of this assay is the binding of primary antibody from the patient's serum, aspirate or faeces to bacteria and the quantitation of the
amount of antibody bound by using radio-labelled anti heavy chain specific antisera. Having ensured that both bacteria and radio-labelled antisera are in excess, it is then valid to equate radioactivity bound with primary binding antibody present in the fluid tested. Using this technique antibody was shown in the serum and intestinal fluid of every patient with cholera and in the faecal samples of most of them (Fig.II - 1). Only rarely were rises seen in the patients without cholera. The serum antibody response was predominantly in the IgG class and the intestinal antibody mainly IgA. The results of the antibody estimations were expressed in cpm bound per mg, of Ig. thus overcoming the variation induced by varying dilution factors of the intestinal antibody. A mean thirty-fold rise in IgA antibody between the first and third sampling was seen in the intestinal fluid with only a two-fold rise in serum IgA antibody, a result which must mean that the IgA antibody in intestinal secretions is locally synthesised. Another interesting observation made was that the anti cholera antibody levels in initial samples of jejunal fluid from patients with cholera were lower than in controls with non choleraic diarrhoea. This posed the question of whether the lower antibody predisposed to infection or whether the cholera vibrios present in the gut at the time of first sampling absorbed up the antibody. The intestinal and serum antibody responses were of the same order of magnitude, a twenty five-fold rise in serum IgG antibody and intestinal IgA antibody. Data were not published on the antibody responses in individual patients.
Figure II-1

ANTIBODY RESPONSE TO CHOLERA
(from Waldman et al 1972)

Int fluid IgA
Int fluid IgG
Serum IgA
Serum IgG

mean cpm x $10^{-3}$ per mg Ig

acute 2 weeks 4 weeks
The impression given by this study was that over the period of observation, serum antibody reflected secretory antibody at any rate in the group as a whole, in spite of the theory that in cholera, bacteria are confined to the intestinal lumen.

The only other studies in humans of intestinal antibody responses to bacterial infection are two studies that used agglutination assays. Reed and Williams prospectively studied a group of patients with Shigellosis comparing their copro-antibody responses with subjects presenting with Shigella negative diarrhoea (Reed and Williams, 1971). As mentioned earlier, haemagglutination did not prove to be a feasible assay system in this study and bacterial agglutination was used. An attempt was made to determine the class specificity of the antibody response by absorbing out antibody with heavy-chain specific anti-sera and taking a three well reduction in agglutinin titre as evidence of the agglutinins belonging to the immunoglobulin class of the absorbing antiserum. The authors claim that the results obtained were reproducible, and appropriate tests of specificity were carried out using Shigella antigen and unrelated antigens such as E.coli antigen. The results indicate the presence of a copro-antibody response in patients with Shigella. The median of the highest titres for each patient in the Shigella group was 1:128 and for the control group was between 1:16 and 1:132. Six patients with Shigellosis had duplicate specimens; in only two of them was there a four-fold rise in titre. In three there was a two-fold rise. Duplicate
samples were not taken in the control group. The agglutinins appeared to belong to different classes of immunoglobulins in the various patients though IgA was the predominant class. Some confusion arises from the observation that in one sample with high agglutinin titres no reduction was obtained after absorption with any of the anti-heavy chain sera. The authors comment that this may reflect agglutination caused by a substance that is not an immunoglobulin. In essence, this study shows the presence of copro-antibody to Shigella after an attack. The data does not allow a meaningful estimate of the time course of this antibody response and there must be reservations with regard to the immunoglobulin class of the antibody as defined by the techniques used in this study.

The other study is that carried out by McNeish and colleagues in children with E.coli gastroenteritis (McNeish et al., 1975). The published methodology leaves critical unanswered questions that must undermine any conclusions reached. This is accentuated by the lack of a control group. Agglutination was the method used to assay for antibodies and immunoglobulin class was determined by absorption with heavy chain antisera as described by Reed and Williams. The reliability of the agglutination technique was accepted on the basis of negative saline controls and consistent titres obtained against commercial typing antisera. In view of the great difficulty other workers have had with reproducibility of results using intestinal fluid as compared with serum, this would seem an inadequate control. The results described by the authors did however fit into a pattern. Essentially they
showed a consistent antibody response in duodenal aspirate (assuming that the fluid would give a low back ground in the absence of antibody) that appeared to peak in the second week of infection and fell in the third and fourth weeks. The antibodies were mainly in the IgA class though an IgM antibody response was seen in some. Serum antibody responses were detected in eight out of the fourteen patients and correlated poorly with intestinal antibody titres. This study and Davies' observations in 1922 are the only ones comparing serum and intestinal antibody titres in infection that suggest that the intestinal antibody titres are comparable in absolute terms to the serum antibody titres. No data are given on what the pathogenetic mechanism of diarrhoea caused by the responsible E. coli in this study were. There must however be considerable reservation in accepting these results at their face value (see comments earlier in this chapter on Assay Techniques).

In summary what has been demonstrated in these studies of intestinal antibody response to bacterial infection in the gut is a consistent local antibody response to cholera. There is evidence of a consistent intestinal antibody response following Shigella and E.coli enteritis, but the methodology used in these studies is clearly suboptimal. All the studies suggest that the antibody response in the intestine occurs within a few days of the start of the infection. In Waldman's study (Waldman et al., 1972) with the adults, the antibody response, at least in terms of immunoglobulin commitment to specific antibody, appeared to be rising still four weeks after the start of the infection. On the other hand, the study by McNeish (McNeish et al., 1975) in infants suggested
that the antibody titres declined after two weeks. The studies of Northrup and Hossain (Northrup and Hossain, 1970) on cholera and Reed and Williams (Reed and Williams, 1971) on Shigella suggested a major contribution of IgG and IgM antibodies to the response seen in the gut. Waldman's study on adult patients similar to those studied by Northrup and Hossain unequivocally pointed to a local IgA antibody response. McNeish's study in infants with E. coli found evidence of an IgM response in addition to the predominant IgA response locally. Considerable variation between the studies also existed with regard to the relationship between serum and intestinal antibody. The studies of Davies, Freter and McNeish suggested dissociation between them but only in McNeish's was there clear evidence for this in terms of individual responses and the strong reservations with regard to this study have been emphasised. In Waldman's study, data was not published with regard to individual patients but in the group results, the nett serum antibody response was not dissimilar to that of the intestine.

Aims of this Thesis.

In view of the inadequacy of data on the intestinal antibody response to bacterial gastroenteritis, this study was undertaken to document this response more clearly.

As a preliminary to the studies, further information was sought on the stability of antibodies of the IgG and IgM classes in intestinal fluid at -20°C and -80°C. This was done to determine whether reducing the temperature of storage below -20°C would stop the degradation of immunoglobulin described at that temperature. These experiments are described in Chapter 4.
As uncertainty regarding the published work on intestinal antibody largely arises from the limitations of the assays used, development of a suitable assay for the purpose was a prerequisite for these studies. Ideally this assay had to be sensitive and specific. It had to be capable of measuring partially degraded antibody provided this antibody retained its primary function of binding. Finally the ability to distinguish the immunoglobulin class of antibody using the assay would be an advantage. In chapter 5 the development of an assay meeting most of these demands is described.

The only clearly defined bacterial gastroenteritides available for study were Salmonella and Shigella infections. Adults and children with these infections were studied. The controls for those infected patients were mainly healthy volunteers from among laboratory staff and post-graduate students. No separate paediatric controls were studied. It had been proposed to use children with rota-virus gastroenteritis as controls but no suitable subjects became available during the period of study. The protocol of the study is outlined in Chapter 6, and the methods of obtaining and storing samples outlined in Chapter 3.

In the study of antibody responses to bacterial gastroenteritis the focus was on intestinal antibody. The pattern of intestinal antibody response to infection was compared with the response in serum. Paired samples 4-8 weeks apart were taken to gain insight into the temporal pattern of the response. Estimates of the IgA and IgG contributions to the antibody responses were made. Problems with development of a suitable anti-μ serum
prevented the study of IgM antibody. The possibility that the intestinal antibody was mirrored at some other site such as in parotid saliva or serum antibody of the IgA class was examined. The pattern of response in adults and children were compared. The results of these studies are contained in Chapter 6. Chapter 7 is a discussion of these results and their implications for future studies in the area of intestinal immunity to bacterial infections.
CHAPTER 3
MATERIALS AND METHODS

Bacterial Strains

The bacterial strains used in these studies were stored on nutrient agar slopes at room temperature and also lyophilised in skimmed milk at 8°C. They were grown up when required in nutrient broth (Difco Mich.) on a shaker at 37°C.

The strain of cholera used in the antibody preservation experiment was Vibrio cholerae 569B, a classical Inaba strain. This was obtained from the Cholera Research Laboratories, Dacca. The Salmonella and Shigella strains were obtained from the bacterial typing laboratory of the Institute of Medical and Veterinary Science in Adelaide. They were strains isolated from the stools of the patients studied (see appendix).

Tetanus Toxoid

The Tetanus Toxoid used in the antibody preservation experiment (Chapter 5) was originally manufactured by C.S.L. Australia (Batch No. 053). It was obtained from Miss P. Cowl of the Queen Elizabeth Hospital, Woodville. This had a concentration of 1100 LF units/ml and its protein content was found to be 6mgs/ml in a micro-Folin assay using a bovine serum albumin standard.
Gel Chromatography

Sephadex G-75, G-100, G-200 and Sepharose 6B (Pharmacia) were used for fractionating proteins by gel-chromatography. Columns 100 cms long with diameters of 1.5 to 3.5 cms were used. The Pharmacia handbook on gel-chromatography was followed in preparing the columns. The gel was swollen in the appropriate buffer at 4°C for 72 hours before use. After degassing it was poured into the column, with precautions to prevent any trapping of air, swirling of the beads, and uneven layering. After running buffer through it for 48-72 hours, first downwards and then upwards, the exclusion volume and bed volume of the column was determined using Dextran blue (excluded by beads) and para-nitro-phenol (totally trapped by beads), or using normal human serum. The volume of the sample applied was always less than 2% of the volume of the column. The column was used with an LKB fraction collector, ultra-violet absorption scanner and printer so that the protein content of the fractions were visually displayed and recorded.

Ion-Exchange Chromatography

Whatman's DE 52 pre-swollen micro-granular cellulose was used for ion-exchange chromatography in the preparation of immunoglobulins. The gel was prepared and the column poured according to instructions in the Whatman booklet on ion-exchange chromatography. The main steps in this were repeatedly washing the gel in the initial buffer to remove fines and allowing the gel to equilibrate with the starting buffer. When the pH and conductivity of the supernatant
after the wash was the same as the initial buffer, the gel was degassed and poured into the column carefully avoiding swirling and trapping of bubbles. An open system was used with a 5cm head of buffer above the gel at the upper end of the column and using downward flow. That equilibration of the gel had occurred was checked by measuring the pH and conductivity of the effluent and ensuring that it was the same as the initial buffer. The sample, after extensive dialysis against the initial buffer, was then applied carefully on to the column after removing excess head of buffer. This was washed through with the initial buffer. Stepwise changes of buffer were used for eluting off proteins bound to the column. The column was connected to an LKB fraction collector and U-V absorption scanner and printer so that the protein content of the fractions was visually displayed. The stepwise changes of salt concentration in the eluting buffer were only made after the absorption profile had reached a steady base-line.

Affinity Chromatography

Affinity chromatography was performed using columns of protein coupled to commercial Cyanogen bromide activated Sepharose 4B(Pharmacia) according to the instructions issued by Pharmacia with the gel. 2-3 mls of gel usually had 20-30mgs of purified protein attached to it. The gel was swollen and washed in 10^{-3}M Hydrochloric acid. The substance to be coupled was dissolved at a concentration of 10mgs/ml in a solution of 0.5M sodium chloride 0.1M sodium bicarbonate. This was mixed with the gel in a test-tube and rotated end over end for two hours at room temperature.
The ratio of gel to protein during this process was 1 G of swollen gel to 5 mls of protein solution. Unbound protein was washed away with coupling buffer on a sintered glass funnel. Remaining active sites on the gel were quenched with 1 M Ethanolamine at pH 8 for 2 hours. Three washing cycles to remove non-covalently adsorbed protein were used, consisting of a wash at pH4 (0.1 M Acetate buffer with 1 M sodium chloride) followed by a wash at pH8.0 (0.1 M Borate buffer containing 1 M sodium chloride). The effectiveness of the coupling procedure was checked by determining protein losses in the various washing steps. The efficiency of coupling ranged between 75% and 95%.

On using the columns, protein was run on and washed through with 0.1 M Phosphate buffer at pH 7.4 with 0.1% Sodium azide. Elution of bound protein was with 3M Sodium thiocyanate in 0.1 M phosphate buffer pH 7.4. The whole procedure was carried out at room temperature, using an LKB fraction collector, absorption scanner and printer. The eluate was immediately dialysed in the cold against repeated changes of phosphate buffered saline (.04M phosphate, .15M sodium chloride pH 7.4; P.B.S.) and the column washed through with 0.1 M Phosphate buffer.

PROTEIN ESTIMATIONS

Extinction Co-efficients

Quantitation of protein in pure preparations of immunoglobulins was based on determination of the optical density of the solutions at a wavelength of 280 μm using either a Zeiss or a Hitachi-Elman U-V spectro-photometer.
The extinction co-efficient used for calculating immunoglobulin content were $\varepsilon_{280}^{1%}$ 13.4 for IgA (Heremans 1974), 13.8 for IgG (Heremans 1974) and 11.8 for IgM (Williams and Chase, 1968).

**Quantitative Folin Assay**

The protein concentration of non-Ig proteins or mixtures of protein was determined using Lowry's modification of the Folin Ciocalteau assay (Lowry et al., 1951) and bovine serum albumin as a standard.

**Single Radial Immuno-Diffusion (S.R.I.D.)**

The immunoglobulin concentrations in various body fluids and preparation of mixtures of immunoglobulins was determined by single radial immuno-diffusion (SRID) as described by Mancini, Carbonara and Heremans (1965) using commercial heavy-chain specific rabbit anti-sera (Hoechst). The basis of this technique is that immunoglobulin diffuses out of a well and reacts with heavy chain specific antiserum in the agarose, resulting in the formation of a precipitin ring at the point of equivalence. A plot of the square of the diameter of the rings against the concentration of the immunoglobulin gives a straight line in the range of concentrations assayed.

Ten centimetre square glass plates were cleaned and coated with agar by dipping them into molten 0.5% agar. These were stored wrapped in tissue paper till used. When pouring the plates 15 mls of 1% agarose (Calbiochen Grade A) in 0.05 Molar Veronal pH 8.0 buffer with anti heavy-chain
specific serum at 60°C was layered on each of them avoiding trapping air bubbles. After this set, excess agarose was trimmed off from round the plate, 64 wells 2mms in diameter were cut (8 rows of 8 wells 1cm apart) and the agarose sucked out. Each well had a capacity of 3 μls. The amount of anti heavy-chain antisera required to give clear rings of diameters ranging from 3-10 mms with the range of immunoglobulin concentrations being measured were 0.3 mls anti-γ serum and 0.2 mls anti-α and anti-μ serum. 3μl sample volumes were put in the wells using a micro-pipette. All samples had over 48 hours diffusing time in the plates before the plates were washed, which is adequate time for equilibrium to be reached. (R.Samson - 1972). After washing out non-precipitated protein in normal saline, which was repeatedly changed over forty-eight hours, and water, for three hours, the plates were dried in an oven and the precipitin rings stained with 1% Xylene Brilliant Green in methanol, distilled water and acetic acid (5 parts: 5 parts: 1 part). The destainer used to remove excess stain was methanol, distilled water and acetic acid (5 parts: 5 parts: 1 part). Five known standards for each immunoglobulin measured were added to the plates. The range of immunoglobulin standards measured was 1-30 mgs% for 7s IgA and IgG and 1-100 mgs% for secretory IgA and IgM. For serum immunoglobulin determinations, appropriate dilutions of serum were put into the wells. The IgA concentration in saliva and intestinal fluid was measured in terms of secretory IgA standard. By plotting the diameter squared of the rings of the standards against the concentration of
the standards, a standard curve was constructed. The concentration of the unknowns were read off this.

The rationale for the use of Secretory IgA standards for determining IgA concentrations in secretions is that molecular size is important in determining migration of antigen in the gel-layer. Secretory IgA would be underestimated by a factor of 3 if monomeric standards are used (Tomasi 1976). While a more meaningful estimate of IgA content in intestinal aspirate and saliva is obtained by using Secretory IgA standards rather than monomeric IgA standards, it must be remembered that the significant but varying amount of monomeric IgA in these fluids introduces error into these estimates. Degradation of immunoglobulins by intestinal enzymes can result in both over and under estimation of their amounts by single radial immuno-diffusion. Samson and colleagues (1973) studied some of these problems. There does not however appear to be any more meaningful a method of measuring immunoglobulins in gastrointestinal secretions than the technique outlined above.

Qualitative Determinations of Protein Purity

The nature of proteins and their purity after various steps in preparation were determined by immuno-electrophoresis and Ouchterlony analysis (Ouchterlony and Nilsson, 1973). On some occasions further analysis using S.D.S Polyacrylamide gel electrophoresis were carried out by Dr. Peter Ey of the department of Microbiology and Immunology, University of Adelaide.
Immuno-electrophoresis (I.E.P.)

Glass slides were cleaned and coated with agar. Using a Gelman I.E.P. analysis kit, the slides were prepared with a 1mm layer of 1% agarose in .05 Molar Veronal buffer pH 8.0 and wells and troughs cut out. The protein antigens were loaded into the wells using a 5 µl micro-pettor and then electrophoresed for 90 minutes with a 20m.-amp current. 100 µls of antisera were then run into the adjacent trough and left to react for 48 hours. The slides were washed, stained and destained as outlined for the Mancini assays (p.62). Controls run in the assays were previously purified antigens and commercial heavy-chain specific and anti-whole-human-serum antisera. The protein concentration of purified protein antigens or antisera was in the range of 1-5mgs/ml.

Ouchterlony Analysis.

For Ouchterlony analysis, slides with a 1mm layer of 1% agarose in 0.05 M. Veronal buffer (pH 8.1) were used. A rosette of wells was distributed around a central well each well having a capacity of 5 µls. The antiserum was usually placed in the central well and the antigens in the outer wells, though this pattern was reversed when indicated. The washing procedures and staining were as for the Mancinis. By using known antigens and antisera over a range of concentrations and determining whether adjacent immuno-precipitin lines gave lines of identity, partial identity or non-identity, the proteins and antigenic specificity of the antisera were defined.
Precipitin Analysis of Antisera

The antibody content of the crude goat antisera was determined by precipitin analysis. The purified immunoglobulins prepared for injection of the goats were used as the antigens in the precipitin reaction. Doubling dilutions of antigen in 0.5ml volumes (1mg in the first tube) were mixed with 0.5ml volumes of antiserum. These were well mixed and incubated at 37°C for 1 hour. After a further 48-hour incubation at 4°C, the precipitates were spun down. They were then washed with ice-cold normal saline on two more occasions and the final precipitate dissolved in 1 ml of 1N. sodium hydroxide. The protein content of the precipitates was determined using optical density at 280nm. and plotted against the known antigen content of the tubes. At equivalence complete antigen and antibody precipitation is expected with the largest precipitate.

By subtraction of the weight of antigen from the precipitated complex, the antibody content of 0.5 mls of serum was determined. From this the antibody content of the serum was calculated.

Immunoglobulin and Light Chain Preparation

Immunoglobulin A

Serum was obtained from a patient with IgA Myeloma. The concentrations of the major immunoglobulins classes in this serum were IgA 77G/L, IgG 2.8 G/L and IgM 0.2 G/L as determined by single radial immuno-diffusion. After centrifugation at 20,000G for 1 hour, the super-natant lipo-protein layer and any precipitate was discarded.
20 mls of serum was subjected to gel chromatography on a Sephadex G200 column (4cm in diameter, 100 cm high). The 7S peak off the column was pooled and dialysed against 0.02M sodium hydrogen phosphate buffer (pH 6.3). This was centrifuged at low speed to remove the fine precipitate which appeared during dialysis.

The next step in purification was DE52 ion exchange chromatography. The protein was run on to the column (3cms x 40cms) and washed through with 0.02M sodium hydrogen phosphate buffer at pH 6.3. After the OD sub of the common effluent had dropped to 0.05, .05M sodium chloride .02M sodium hydrogen phosphate pH 6.3 was run onto the column. The protein eluted was pooled to give 20mls with an OD sub of 12.8. Using SRID, its IgA concentration was 9.6G/L with less than 0.1G/L IgG and IgM. Immunoelectrophoresis (Fig. III.-1) and Ouchterlony analysis of this did not reveal any contaminating protein.

This protein was used for immunisation of goats in raising the anti-alpha chain serum.

**Immunoglobulin G**

10mls of normal human serum was used. The globulin fraction was precipitated with ammonium sulphate at a 40% concentration (W/V). This was harvested after standing for 24 hours in the cold and re-dissolved in 10mls of .01M sodium hydrogen phosphate buffer at pH 7.0. The globulin was re-precipitated using 35% ammonium sulphate. This precipitate was redissolved in 5mls of the phosphate buffer and dialysed against two changes of 100 volumes of the buffer over 24 hours.
The IgG was obtained from this mixture of protein by DE 52 ion-exchange chromatography. The protein was run onto the column which had been equilibrated with 0.01M phosphate buffer pH 7.0. The column was washed through with the same buffer. The fractions of eluate with unbound protein were pooled and concentrated using aquacide to 10mls. This had an O.D. 280 of 19.8.

Using SRID its IgG concentration was found to be 15 mgs/ml with less than 0.1 mg/ml contamination with IgA and IgM.

Immuno-electrophoresis (Fig III-2) and Ouchterlony analysis confirmed that the protein was IgG. No contamination by IgA and IgM was shown on Ouchterlony analysis, and only an IgG line was obtained on immuno-electrophoresis and development with anti-whole human serum.

Preparation of Light-Chain.

Light chains were prepared from IgG based on the method given in Williams and Chase (1967). 200 mgs of IgG at a concentration of 20 mgs/ml were reduced under nitrogen with 0.75M 2-mercaptoethanol for 90 minutes at 20°C. This was cooled on ice. An equal volume of 0.8M iodoacetamide was added with the pH kept over 8 by dropwise addition of triethylamine. After extensive dialysis against saline at 4°C, the reduced IgG was concentrated using an Amicon diaflo apparatus to 10mls and dialysed against 1 N propionic acid at 4°C. This was then fractionated on a Sephadex G75 column (3cms x 65 cms) previously equilibrated with 1 N propionic acid. Three peaks came through and analysis of these peaks using
polyacrylamide gel electrophoresis techniques by Dr. Peter Ey, confirmed that the first peak corresponded to the molecular weights of heavy-chain dimers, the second to a mixture of heavy and light chains, and the third to light chains only. After dialysis against phosphate buffered saline the pooled light chains were concentrated to 10 mgs/ml and used for affinity chromatography after attachment to activated sepharose beads. (I.E.P. analysis in Fig. III-3).

Preparation of Secretory IgA.

Secretory IgA was prepared according to the method of Newcomb et al (1968), using colostrum obtained from mothers in the first three days after delivery.

14 mls of pooled colostrum was diluted one in two with an equal volume of .15M sodium chloride. This was centrifuged at 20,000 rpm for 60 minutes. The middle layer was harvested leaving the supernatant fat and the precipitated cells. The casein was precipitated from this by lowering its pH to 4.6 with 1M acetic acid. The supernatant was harvested by centrifuging out the casein. After dialysis against .01M tris-HCl pH 8.0, this was subjected to ion-exchange chromatography on DE 52.

Stepwise elution with .01M tris pH 8.0, .05M sodium chloride in .01M tris pH 8.0, and 0.1M sodium chloride in .01M tris pH 8.0 was carried out. The .1M sodium chloride eluate was pooled, concentrated to 10 mls using an Amicon diaflo apparatus and dialysed against PBS. This was subjected to gel chromatographic separation on a Sephadex G 200 column (100 cms by 2.5 cms). The second half
FIGURE III - 1. ANALYSIS OF IgA USING IMMUNO-ELECTROPHORESIS

<table>
<thead>
<tr>
<th></th>
<th>Top trough</th>
<th>Bottom trough</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Anti light-chain serum</td>
<td>Anti α serum (Hoechst)</td>
</tr>
<tr>
<td>B</td>
<td>Anti μ serum (Hoechst)</td>
<td>Anti whole human serum (Hoechst)</td>
</tr>
<tr>
<td>C</td>
<td>Anti α serum (Hoechst)</td>
<td>Anti γ serum (Hoechst)</td>
</tr>
</tbody>
</table>

FIGURE III - 2. ANALYSIS OF IgG USING IMMUNO-ELECTROPHORESIS

<table>
<thead>
<tr>
<th></th>
<th>Top trough</th>
<th>Bottom trough</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Anti α</td>
<td>Anti γ</td>
</tr>
<tr>
<td>B</td>
<td>Anti μ</td>
<td>Anti light-chain</td>
</tr>
<tr>
<td>C</td>
<td>Anti whole human serum (Hoechst)</td>
<td>Anti whole human serum (Hoechst)</td>
</tr>
</tbody>
</table>
Figure III-1

A

B

C

Figure III-2

A

B

C
FIGURE III - 3  ANALYSIS OF LIGHT-CHAINS USING I.E.P.

A  Top well  Light-chains
    Top trough  Anti α
    Bottom trough  Anti γ
    Bottom well  Light-chains

B  Top well  Light-chains
    Top trough  Anti γ
    Bottom trough  Anti μ
    Bottom well  Light-chains

C  Top trough  Anti light-chains
    Middle well  Light-chains
    Bottom trough  Anti whole human serum (Hoechst)

FIGURE III - 4  ANALYSIS OF SECRETORY IgA USING I.E.P.

A  Top well  Secretory IgA
    Top trough  Anti α
    Middle well  IgG
    Bottom trough  Anti γ
    Bottom well  Secretory IgA

B  Well  Secretory IgA
    Bottom trough  Anti secretory component (Hoechst)
    Bottom well  Secretory IgA

C  Top trough
    Middle well  Secretory IgA
    Anti whole human serum (Hoechst)
of the first protein peak containing the secretory IgA was harvested, concentrated and re-run on the column on two more occasions. Concentration of the second half of the protein peak, after the third run, to 2 mgs/ml and analysis using Ouchterlony and immuno-electrophoresis (Fig. III - 4). with commercial anti-whole-human serum, anti-secretory component and anti-alpha serum confirmed that it was secretory IgA. There was no contamination discernible by these techniques at that concentration. Polyacrylamide gel-electrophoresis (done by Dr. P. Ey) revealed only one protein band. The final yield of secretory IgA was 20 mgs.

Preparation of Antisera

Antisera were raised by immunising goats held at the Waite Institute with the purified IgA and IgG. The primary inoculation was with 5 mgs of immunoglobulin in 2.5 mls normal saline emulsified with 2.5 mls of complete Freund's adjuvant. Injections were given in multiple subcutaneous sites. Booster injections of 1 mg immunoglobulin emulsified in incomplete Freund's adjuvant were given at intervals of one to two months in multiple subcutaneous sites. Test-bleeds were performed after 3 injections to confirm that the animals had produced an antibody response to the immunogens. Eight months after the immunisations were started clear precipitin lines were obtained even when using a 1 in 10 dilution of the antisera in the immuno-electrophoretic analyses. The animals were then anaesthetised and bled out through their carotids. After separation, the serum obtained was stored at -20°C.
Analysis of the Anti-IgA Serum.

The Ouchterlony and immuno-electrophoretic analysis of this antiserum resulted in immunoprecipitin lines forming strongly against IgA, and weakly against IgG and IgM (Figure III-5). It was assumed that the lines against IgG and IgM were a consequence of light-chain cross-reactivity. This was not investigated but as will be described below, affinity chromatography using a Sepharose linked secretory IgA column and a Sepharose linked IgG column allowed the preparation of anti α-heavy-chain specific antiserum.

Quantitative precipitin analysis using the monomeric IgA gave the specific anti-IgA antibody content of the serum as 0.05 mg/ml.

Analysis of the Anti-IgG Serum.

This serum reacted very strongly with IgG and weakly with IgA and IgM on immunoelectrophoresis (Fig. III -6) and Ouchterlony analysis. As the activity against IgA and IgM was removed on running the serum through a Sepharose linked light-chain affinity column it seems likely that these lines were a consequence of light-chain cross-reactivity.

Quantitative precipitin analysis of the goat serum using purified IgG gave the specific IgG antibody content of serum as 4mgs/ml.

Columns for Affinity Chromatography.

Light chains, IgG and secretory IgA were attached to activated Sepharose 4B as outlined above (page 65 ). 20-40 mgs of each protein was used.
Purification of Specific Antibodies from Goat Antisera.

Anti-gamma antibodies were prepared by separating anti IgG antibodies from the crude anti IgG antiserum run on the Sepharose linked IgG column in 4ml aliquots. After elution with 3M sodium thiocyanate and dialysis against PBS, light-chain cross reactivity was removed by running the anti IgG serum through the Sepharose linked light-chain column. This step was repeated to ensure that there was no residual light-chain cross-reactivity. After concentration of the presumptive anti-gamma antibodies to 1mg/ml, their activity was confirmed by immuno-electrophoresis and Ouchterlony analysis which showed activity only against IgG and not against IgA and IgM (Fig. III-6).

Anti-alpha antibodies were prepared using the secretory IgA affinity column for obtaining the anti-IgA activity and the IgG affinity column for removing light-chain cross-reactivity. I.E.P. and Ouchterlony analysis confirmed the anti-alpha activity of the serum and the absence of light-chain cross-reactivity. (Fig.III-5).

The anti LC antibodies were obtained by eluting off the protein left on the light chain affinity column after running the crude anti IgG antiserum through it. This reacted with purified IgA, IgG and IgM in immuno-electrophoresis and Ouchterlony analyses (Fig. III.-7). These purified specific antibody containing antisera were stored at 4°C in PBS with 0.1% sodium azide. They were at a concentration of 2 mg/ml.
FIGURE III - 5. ANALYSIS OF ANTI IgA AND ANTI - α SERUM USING IMMUNO-ELECTROPHORESIS

A  Top well  Whole human serum
    Top trough  Crude anti IgA (neat)
    Middle well  IgA
    Bottom trough  Crude anti IgA (1/10 dilution)
    Bottom well  Whole human serum

B  Top well  IgG
    Top trough  Crude anti IgA (neat)
    Middle well  'IgM'
    Bottom trough  Crude anti IgA (1/10 dilution)
    Bottom well  IgG

C  Top well  Secretory IgA
    Top trough  Anti α
    Middle well  IgG

D  Top well  IgM
    Top trough  Anti α
    Middle well  Whole human serum

E  Top well  Light-chains
    Top trough  Anti α
    Middle well  Secretory IgA
    Bottom trough  Anti α(Hoechst)
    Bottom well  Whole human serum
FIGURE III - 6. ANALYSIS OF ANTI IgG SERUM AND ANTI-\( \gamma \) ANTIBODY USING IMMUNO-ELECTROPHORESIS

A  Top well  Whole human serum  
   Top trough  Crude anti IgG  
   Middle well  Secretory IgA  
   Bottom trough  Crude anti IgG  
   Bottom well  Whole human serum  
(1/10 dilution)

B  Top well  IgG  
   Top trough  Crude anti IgG  
   Middle well  IgM  
   Bottom trough  Crude anti IgG  
   Bottom well  IgG  
(1/10 dilution)

C  Top well  Light-chains  
   Top trough  Anti \( \gamma \)  
   Middle well  IgG  
   Bottom trough  Anti \( \gamma \) (Hoechst)  
   Bottom well  Whole human serum

D  Top well  Secretory IgA  
   Top trough  Anti \( \alpha \)  
   Middle well  IgG  
   Bottom trough  Anti \( \gamma \)  

E  Middle well  Whole human serum  
   Bottom trough  Anti \( \gamma \)  
   Bottom well  IgM
Figure III - 6

A

B

C

D

E

WHS

Agk'G

61A

Agk'G ko

WHS

Lc

Agk'G

15A

Agk'G ko

WHS

61A

Agk'G ko

WHS

15A

Agk'G ko

WHS

15A

Agk'G ko

WHS

15A

Agk'G ko

WHS

15A

Agk'G ko

WHS
**FIGURE III - 7  ANALYSIS OF ANTI LIGHT-CHAIN ANTIBODY**

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>Description</th>
</tr>
</thead>
</table>
| A      | Top well: Whole human serum  
Top trough: Anti light-chain  
Middle well: Light-chains  
Bottom trough: Anti whole human serum (Hoechst)  
Bottom well: Whole human serum |
| B      | Top well: IgG  
Bottom trough: Anti light-chain  
Bottom well: Secretory IgA |
| C      | Top well: IgM  
Top trough: Anti \( \mu \)  
Middle well: Whole human serum  
Bottom trough: Anti light-chain  
Bottom well: IgM |
Figure III - 7

A

B

C
Preparation of Lipo-polysaccharide from Salmonella bovis-morbificans.

This was required for quantitation of the radio-immuno-assay described in Chapter 4.

Salmonella bovis-morbificans isolated from the stools of the patient whose serum was used for the quantitation experiment was grown from a freeze-dried ampoule in which it had been stored.

A modification by Dr. S.H. Neoh of the Department of Microbiology and Immunology of Westphal's Phenol-water extraction process (Westphal et al 1952) was used for obtaining the lipo-polysaccharide. In brief, bacteria harvested from 50 nutrient agar plates and suspended in distilled water were extracted by 45% Phenol for 20 minutes at 65°-66°C. After cooling, the aqueous layer was harvested by centrifugation and precipitated with 5 volumes of cold ethanol. The resulting precipitate was harvested, re-dissolved in distilled water and the nucleic acid in it precipitated by adding an equal volume of ethanol. The precipitate was spun out and discarded. More alcohol was added to the supernatant to bring the proportion of alcohol in it up to 5 volumes. The resulting precipitate was spun out, redissolved in distilled water and ultracentrifuged at 100,000G on two occasions. The final pellet was dissolved in 5mls of distilled water.

The dry weight of this final preparation was 12.4 mg/ml and the lipo-polysaccharide concentration as calculated on the basis of 2-Keto 3-deoxyoctanoic acid (K.D.O.) content was 12 mg/ml. The protein content as determined by a micro-Folin assay was 0.12 mg/ml.
**Commercial Antisera and Standards.**

In the Mancini radial immunodiffusion assays of immunoglobulin concentration, and Ouchterlony and immunoelectrophoresis analyses of proteins, commercial heavy chain (α, γ, and μ) specific rabbit antisera from Behringwerke were used. Antisera against whole human serum and against secretory component were obtained from the same source. In the Mancini assays commercial standards from Behringwerke were used for all the quantitative estimations except for measuring IgA in secretions, where secretory IgA (p.74) standards were used.

**Radio-Iodination of Antisera.**

Radio-iodination of secondary antibody was carried out using the method of McConahey and Dixon (1966). The specific goat antibodies to human immunoglobulins were iodinated at a concentration of about 2 mgs/ml, in PBS. 50 μl of protein solution was reacted with 250 μ Ci of I$^{125}$ in 10 μls of de-ionised water in the presence of 10 μgs of Chloramine T in 10 μls of de-ionised water for 9 minutes on ice. The reaction was terminated with 12 μgs of sodium meta-bi-sulphite in 12 μls de-ionised water and the free iodide separated by chromatography on Sephadex G 25. The specific activities were approximately 1000 cpm/ng. protein.

**Haemagglutination Assay for Anti-Tetanus Toxin.**

In this assay, antibody ability to agglutinate red cells coated with Tetanus Toxoid was titrated.

Ten mls of human O-positive red cells from three individuals were pooled and washed in normal saline three
times. Toxoid coated red cells were made by reacting 1 ml of washed red cells with 5 mls 0.25% bovine serum albumin (BSA) in normal saline in the presence of three mls Tetanus Toxoid (at a concentration of 80 L.F. units/ml) and 1 ml of 0.1% chromic chloride for 10 minutes at room temperature.

After two washes in normal saline, the cells were re-suspended to 1%. Uncoated red cells for use as controls were prepared with 8 mls 0.25% BSA, 1 ml of 0.1% chromic chloride and no Tetanus Toxoid. Serial doubling dilutions of serum and intestinal juice were made in 25 µl volumes in micro-titre trays with V-shaped wells. Paired series, with 25 µls of coated red cells and 25 µls of uncoated red cells added to each dilution, were first incubated at room temperature for two hours and then held at 4°C overnight. Settling patterns were examined the following morning. A small button of cells and a clear surrounding plastic floor were counted as negative, a mat of cells obscuring the plastic floor of the well as positive and the presence of both a mat of red cells and a button being read as +.

The assay was based on the assay being used in the Clinical Immunology Laboratory at the Queen Elizabeth Hospital, Adelaide, with minor modifications to prevent auto-agglutination of the coated red cells in intestinal aspirate, even in the absence of antibody.

Vibriocidal Assay (Rowley, 1968)

Doubling dilutions of the biological fluid being assayed were made in 0.4 ml volumes of peptone saline.
(Peptone saline is a sterile solution of 0.1% Difco Proteose peptone in isotonic saline). An equal volume of 10% guinea pig serum (as a source of complement) in peptone saline containing about $10^4$ viable Vibrios (V.cholerae 569B) was added to each tube. Antibody free controls were included in each assay. All these steps were carried out on ice. The tubes were then incubated at $37^\circ$C for 1 hour and returned to the ice bath to prevent further killing or further growth of organisms.

The number of organisms in a drop from a Pasteur pipette (about 25 µl) was determined by averaging the number of organisms growing up in three separate drops spotted out on nutrient agar plates and grown up overnight at $37^\circ$C. The vibriocidal titre of a biological fluid was defined as the dilution which gave 50% reduction in the control level of organisms. This 50% end point was determined by interpolation from a plot of percentage surviving organisms against antibody dilutions.

Radio-Immuno Assay.

Development of this assay and details of the methods used are given in Chapter 5.

Sampling of Subjects.

Blood obtained by venepuncture was allowed to clot in a sterile tube. After 1 hour at $37^\circ$C, the tube was held at $4^\circ$C for 6-20 hours to facilitate clot retraction. The serum was then harvested and spun on a desk centrifuge to remove any red cells. After an hour at $56^\circ$C to heat-inactivate complement, the serum was stored at $-20^\circ$C.
Intestinal fluid was obtained from the upper jejunum of subjects studied through a double lumen Salem sump tube (Argyle; 12 French gauge) which incorporates a radio opaque marker. A small mercury bag was sewn onto the end to give it weight. The tube was swallowed after a fast of at least 8 hours. Local anaesthetic was sprayed onto the subject's pharynx to reduce gagging. The position of the tube was checked fluoroscopically and aspiration was commenced when the tube was beyond the duodeno-jejunal flexure. The aspirated fluid was collected into a sterile container held on ice. The pH of the fluid stored was over 6.0 unless otherwise stated. The pH was determined using pH papers. The pooled fluid was transferred as soon as possible (10 minutes to 45 minutes) to small bottles and stored in 0.5 ml to 1.0 ml aliquots at -70°C.

Parotid saliva was collected by applying a Curby cap to the inside of the subject's cheek over the orifice of the parotid duct and holding it there by suction. The flow of parotid saliva was stimulated by placing a few crystals of citric acid on the patient's tongue. This was stored at -70°C in 0.3 to 1.0 ml aliquots.
BACKGROUND

A preliminary question that arose in relation to the studies of intestinal antibody was whether there was any advantage to be gained from storing samples of intestinal fluid in a refrigerator at -80°C rather than in the more commonly available -20°C refrigerators.

Samson and colleagues (1973) had reported that at -20°C intestinal IgA was stable, intestinal IgM decreased by 20% in 1 month, and IgG decreased to undetectable levels. These conclusions were based on estimates of concentration using Mancini SRID assays. These estimates depend on the preservation of the antigenicity of the heavy chain part of molecule and on molecular size. It was conceivable that the FAB binding ability of the molecule could be damaged during storage by enzyme activity in the intestinal fluid to a greater extent than was suggested by its concentration on SRID assay.

Design of Experiment

The aim of the experiment was to determine the stability of an IgG antibody and an IgM antibody in intestinal aspirate over a period of two months.

In order to determine the stability of IgG antibody, serum from a volunteer hyper-immunised with Tetanus Toxoid was obtained (from Miss P. Cowled, Scientific Officer, Department of Medicine, Queen Elizabeth Hospital Woodville).
Figure IV-1

Vibrio-cidal activity in fractions of serum off a Sephadex G200 column

![Diagram showing % Transmission 280nm vs Fraction No. for IgM and IgG, as well as Bacteriocidal Titres against 1:100 to 1:5000.]
Diluted 1/100 in normal saline this gave a haemagglutination titre of 1/2048 (11 wells) to 1/8192 (13 wells) in the haemagglutination assay described in Chapter 3. (page 79). That this haemagglutination activity was in the IgG antibody class was shown by its complete resistance to 0.1M mercapto-ethanol at 37°C for 1 hour. On adding it in a dilution of 1/100 to jejunal aspirate obtained from 5 healthy volunteers it caused haemagglutination to the same extent or to 1 well less than that of the control (1/100 dilution of the serum in saline). The jejunal aspirate without antibody haemolysed the red cells to 3 or 4 wells (1/8 to 1/16) and caused non-specific agglutination to 5 wells (1/32).

As a source of predominantly IgM antibody, serum from a colleague given two cholera vaccinations, was obtained. This had a vibriocidal titre of between 1/64,000 and 1/100,000 in repeated assays. That this vibriocidal activity was mainly in the IgM class was shown by a decrease of over 75% after exposure to 0.1M mercapto-ethanol for 1 hour and by the distribution of antibody activity in the fractions obtained after a Sephadex G 200 fractionation of some of this serum (Figure IV-1). On adding it in a 1/100 dilution to the pooled jejunal aspirate of 5 normal volunteers its activity remained unchanged (i.e. the jejunal fluid had a vibriocidal titre of 1/100) on immediate assay.

Samples of the intestinal fluid were stored at -80°C, -20°C and 4°C. Individual samples were retrieved and assayed over the ensuing two months, in each case using a 1/100 dilution in saline of the original serum as a control.
TABLE IV - 1. STABILITY OF IgM ANTIBODY ON STORAGE

(Vibriocidal titre of control serum and seeded aspirate)

<table>
<thead>
<tr>
<th>Time after seeding</th>
<th>Control</th>
<th>Intest. Aspirate -80°C</th>
<th>Intest. Aspirate -20°C</th>
<th>Intest. Aspirate +4°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 days</td>
<td>1/1000</td>
<td>1/600</td>
<td>1/700</td>
<td>1/500</td>
</tr>
<tr>
<td>1 week</td>
<td>1/800</td>
<td>1/1000</td>
<td>1/1200</td>
<td>1/600</td>
</tr>
<tr>
<td>2 weeks</td>
<td>1/640</td>
<td>1/640</td>
<td>1/640</td>
<td>1/200</td>
</tr>
<tr>
<td>1 month</td>
<td>Failed</td>
<td>Failed</td>
<td>Failed</td>
<td>Failed</td>
</tr>
<tr>
<td>2 months</td>
<td>1/700</td>
<td>1/1000</td>
<td>1/900</td>
<td>1/50</td>
</tr>
</tbody>
</table>
TABLE IV - 2 STABILITY OF IgG ANTIBODY ON STORAGE

(Haemagglutination titres using red cells coated with tetanus toxoid)

<table>
<thead>
<tr>
<th>Time after seeding</th>
<th>Control</th>
<th>Intest. Aspirate -80°C</th>
<th>Intest. Aspirate - 20°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wells Titre</td>
<td>Wells Titre</td>
<td>Wells Titre</td>
</tr>
<tr>
<td>1 day</td>
<td>11 1/2048</td>
<td>10 1/1024</td>
<td>10 1/1024</td>
</tr>
<tr>
<td>4 days</td>
<td>12 1/4096</td>
<td>11 1/2048</td>
<td>10 1/1024</td>
</tr>
<tr>
<td>1 week</td>
<td>11 1/2048</td>
<td>10 1/1024</td>
<td>10 1/2048</td>
</tr>
<tr>
<td>2 weeks</td>
<td>12 1/4096</td>
<td>11 1/2048</td>
<td>11 1/2048</td>
</tr>
<tr>
<td>1 month</td>
<td>12 1/4096</td>
<td>11 1/2048</td>
<td>8 1/ 256</td>
</tr>
<tr>
<td>2 months</td>
<td>8 1/ 256</td>
<td>9 1/ 512</td>
<td>BG BG</td>
</tr>
</tbody>
</table>

TABLE IV - 3 DECAY OF IgG ANTIBODY AT ROOM TEMPERATURE

<table>
<thead>
<tr>
<th>Time at 20°C</th>
<th>0 hours</th>
<th>1 hour</th>
<th>3 hours</th>
<th>6 hours</th>
<th>18 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample (previously at -80°C)</td>
<td>Wells Titre</td>
<td>Wells Titre</td>
<td>Wells Titre</td>
<td>Wells Titre</td>
<td>Wells Titre</td>
</tr>
<tr>
<td>11 1/2048</td>
<td>8 1/ 256</td>
<td>7 1/ 128</td>
<td>6 1/ 64</td>
<td>BG BG</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>12 1/4096</td>
<td>12 1/4096</td>
<td></td>
<td></td>
<td>11 1/2048</td>
</tr>
</tbody>
</table>

BG: Background (The intestinal fluid without added antibody caused agglutination to 5 wells (1/32)).
Results

There was no evidence of any difference in the stability of IgM antibody, held at -20°C from that held at -80°C during the two months storage. There was gradual but fairly complete loss of activity of the IgM antibody held at 4°C (in the presence of sodium azide 0.1% to stop bacterial growth) over the two month period. (Table IV-1).

In the case of IgG antibody a clear-cut difference became apparent after 1 month with loss of antibody activity in the sample stored at -20°C and no detectable loss of activity in the sample stored at -80°C. (The lower control value at two months probably reflects sub-optimal coating of the red cells with Tetanus Toxoid). Complete loss of this IgG antibody activity in intestinal fluid at room temperature was seen within a few hours. (Tables IV, 2 and 3).

Conclusions

The results obtained suggest that the stability of IgG and IgM as determined in the Mancini SRID assay was very similar to the stability of antibody activity in these two immunoglobulin classes, in terms of the assays used in this experiment.

A corollary to these results is that storage of intestinal aspirate at -80°C has some advantage over storage at -20°C in preventing degradation of antibody activity of particularly IgG molecules.
CHAPTER 5

RADIO-IMMUNO ASSAY

The basic demands that an assay had to meet to be of use in the studies planned were that it had to be sensitive, capable of measuring even partially degraded antibody provided it could bind to antigen, and to be not dependent on complement activation. Among such assays that had been used in other areas was a radio-immuno-sorbent assay developed by Nielsen, Parratt and White (1973) for measuring small amounts of serum antibody to Microspora polyfaeni, aetiological agent in the allergic disease known as Farmer's Lung. It had, as an added advantage, the potential to estimate the immunoglobulin class-distribution of the antibody being measured. This assay was modified and developed for the studies described in this thesis.

The basic principles of the assay were as follows:
Antibody in a biological fluid was absorbed onto whole bacteria against which it was specifically directed; radio-labelled anti human immunoglobulin was used to measure the amount of specific antibody to the bacteria. (The specific antibacterial antibody that bound in the first stage will be referred to as 'primary antibody' and the radio-labelled anti human immunoglobulin antibody that bound to the primary antibody as 'secondary antibody'.) Serial dilutions of the biological fluid containing the primary antibody were made and the titre of the fluid taken as the dilution of the biological fluid that resulted in the binding of a certain proportion of secondary antibody.
The secondary antibody was prepared by affinity chromatography as outlined in Chapter 3. Anti-light-chain antibody was used to measure all classes of intact primary antibody and also antibody fragments that bound. Primary antibody of the IgA and IgG classes were measured by using anti-alpha and anti-gamma heavy-chain-specific antibodies respectively as the secondary antibody.

Description of Assay

The assay in its final form will first be described. Following that, experiments carried out during the development of the assay will be discussed.

The bacteria used as the solid phase for absorbing primary antibody were grown from single colonies in 1 litre volumes of nutrient broth overnight at 37°C on a shaker. Two or three litres of this culture which usually had a density of $2 \times 10^3$ organisms/ml were harvested by centrifuging the organisms in 750 ml flasks at 5,000 rpm for 20 minutes. They were washed in phosphate buffered saline (0.04 molar phosphate pH 7.4, 0.15 molar sodium chloride: P.B.S.) in about a fifth of their original volume on two occasions, centrifuging the bacteria to a pellet after each wash and discarding the supernatant. They were then fixed for 20 minutes in 0.2% glutaraldehyde at a concentration of approximately $5 \times 10^9$/ml (about half their original volume). After three more washes they were stored in phosphate buffered saline with 0.1% sodium azide at $10^{-11}$/ml. Prior to use in the assay they were again centrifuged and resuspended in phosphate buffered saline to remove any antigen that had escaped into solution.
The secondary antibody was prepared and radio-labelled as described in Chapter 3. Between 4 and 40 ng of specific antibody approximately (10,000 cpm) were added per tube.

0.1% bovine serum albumin in phosphate buffered saline (PBS.BSA) was used for diluting the biological fluids and the radio-labelled antiserum, and also for the washes.

Detailed Steps in Assay

All procedures were done in the cold - on ice or in a refrigerated centrifuge (4°C).

1) Serial doubling dilutions of biological fluid in PBS.BSA in 100 μl volumes in plastic tubes were made.

2) 50 μls of bacteria (10^10/ml) against which antibodies were being sought were added to each tube.

3) Incubation for 30 minutes (binding of primary antibody).

4) Washed with 2.5mls PBS.BSA - vortex mixed and centrifuged at 2,500 rpm for 15 minutes. Supernatant decanted and washing procedure repeated three times. This was to remove non-specific immunoglobulin.

5) Radio-labelled secondary antibody added to each bacterial pellet, mixed well and allowed to stand for one hour (binding of secondary antibody).

6) Washed with 2.5mls PBS.BSA as described in step 4 three times.

7) Tubes counted in a gamma counter.
Shape of Immune Curve

The results were plotted on semi-log paper with counts per minute as the ordinate on a linear scale and the reciprocal of the dilution as the abscissa on a log scale.

A representative plot of results from an immune serum are shown in Figure V-1.

As will be discussed below, the antigenic surface provided by the bacteria under the conditions of the assay is in excess of that needed for maximum binding of secondary antibody. In zone A, all active secondary antibody is taken up by the excess of primary antibody bound to the bacteria. In zone B, the amount of primary antibody bound to the bacteria is less than that needed to take up all the active radio-labelled secondary antibody. The radioactivity bound to the bacterial pellet falls off exponentially in this zone till it reaches zone C where in effect the antibody in the biological fluid has been diluted out to the extent that the radio-activity bound after the second incubation is no different from the background binding. The background binding is the residue of radio-activity after adding secondary antibody to the bacteria without prior exposure to primary antibody. This varied between 0.5% and 2% of added radio-activity. The titre of the biological fluid was taken as the dilution at which there was binding of 10% of the added radio-activity.

Experiments in Development of the Assay

Effects of Washes

In many of the earlier experiments a marked pro-zone was seen in titrations of some samples. This pattern decreased or disappeared on increasing the number of washes
Figure V - 1

Representative Binding-curve in Radio-immuno-assay
the bacteria had, prior to adding the radio-labelled antiserum (Figure V-2). The prozone probably reflects the presence of unbound immunoglobulin with no specific antibody activity remaining behind, in spite of washes to remove it. This binds some of the radio-labelled secondary antibody, and then is washed off with it, prior to counting. This leads to reduction in maximum binding in low dilutions of the sample.

**Effects of Time on Binding**

In this assay the times of incubation were fixed at 30 mins for the primary reaction, and 60 mins for the second. As shown in figure V-3 the uptake of primary antibody appears to have reached equilibrium in 30 minutes but the second antigen antibody reaction had clearly not reached equilibrium within one hour (Fig.V-4).

**Effect of Temperature on Binding**

In the case of serum antibody there was no difference apparent in the amount of primary antibody bound to the bacteria whether the incubation was at 20°C or at 4°C. Intestinal antibody on the other hand bound better at 4°C than at 20°C. (Fig. V-5). A possible explanation for this may be that the intestinal antibodies are of low avidity and thus bind better in the cold.

**Effect of Carrier Protein**

In the assays, initially normal goat serum (NGS) and later bovine serum albumin were used as inert protein with the aim of reducing the non-specific binding of primary and secondary antibody to the bacteria and the tubes. While
FIGURE V - 2

Effects of washing bacteria prior to adding secondary antibody

Identical series of a serum, given different numbers of washes after primary incubation and prior to adding radio-labelled secondary antibody.

● 2 washes
■ 4 washes
◆ 5 washes
Figure V-2

EFFECT OF WASHING BACTERIA PRIOR TO ADDING SECONDARY ANTIBODY

Radio-activity bound x 10^3 cpm

Dilution

○ 2 washes
□ 4 washes
● 5 washes
Figure V-3

TIMES OF INCUBATION (PRIMARY)

Primary incubation (secondary - 60')

- 30 minutes
- 60 minutes

Dilution

$\gamma_{10}$ $\gamma_{200}$ $\gamma_{800}$ $\gamma_{3200}$ $\gamma_{12800}$

cpm bound

- 3000
- 2000
- 1000

$\gamma_{700}$
FIGURE V - 4.

Times of Incubation (Secondary)

The effect of different times of incubation on binding of secondary antibody to identical series of a serum. (The primary incubation was for thirty minutes).

<table>
<thead>
<tr>
<th>Time of secondary incubation</th>
<th>Titre (10% binding)</th>
</tr>
</thead>
<tbody>
<tr>
<td>● 15 minutes</td>
<td>1/260</td>
</tr>
<tr>
<td>X 30 minutes</td>
<td>1/480</td>
</tr>
<tr>
<td>O 60 minutes</td>
<td>1/700</td>
</tr>
<tr>
<td>◊ 4 hours</td>
<td>1/1400</td>
</tr>
<tr>
<td>□ 18 hours</td>
<td>1/7600</td>
</tr>
</tbody>
</table>
Figure V-4

Times of Incubation (Secondary)

![Graph showing times of incubation with end-point at 1000 cpm bound.](image-url)
Figure V-5

VARIATION IN BINDING OF INTESTINAL ANTIBODY WITH TEMPERATURE

Sample A
- $4^\circ$C
- $20^\circ$C

Sample B
- $4^\circ$C
- $20^\circ$C

Dilution

cpm bound

2000
1000

$\frac{1}{10}$ $\frac{1}{20}$ $\frac{1}{40}$ $\frac{1}{80}$ $\frac{1}{160}$ $\frac{1}{320}$
TABLE V - 1

REDUCTION OF NON-SPECIFIC BINDING OF SECONDARY ANTIBODY BY COATING TUBES WITH NORMAL GOAT SERUM.

<table>
<thead>
<tr>
<th>Tube</th>
<th>Added Radio-activity (c.p.m.)</th>
<th>Coated tube</th>
<th>Uncoated tube</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>cpm bound</td>
<td>% bound</td>
</tr>
<tr>
<td>1</td>
<td>200</td>
<td>30</td>
<td>15%</td>
</tr>
<tr>
<td>2</td>
<td>400</td>
<td>36</td>
<td>9%</td>
</tr>
<tr>
<td>3</td>
<td>800</td>
<td>85</td>
<td>11%</td>
</tr>
<tr>
<td>4</td>
<td>1000</td>
<td>107</td>
<td>11%</td>
</tr>
<tr>
<td>5</td>
<td>2000</td>
<td>310</td>
<td>15%</td>
</tr>
<tr>
<td>6</td>
<td>4000</td>
<td>795</td>
<td>20%</td>
</tr>
<tr>
<td>7</td>
<td>10000</td>
<td>2729</td>
<td>27%</td>
</tr>
<tr>
<td>8</td>
<td>20000</td>
<td>5789</td>
<td>29%</td>
</tr>
</tbody>
</table>
there was no direct evidence for this on comparing sera titrated, using PBS as diluent with and without inert protein, the ability of inert protein (normal goat serum) to reduce non-specific binding of secondary antibody was shown indirectly.

Radio-labelled anti LC serum was added to one set of 8 tubes coated with normal goat serum in the amounts shown, (Table V-1) and to another set of tubes that had no pre-treatment. After incubation for ten minutes these were washed three times with PBS containing 1% normal goat serum. The amount of radio-activity bound non-specifically was much less in tubes 7 and 8 of the series coated with normal goat serum than in the series that had no pre-treatment.

The assay was finally performed with PBS containing 0.1% bovine serum albumin as diluent for washing non-specific immunoglobulin and unbound secondary antibody off the bacteria and tubes, for diluting the intestinal fluid and sera and the radio-labelled antisera.

**Volumes of Antibody Containing Fluid.**

Increasing the amount of antibody presented to the bacteria by increasing the volume of fluid incubated with the bacteria resulted in an increase in the amount of antibody bound by the bacteria. In an experiment using 0.05 ml, 0.1 ml, 0.5 ml and 1.0 ml volumes of the same dilutions of fluid, an apparent increase in titre of the sample was seen proportionate to the increase in antibody presented to the bacteria for binding. (Fig. V-6).
FIGURE V - 6

Volumes of Antibody containing fluid

4 series with different volumes of dilutions of a serum at the stage of primary incubation with bacteria.
Figure V-6

VOLUMES OF ANTIBODY CONTAINING FLUID

<table>
<thead>
<tr>
<th>Volumes incubated</th>
<th>Titres</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05 ml</td>
<td>1/600</td>
</tr>
<tr>
<td>0.1 ml</td>
<td>1/100</td>
</tr>
<tr>
<td>0.5 ml</td>
<td>1/5600</td>
</tr>
<tr>
<td>1.0 ml</td>
<td>1/1500</td>
</tr>
</tbody>
</table>

- cpm bound vs Serum Dilution

- Various volumes of fluid incubated and their corresponding titres are shown in the graph.
This is clearly dependent on the bacteria providing a great excess of antigen binding sites relative to primary antibody in zone B (Fig. V-1) of the binding curve.

**Excess of Bacteria.**

In the region (B) of the curve which is used for determining the titre of the fluid being assayed, one possible variable was the amount of antigen provided for binding of primary antibody. In an experiment (Fig. V-7) using 1/500 dilution of serum which had a titre of greater than 1/500, 100 µl volumes of this serum dilution were incubated with 10 µls, 25 µls, and 100 µls of bacteria in a concentration of $10^{10}$/ml. Varying amounts of radio-labelled (secondary) anti-light-chain antibody emitting 1,000 cpm, 5,000 cpm, and 2,000 cpm were added to tubes with each concentration of bacteria. Increasing the antigenic surface provided by the bacteria made no difference to the proportion of added radio-activity bound. Increasing the amount of secondary antibody added increased the amount bound proportionately between the range of 1,000 to 20,000 cpm. This indicates the binding of primary antibody was not limited by even a five-fold reduction in the antigenic surface provided by the amount of bacteria used in the assay.

**Method of Preparing Bacteria for Use in Assays**

Different methods of killing and preserving bacteria have different effects on antigen preservation which could clearly affect the ability of the bacteria to have antibody bind to them. Among the methods used to kill bacteria are
Excess of bacteria used as an immuno-adsorhent.

3 series of 100 μls of a 1/500 dilution of a serum (with a titre > 1/500) incubated with different amounts of bacteria during primary incubation.
Figure V-7

ABSORPTIVE SURFACE PROVIDED BY $10^8$ BACTERIA IS NOT LIMITING

Bacteria ($10^{10}$/ml added)
- $10 \mu$l
- $25 \mu$l
- $100 \mu$l

cpm bound


cpm added (Anti LC Serum)
Glutaraldehyde fixation, boiling and alcohol fixation. Experiments were done using these methods, and also with live organisms to determine how the different methods of fixing affected the binding of antibody from an immune serum.

Fig. V-8 shows the result of one such experiment. In series A live organisms were used. In series B organisms fixed with 0.2% glutaraldehyde (as described on page 87) were used. In series C, boiled organisms which had been held at 100°C on a water bath for one hour, cooled, and held at 100°C for another hour, were used after washing. In series D, organisms at $10^{10}$/ml in five times their volume of absolute alcohol were held at 4°C overnight. They were washed three times before use. The bacteria used in all the series were prepared from equal volumes of the same starting pool. They were made up to a final concentration of $10^{10}$/ml. The titre of the serum as assessed using the ethanol killed bacteria was 50% less than when using the glutaraldehyde fixed organisms. The live and the boiled organisms were very similar to the glutaraldehyde fixed organisms Fig. (V-8).

Ethanol fixation of bacteria denatures protein antigens and this may explain the poorer antibody binding capacity of bacteria fixed in this way. The marginally higher binding with the glutaraldehyde fixed bacteria may have been a consequence of better recovery of organisms fixed in this way than of live or boiled organisms after centrifuging. This difference was only marginal.
**Figure V-8**

Different Preparations of Bacteria

<table>
<thead>
<tr>
<th>Series</th>
<th>Bacterial Preparation</th>
<th>Titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Live</td>
<td>1/5000</td>
</tr>
<tr>
<td>B</td>
<td>Glutaradehyde fixed</td>
<td>1/6400</td>
</tr>
<tr>
<td>C</td>
<td>Boiled</td>
<td>1/5000</td>
</tr>
<tr>
<td>D</td>
<td>Ethanol fixed</td>
<td>1/2400</td>
</tr>
</tbody>
</table>

![Graph showing cpms bound against dilutions for different bacterial preparations]
In the original description of the assay (Nielsen, Parratt and White 1973) the need to wash off antigen that went into solution before using the micro-organisms each time was emphasised. Though preliminary experiments did not confirm that this was necessary when using glutaraldehyde fixed bacteria in the assay, the stock bacteria were washed before use in each assay.

Specificity of Assay

The specificity of this assay in detecting primary antibody binding was examined with immune sera and aspirates.

Serum from a patient with a recent Salmonella typhi-murium infection was titrated using Salmonella typhi-murium, E.coli 0134 and Vibrio cholerae. As seen in table V-2, the titre of the serum was much higher against the infecting organisms than against the other two organisms. That this high titre represented specific antibody was confirmed by absorption experiments using $10^{11}$ organisms in 1.5 mls of a 1/100 dilution of the serum for twenty minutes on ice.

In another experiment sera from two patients who had had Salmonella gastroenteritis, one with S. typhi-murium (Fig. V-9) and one with S. newport (Fig. V-10) were assayed using 4 different species of Salmonella and Citrobacter, all fixed with glutaraldehyde. The known antigenic constituents of the bacteria used are shown (Table V-3) as defined by F. Kaufmann (ref. F. Kaufmann's "Classification of Bacteria" - Munksgard 1975). Citro-bacter shares two major O-somatic antigens with Salmonella typhi-murium (5 + 12)
TABLE V - 2. TITRES OF SERUM PRE- AND POST ABSORPTION, FROM
A PATIENT WITH SALMONELLA TYPHI-MURIIUM INFECTION.

<table>
<thead>
<tr>
<th></th>
<th>Absorbed with S. typhi-murium</th>
<th>Absorbed with E. coli</th>
<th>Absorbed with V. cholerae</th>
<th>Unabsorbed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Titrated vs. S. typhi-murium</td>
<td>110</td>
<td>1100</td>
<td>1800</td>
<td>1100</td>
</tr>
<tr>
<td>Titrated vs. E. coli</td>
<td>170</td>
<td>120</td>
<td>200</td>
<td>240</td>
</tr>
<tr>
<td>Titrated vs. V. chol.</td>
<td>110</td>
<td>120</td>
<td>160</td>
<td>120</td>
</tr>
</tbody>
</table>
**TABLE V - 3**

ANTIGENS OF BACTERIA USED IN CHECK ON SPECIFICITY OF ASSAY (Kaufmann - White Classification).

<table>
<thead>
<tr>
<th>Organism</th>
<th>O Antigens</th>
<th>H Antigens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmonella typhi-murium</td>
<td>1,4,5,12</td>
<td>i: 1,2</td>
</tr>
<tr>
<td>Salmonella senftenberg</td>
<td>1,3,19</td>
<td>g [s]t</td>
</tr>
<tr>
<td>Salmonella newport</td>
<td>35</td>
<td>f, g</td>
</tr>
<tr>
<td>Salmonella adelaide</td>
<td>6,8</td>
<td>eh:1,2</td>
</tr>
<tr>
<td>Citrobacter</td>
<td>5,12</td>
<td></td>
</tr>
</tbody>
</table>
Figure V-9

Serum from a patient with *Salmonella typhi-murium* gastro-enteritis

<table>
<thead>
<tr>
<th>Bacteria used in assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Salmonella typhi-murium</td>
</tr>
<tr>
<td>■ &quot; senftenberg</td>
</tr>
<tr>
<td>□ &quot; newport</td>
</tr>
<tr>
<td>○ &quot; adelaide</td>
</tr>
<tr>
<td>◇ Citrobacter</td>
</tr>
</tbody>
</table>

Dilution

- $\frac{1}{50}$
- $\frac{1}{200}$
- $\frac{1}{800}$
- $\frac{1}{3200}$

cpm bound
Serum from a patient with *Salmonella newport* gastro-enteritis

**Figure V-10**

- **Bacteria used in assay**
  - *Salmonella typhi-murium*
  - " senftenberg
  - " newport
  - " adelaide
  - ◊ Citrobacter
(Rowley -1977). As seen from the figures the titres of antibody demonstrated by the different organisms closely reflect the degree with which the antigens of the infecting strain are carried by the organisms used in the assay.

These results provide strong evidence for the specificity of the assay.

Mel and colleagues (1965) claimed that minor differences in antigenic composition of strains of Shigella used for measuring copro-antibody responses after vaccine trials made a major difference to the estimated amounts of antibody. In view of this report the possibility that the strain of organism (e.g. Salmonella typhi-murium) isolated from a subject with gastroenteritis would prove a better absorbent of antibody from that particular patient than isolates (also Salmonella typhi-murium) from other sources, was examined. This was not found to be the case in the subjects studied using this assay. Hence though the organisms fixed as immunoabsorbents were initially isolated from patients (p. 63), the assay of fluids from each subject was not always performed with the subject's own isolate.

Natural Antibody or Non-Specific Binding in Non-Immune Sera.

Sera from patients not known to have had an infection with Salmonella gave a low but definite titre in the assay. The two possible explanations for this were that they had small amounts of antibody or that this binding was a non-specific effect that became apparent when using higher
FIGURE V - II

Reduction of binding of normal control serum
by serial absorptions.

Effect of 3 successive absorptions using $10^{10}$
bacteria (Salmonella typhi-murium) on sera from
children not known to have had Salmonella infection.
Figure V-11

HARRISON
- Pre absorption
- Post 1 absorption
- " 2 absorptions"
- " 3 "

MARTIN
- Pre absorption
- Post 1 absorption
- " 2 absorptions"
- " 3 "

[Graph showing cpm bound vs. dilution for both HARRISON and MARTIN, with different symbols representing various absorptions and an end-point marker.]
concentrations of serum. If it was due to antibody, the antibody could be either 'natural antibody' (see page 2) or antibody from a previous mild infection.

An experiment was done with sera from two children who had presented to a Paediatric unit because of short stature but with no history of gastrointestinal disease. These were sera giving the lowest titre out of a batch of six. They were diluted 1 in 10 and 5 mls absorbed three times with $10^{10}$ organisms. A 200 ul aliquot was removed from the 5mls after each absorption and held for assay with a preabsorption sample. As shown in the graphs (V-II), there was increasing loss of antibody with successive absorptions suggesting that the low levels of binding in non-immune sera do represent antibody. On other occasions though there was little or no change in the low antibody titre of non-immune sera after repeated absorptions. While this may be an effect of natural antibody that is of low affinity and difficult to remove by absorption, the specificity of the binding at low serum dilutions was not conclusively demonstrated.

Radio-Labelled Secondary Antibody

The secondary antibody used in this assay was anti human immunoglobulin (specific for light chains, alpha and gamma heavy-chain) prepared by affinity chromatography (page 77). This was radio-labelled using $^{125}$I to a specific activity of 1,000 cpm/ng protein, (range of 400-3,000 cpm/ng protein during use), as described in Chapter 3.
Maximum Binding of Secondary Antibody

Theoretically in Zone A of the immune curve (Fig. V-1) complete binding of the radio-labelled secondary antibody would have been expected as in this zone there is excess primary antibody relative to the secondary antibody. This expectation was never realised with any of the batches of secondary antibody used and with any of the I\textsubscript{125} labellings. The maximum binding obtained was in the range of 40% to 60% of added radio-activity.

Two sets of experiments were done to attempt to ascertain the reason for this phenomenon.

1. Determination of Antibody Activity of Secondary Antibody

One possible explanation for the sub-maximal binding of the secondary antibody was that some of it had lost its binding ability in the course of elution from the affinity column and further preparation. If this was so it would no longer bind on being re-run on the affinity column. That this was not so was shown by the fact that over 90% of radio-labelled anti light-chain antibody bound to a Sepharose IgG column although its maximum binding in the assay was 55%.

2. Determination of Maximum Binding of Secondary Antibody to Polymerised Immunoglobulin Under the Same Conditions as in the Assay.

Serum from a patient with IgA myeloma was insolubilised using glutaraldehyde (Avrameas and Ternynck, 1969). Two series of doubling dilutions
of this insolubilised serum was made in 0.1 ml volumes in PBS 1% NGS. 50 µls of Salmonella typhi-murium (10^{10}/ml) were added to each tube to provide a visible pellet. Series A had anti-alpha secondary antibody (9600 cpm on 44 ngs antibody/tube) and series B had an anti LC secondary antibody (9500 cpm on 19 ngs antibody/tube) added to each tube. After one hour's incubation, the pellet of bacteria and insolubilised serum was washed three times as in the assay. Maximum binding on counting the pellets, was still around 60%, as shown in the figure of the results. (Fig. V-12).

These experiments left the question of why maximum binding of secondary antibody could not be achieved, unanswered. Other experiments checking O.D. 650 of the resuspended bacterial pellet, suggested there was no loss of bacteria during the experiments. The possibility that the physical forces generated by centrifuging repeatedly broke off some of the secondary antibody on its own or along with the primary antibody remains a reasonable but untested hypothesis for this phenomenon. Another possibility is that some of the antigenic sites on the primary antibody against which the secondary antibody is directed become inaccessible when the primary antibody itself binds to antigen or is insolubilised with glutaraldehyde.
Figure V-12

Maximum binding of Secondary Antibody

- Anti IgA Serum (9600 cpm added)
- Anti Light-chain Serum (9500 cpm added)

Doubling dilutions of insolubilised IgA

Controls
Saturation of Antigenic Surface of Primary Antibody by Secondary Antibody.

Implicit in the assumption that the amount of primary antibody bound to bacteria could be quantitated by adding secondary antibody (with activity against the first antibody) is that the association of secondary antibody with primary antibody is specific. It would therefore be expected that the capacity of a given quantity of primary antibody to allow secondary antibody to bind to it was finite.

Experiments were done to confirm this with immune sera. An immune serum was set up in three series (A-C) of 4 tubes (Fig.V-13). Series A was 100 μls of 1/500 dilution; series C was 100 μls of 1/2000 dilution; series B was 100 μls of a 1/200 dilution that had been absorbed with an excess of killed Salmonella typhi-murium. Each tube had its control with bacteria but no serum. After incubation of primary antibody and repeated washing, secondary anti LC antibody was added: 5.3 ngs (1,000 cpm) to tube 1, 26.5 ngs (5,000 cpm) to tube 2, 53 ngs (10,000 cpm) to tube 3 and 106 ngs (20,000 cpm/tube) to tube 4, in each series and its control. After the secondary incubation and repeated washing the pellets were counted and secondary antibody bound specifically, determined by subtracting the control value. As shown in fig. V-13, a plateau was reached for uptake of secondary antibody by the constant amount of primary antibody and the amount of secondary antibody bound was proportional to the amount of primary antibody.
Saturation of Primary Antibody by Secondary Antibody

Series A (1/500 dilution), Series B (1/200 dilution, post absorption with S. typhi-murium) and Series C (1/2000 dilution) were incubated with varying amounts of anti light-chain antibody (1000 cpm - 20000 cpm) during second incubation. A plateau of uptake of secondary antibody is seen.
Figure V-13

SATURATION OF PRIMARY ANTIBODY BY SECONDARY ANTIBODY

<table>
<thead>
<tr>
<th>Radio-activity added</th>
<th>cpm bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000 cpm added</td>
<td>500 cpm</td>
</tr>
<tr>
<td>5000 cpm</td>
<td>1000 cpm</td>
</tr>
<tr>
<td>10000 cpm</td>
<td>1500 cpm</td>
</tr>
<tr>
<td>20000 cpm</td>
<td>2000 cpm</td>
</tr>
</tbody>
</table>

A 1/500
B 1/200 (absorbed)
C 1/2000

cpm bound
A similar result was obtained with another serum in which series A was diluted 1/100, B 1/200, C 1/400, D 1/800 and E 1/1600. Each series had 6 tubes. Anti LC antibody was added in increasing amounts to each tube (from 18.7 ngs and 4350 cpm in tube 1 to 1233 ngs carrying 286800 cpm in tube 6). As shown in fig. V-14, there was saturation of primary antibody again and the amount of primary antibody bound was proportional to secondary antibody bound.

In both these experiments the vast excess of secondary antibody that was added prior to saturating the primary antibody should be noted. This reflects the varying proportion of secondary antibody to primary antibody molecules in the immune complex that occurs with varying proportions of primary antibody and secondary antibody in this antigen-antibody complex.

In the routine use of this assay the end-point binding of secondary antibody to primary antibody would not have been in the area of saturation.

**Background Binding**

Under the conditions of the assay, the background binding of secondary antibody (binding of secondary antibody to bacteria with no primary antibody) varied between 0.5% and 2.0%.

**End-Point**

The initial end-point chosen for use in this assay was where the radio-activity bound was twice the 'background' binding ('binding' of radioactivity by bacteria not exposed to serum). Using this end-point proved difficult because of the narrow margin between background and end-point particularly
when the background was low (0.5%). Also as this end-point tended to be on the flattened part of the sigmoid curve (zone C), this led to slight variations in counts giving great apparent differences in 'titre'.

As an alternative to this the binding of a fixed amount of secondary antibody was considered as a possible end-point. As discussed above this would have involved using much larger amounts of secondary antibody so as to saturate the primary antibody bound at the end-point dilution of the fluid being tested.

As the amount of secondary antibody used was less than that required to saturate the primary antibody, the possibility of defining the end-point as the binding of a proportion of the radio-activity added was considered. This approach would only be valid if the amount of secondary antibody bound by any fixed amount of primary antibody was proportional to the amount of secondary antibody added to the second Ag-Ab reaction. Experiments done with amounts of secondary antibody that did not saturate the primary antibody confirmed that this was so over the range of secondary antibody used. In an experiment (Fig V-15) two identical series of an immune serum were incubated with bacteria and washed. To one series 10 ngs of anti LC antibody (2,000 cpm/tube) was added to each tube and to the other series 100 ngs of anti LC antibody (20,000 cpm/tube) was added to each tube. As seen from the plots of the final results, the proportion of secondary antibody bound and not the absolute amount of antibody bound was similar for the two series. In initial experiments done to check on the antigenic surface provided by bacteria and the possibility of saturating the primary
Figure V-14

SATURATION OF PRIMARY ANTIBODY BY SECONDARY ANTIBODY

- A 1/100
- B 1/200
- C 1/400
- D 1/800
- E 1/1600

$10^3$ cpm specifically bound vs. $10^3$ cpm added (Anti LC)
Figure V-15

Identical Series of a Serum with

A) 10 ng (2000 cpm) secondary antibody added

B) 100 ng (20000 cpm) secondary antibody added
FIGURE V - 16

Increasing binding with increasing amounts of secondary antibody.

This experiment was done with identical amounts of primary antibody in all tubes and using amounts of secondary antibody far short of the amount required to saturate primary antibody. It shows no increase in antibody binding with increasing amounts of bacteria (antigenic surface in excess) but increase in secondary antibody bound proportional to amount added.
Figure V-16

Increasing Binding with Increasing Secondary Antibody

[Graph showing the relationship between cpm bound and μls of bacteria added, with lines for 1000 cpm, 4000 cpm, and 20000 cpm added.]
antibody with secondary antibody, a similar increase in binding of secondary antibody proportional to increasing amounts of secondary antibody added (Figs. V-7 & V-16) was seen. This was at a time when the amount of secondary antibody added was far short of that required to saturate the primary antibody present.

It had been noticed that the maximum binding of the various sera and intestinal fluids assayed and their slopes, particularly in the upper part of zone B varied. Experiments to determine a suitable end-point were carried out with various intestinal fluids and sera. A representative experiment illustrated in fig. V-17 shows the binding curves of three immune sera and table V-4 shows the titres obtained at end-points defined by 10%, 15%, 20% and 25% binding of added radio-activity. Table V-4 also shows the variation in relative titres between the 3 sera at the various end-points. A variety of factors including antibody avidity and the varying proportions of different immunoglobulin classes of antibody could theoretically affect the slope of the binding curve. No experiments were performed to probe the reasons for these variations.

As seen from fig. V-17, the 10% binding end-point was still sufficiently above background binding and on the descending zone B of the binding curve to greatly lessen the difficulties experienced with a 'twice background binding' end-point. It was therefore chosen as the end-point to be used in this assay.
<table>
<thead>
<tr>
<th>End-point % age of added radio-activity</th>
<th>Titre (reciprocal)</th>
<th>Ratio of Titres</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>10</td>
<td>1450</td>
<td>470</td>
</tr>
<tr>
<td>15</td>
<td>650</td>
<td>280</td>
</tr>
<tr>
<td>20</td>
<td>470</td>
<td>150</td>
</tr>
<tr>
<td>25</td>
<td>320</td>
<td>88</td>
</tr>
<tr>
<td>30</td>
<td>220</td>
<td>72</td>
</tr>
<tr>
<td>35</td>
<td>50</td>
<td>59</td>
</tr>
</tbody>
</table>

Table V - 4. VARIATION ON TITRE WITH VARYING END-POINTS IN 3 SERA

(Fig. V-17)
Selection of a suitable end-point for assay
Variation Intra-Assay and Inter-Assay

In an experiment to determine intra-assay variation eight series of the same serum were assayed. Taking the reciprocals of these titres the arithmetic mean of these estimations was 1637 with a standard deviation of 210 and a scatter of 1400 to 1900.

To determine interassay variation the titres of the standard serum in a series of 13 consecutive experiments was taken. Taking the reciprocals, the mean of these results was 1233 with a standard deviation of 429 and a scatter of 680 to 2150.

Standardisation of the Assay

As inter-assay variation tended to be much greater than intra-assay variation, a standard immune serum previously divided into 0.2 ml aliquots after dilution 1 in 100, stored at -20°C, was assayed in duplicate with every assay used for titrating samples from patients. This serum had a titre of 1/1000 with anti LC serum. The results of each assay were corrected by a factor that brought the standard back to its original titre.

Quantitation of the Assay

Attempts were made to relate the units of antibody activity determined in the assay to antibody weight determined by precipitation. This only proved possible on acquiring a patient with a very high serum antibody response; two attempts with sera from other patients failed as the protein precipitated was less than could be reliably measured by either OD estimation or by a 'micro-folin'
estimation.

The patient whose serum was used for the quantitation had gastroenteritis caused by Salmonella bovis-morbificans. Lipo-polysaccharide (LPS) from Salmonella bovis-morbificans was prepared as outlined in Chapter 3. The LPS was in a concentration of 12.4 mgs/ml as estimated by dry weight. The protein in the LPS was 120 ugs/ml as determined by a micro-folin assay.

An experiment was done using 4 mls of this patient's serum divided into 2 ml aliquots. 2 mls were incubated with 2 mgs LPS and 2 mls with 4 mgs LPS for 48 hours at 4°C with occasional mixing. The samples were made up to 8 mls with normal saline and the LPS antibody complex spun out. The supernatant was assayed for antibody activity. The pellet was dissolved in 0.5 mls of 1 Normal sodium hydroxide. The protein in the pellet was measured using a micro-Folin assay. The detailed outline of these steps are given on Table V-7. 1500 ugs of protein was precipitated by the LPS out of 1 ml of serum. The same experiment was repeated using 1 ml aliquots of serum and incubating them with 1 mg and 2 mgs of LPS respectively. On this occasion the pellet was given two washes in normal saline. A micro-Folin assay showed 1175 ugs of antibody/ml precipitated. This suggests that the amount of protein in the pellet that was not complexed with the LPS but merely a residue from insufficient washing was negligible.

On assaying the original serum it was found to have a titre of 1/7900 (per 0.1ml volume) with 2% residual activity post absorption. These results equate out at 1 unit of antibody being equal to 19 ngs (on the basis of
TABLE V - 5  QUANTITATION OF ASSAY

Estimate of antibody in serum by absorption with Lipo-polysaccharide

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 mls of Immune Serum</td>
<td></td>
</tr>
<tr>
<td>Ultra-centrifuge (65 rotor) 40,000 rpm - 1 hr.</td>
<td></td>
</tr>
<tr>
<td>2 mls serum + 2 mgs L.P.S.</td>
<td>2 mls serum + 4 mgs L.P.S.</td>
</tr>
<tr>
<td>48 hrs at 4°C occasional mixing</td>
<td></td>
</tr>
<tr>
<td>made up to 8 mls with normal saline</td>
<td></td>
</tr>
<tr>
<td>Ultra-centrifuge, 40,000 rpm-hr</td>
<td></td>
</tr>
<tr>
<td>supernatant assayed</td>
<td></td>
</tr>
<tr>
<td>Pellet</td>
<td></td>
</tr>
<tr>
<td>Dissolved in 0.5 mls IN NaOH</td>
<td></td>
</tr>
<tr>
<td>Micro-Folin Assay for protein</td>
<td></td>
</tr>
<tr>
<td>Protein in pellet = 2950 µgs</td>
<td>3200 µgs</td>
</tr>
<tr>
<td>Protein in L.P.S. = 20 µgs</td>
<td>40 µgs</td>
</tr>
<tr>
<td>Antibody in pellet = 2930 µgs</td>
<td>3160 µgs</td>
</tr>
</tbody>
</table>

Serum contains 1.5 mgs/ml Antibody
1500 ugs/ml precipitated antibody) or 15 ng (on the basis of the 1175 ugs/ml precipitate), when using anti light-chain antibody as the secondary antibody.

Inhibition of Binding of Secondary Antibody by Purified Secretory IgA and IgG

As described in Chapter 3, the anti LC, anti-alpha, and anti-gamma antibodies used in these assays were prepared by affinity chromatography and had been demonstrated to be specific by Ouchterlony and immunoelectrophoretic analysis. The specificity of these antisera were further investigated using IgG and S IgA (preparation described in Chapter 3) to inhibit their binding in the second incubation in the assay (Bacteria-antibody complex and labelled secondary antibody).

An immune serum titred out to 1/40 with anti-alpha serum and to 1/1300 with anti-gamma serum. Two series of ten tubes with 0.1 ml of 1/400 dilution of the immune serum was incubated with bacteria and washed in the usual way. Just prior to adding the labelled anti-gamma antibody doubling dilutions of pure IgG (200 ng down to 0.4 ng) were added to one series, and pure secretory IgA (20,000 ng down to 40 ng) were added to the other series. 50% inhibition of the binding of the anti-gamma serum to the bacterial bound primary antibody was achieved by 13 ng of IgG. 20,000 ng of secretory IgA did not achieve this. This result suggests that the affinity of this anti-gamma serum for IgG compared to IgA is more than 1500 to 1.

A similar experiment with a 1/20 dilution of the serum
and anti-alpha antibody was done: 50 ngs of S IgA gave 50% inhibition of anti-alpha binding with this serum; 20,000 ngs of IgG failed to inhibit anti-alpha binding suggesting a preferential affinity of the anti-alpha for IgA compared to IgG of at least 400:1.

The anti LC serum gave a titre of 1/540 with an immune serum. 50% inhibition of binding with a 1/40 dilution of serum incubated with bacteria was achieved by 160 ngs secretory IgA and 54 ngs IgG. This suggested that the anti LC serum showed greater affinity for IgG than for IgA.

These results emphasised two limitations of the assay in the form it was used. The quantitation of the sensitivity of the assay was only an approximate estimate as 1 unit of antibody determined using the anti light-chain secondary antibody would be quantitatively different certainly in terms of weight and probably in terms of molar amounts depending on the immunoglobulin class of the antibody being measured. The IgA and the IgG antibody estimations using anti-alpha and anti-gamma secondary antibody are not directly comparable as 1 unit of IgG antibody probably represents a smaller weight of immunoglobulin than does 1 unit of IgA.

The Assay and Intestinal Antibody

One of the reasons advanced for the difficulty in measuring intestinal antibody is the effect of intestinal enzymes on the immunoglobulin during the assay itself. One possible means of reducing this effect is to precipitate out the immunoglobulins using ammonium sulphate and to re-dissolve them in an enzyme free milieu prior to the assay.
One ml volumes of two immune intestinal aspirates were diluted 1 in 2 with 1% NGS in PBS with 0.1 ml of NGS added as carrier protein. Saturated ammonium sulphate was added to give a 50% final concentration in the cold. This was left at 4°C overnight. The precipitate was re-suspended in distilled water and dialysed against two changes of PBS. Assay of freshly unthawed aliquots of the same intestinal aspirates gave identical results as the ammonium sulphate cuts, suggesting that there was no advantage to be gained from the initial precipitation.

Another potential reason for difficulty in measuring antibody in the presence of intestinal fluid is interference or inhibition of the assay system by other intestinal fluid contents, such as bile salts. This possibility was tested on one occasion using a 1 in 10 dilution of pooled normal intestinal fluid in PBS with 1% NGS as diluent for a series of an immune serum. The titre of the immune serum was unaffected by the presence of intestinal fluid in this concentration. Further experiments at lower dilutions of intestinal fluid were not performed but it would appear unlikely that non-specific inhibition of antibody binding is likely to interfere with antibody measurements in immune fluid where the titres exceeded one in ten.
Subjects studied

Patients presenting with the clinical features of gastroenteritis, from whose stools at the time of their symptoms Salmonella or Shigella species were isolated, were studied after obtaining informed consent from the patients, and in the case of children, their parents.

Samples of serum and intestinal fluid were obtained (as described in Chapter 3) in every case. In the later stages of the study, samples of parotid saliva were obtained as well. The first sample was obtained as early in the course of the illness as possible. In practice this turned out to be seven days after the onset of symptoms. The second sampling was scheduled for four to six weeks after the first one, though on some occasions this was delayed. Further samples were taken in two patients as will be discussed below.

The controls used for this study were two patients who were admitted to hospital with a diagnosis of gastroenteritis but from whom no bacterial pathogens were isolated and also ten healthy volunteers who had no history of bacterial gastroenteritis.

The collection and storage of samples were described in Chapter 3.
Results on Individual Subjects

The results from individual subjects are given in the appendix on pages 131 to 167.

The date of onset of illness was taken as the first day of the illness on which diarrhoea, vomiting, abdominal cramps, fever or rigors were noticed. In most patients these manifestations were apparent on the same day.

The results of the immunoglobulin estimations were obtained from the Mancini SRID assays (Chapter 3).

Antibody titres, expressed as units/0.1 millilitre (u/0.1ml) were measured using the assay outlined in Chapter 5. The titres were based on a single estimation of each sample using the anti light-chain, anti-alpha and anti-gamma antisera. As described in Chapter 5, the results were adjusted by using two series of a control serum measured with the antilight-chain serum, to correct for inter-assay variation.

The antibody content of the various fluids was also calculated in units per milligramme immunoglobulin (u/mg.Ig.) using the antibody content as measured in the radio-immuno assay and the immunoglobulin concentration as measured in the Mancini SRID assay. The total antibody content (using anti light-chain as secondary antibody) was calculated per milligramme of the total immunoglobulin (IgA, IgG and IgM). IgA and IgG antibody in units per milligramme immunoglobulin (u/mg.Ig.) were calculated using the antibody titres (u/0.1ml) obtained with anti-alpha and anti-gamma as secondary antibody, and IgA and IgG concentrations (mg%) in the fluids respectively.
Various expressions of these individual results grouped together are found in figures VI-1 to VI-20.

Comparison of Antibody Responses in Serum and in Intestinal Aspirate

A clear difference was seen in the total antibody levels in units/0.1ml of the serum and intestinal aspirate of both adults (figures VI-1 and 2) and children (figures VI-8 and 9) compared to controls. This same difference was apparent on expressing the results in units/mg.Ig. (figures VI-5 and 12). These differences were obvious both in the first and second samples in these groups. On examining the results expressed in units/mg immunoglobulin by the Kolmogorov-Smirnov test of equality of distributions these differences were significant at the 1% level (p < .01, Table VI-1).

It is obvious on examining the antibody content in units/0.1ml of serum and intestinal fluid that there are much higher levels of antibody present in absolute terms in serum than in aspirate (compare figures VI-1 and VI-2; VI-8 and VI-9). This difference is of the order of fifty-fold. As has been discussed in the introduction (Chapter 2) the antibody content of fluids expressed in units/mg.Ig. may allow a more meaningful comparison of levels of antibody in different body fluids. On examining the results expressed in this manner (figures VI-5 and VI-12) the antibody response in intestinal fluid is found to be of the same order of magnitude as that in the serum and in point of fact slightly higher than the serum response both in adults and in children. Using the estimate of 20 ng as being the
TABLE VI - 1. COMPARISONS OF ANTIBODY RESPONSES IN GROUPS OF PATIENTS WITH THE CONTROL GROUP USING KOLMOGOROV- SMIRNOV TEST OF EQUALITY OF DISTRIBUTIONS.

<table>
<thead>
<tr>
<th>Groups compared with controls.</th>
<th>ASPIRATE 1st Sample</th>
<th>ASPIRATE 2nd Sample</th>
<th>SERUM 1st Sample</th>
<th>SERUM 2nd Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adults and children</td>
<td>24 1% 1115.4 393 -1360</td>
<td>24 1% 561 333 -1227</td>
<td>25 1% 470.6 282 -640</td>
<td>24 1% 370 182 -591</td>
</tr>
<tr>
<td>Adults</td>
<td>15 1% 667 222 -1200</td>
<td>15 1% 636 333 -1395</td>
<td>16 1% 467 282 -644</td>
<td>16 1% 365 154 -591</td>
</tr>
<tr>
<td>Adults less 2 high-responders (subjects 4 and 6)</td>
<td>13 1% 435 222 -1115</td>
<td>13 1% 577 333 -643</td>
<td>14 1% 439 217 -644</td>
<td>14 1% 316 148 -429</td>
</tr>
<tr>
<td>Adults(Salmonella)</td>
<td>13 1% 667 350 -1200</td>
<td>13 1% 636 333 -1227</td>
<td>14 1% 467 217 -986</td>
<td>14 1% 322 148 -591</td>
</tr>
<tr>
<td>Adults(Salmonella) less 2 high-responders (subjects 4 and 6)</td>
<td>11 1% 435 225 -1115</td>
<td>11 1% 578 333 -643</td>
<td>12 1% 439 190 -986</td>
<td>12 1% 241 145 -429</td>
</tr>
<tr>
<td>Children</td>
<td>9 1% 1329 286 -3077</td>
<td>9 1% 471 273 -1889</td>
<td>9 1% 471 266 -640</td>
<td>8 1% 401 176 -796</td>
</tr>
</tbody>
</table>

N = number in group, P = probability
Med = median, CI = 95% confidence interval.
Analysis by Mr. P. Leppard, Dept. of Statistics, University of Adelaide.

Controls N Med 95% Conf.Int.
Aspirates 12 68 0-273
Serum 10 73 43-128
approximate amount of immunoglobulin antibody measured by 1 unit, it is possible to estimate the proportion of immunoglobulin committed to specific antibody against the enteric pathogen causing disease. Using medians as a measure of the average, one to two percent of the immunoglobulin in serum and in intestinal secretions was specific antibody against the relevant pathogen whereas in the controls it was a tenth of this.

**Pattern of Change in Antibody Response With Time**

By obtaining paired samples with a 4 to 8 week interval between the samples, information on the temporal profile of the antibody response in these subjects was obtained.

In the adults with the three highest intestinal responses on the first sample (Fig. VI-6) the second sample showed a decrease in antibody. There was only little change in the antibody levels (in units/mg.Ig.) of the others during the period of observation (fig.VI-6). In one patient with a definite rise in intestinal antibody, a stool culture at the time of the second sampling revealed persistence of the pathogen (subject 5). Samples of stools were not cultured regularly in subjects at the time the second sampling for the study was carried out; it is therefore not possible to assess whether the occasional rise in antibody or the persistence of antibody at the same level at the time of second sampling resulted from persistence of the pathogen in the flora of the intestine.
The serum antibody in adults (expressed in units/mg.Ig, fig. VI-7) showed little change between the two samplings except for one subject, (subject 6) who had very high levels at the time she was first studied, with a marked decrease over a six week period.

In the children, the most obvious difference from the adults was in the quicker decline in intestinal antibody during the period of study (fig. VI-13 compared to fig. VI-6).

One advantage of expressing the results in terms of antibody units/mg.Ig is apparent on examining the pattern of intestinal antibody response expressed in units/0.1 ml (fig. VI-3). The very variable relationship between the intestinal antibody in the first and second samples is at least partially a result of the variable dilution that affects intestinal immunoglobulin (cf. fig. VI-6).

Relationship Between Intestinal and Serum Antibody in Individual Patients.

The fairly similar degrees of antibody response in serum and intestinal aspirate of the group has been noted. On examining the antibody responses of individuals however, a quite marked dissociation between serum and intestinal antibody in individuals was seen. This is illustrated in figure VI-15. Patient A (subject 1) had a high intestinal response but the lowest serum antibody response among the adults. Patient B (subject 16) with the lowest intestinal response had one of the highest serum responses. Patient C (subject 4) with clearly the highest intestinal antibody response in the group as a whole had a serum response that was very average. This illustrates the fact that serum antibody responses do not reflect the intestinal antibody
response well in the enteric infections studied.

This same phenomenon is illustrated in figure VI-16, where the relationship between intestinal antibody and serum antibody responses in the adult subjects are graphically displayed. It is obvious that there are high and low serum responders in relation to any level of intestinal response. On examining individual responses most individuals do not seem to change greatly with regard to this relationship on two samplings but there are marked differences between different individuals.

**Immunoglobulin Class of Antibody in Serum, Aspirate and Saliva.**

The predominance of IgA antibody over IgG antibody in the intestinal fluid and saliva in terms of concentration (units/0.1 ml) was marked, though there were a few individuals in whose intestinal fluid IgG antibody was found (fig. VI-19). This is in keeping with the immunoglobulin content of these fluids.

In contrast in serum, the amounts of IgA antibody compared to IgG in units/0.1 ml were very small; even in terms of units/mg immunoglobulin there did not appear to be any predominance of IgA antibody over IgG in the serum.

In the serum of the children IgG antibody was clearly the most important in the second sample. Comparison of total antibody (using anti-light-chain as secondary antibody) with IgG and IgA antibody suggested that there was a large amount of non IgA, non IgG antibody in the first serum, probably representing an early IgM antibody response in this age-group (fig. VI-20).
The reservation in comparing the IgA and IgG antibody because of the lack of definite quantitative estimates of 1 unit of antibody as measured with anti-alpha and with anti-gamma serum has been discussed in Chapter 3. The difference seen in the antibody patterns in serum and intestinal fluid is so clear cut however, that this uncertainty could not undermine the conclusions mentioned above.

Correlations of Intestinal Antibody with Salivary and Serum IgA Antibody

The possibility that salivary antibody or serum IgA antibody reflects intestinal antibody was examined.

As will be seen from fig. VI-17, there does not appear to be any correlation on plotting serum IgA antibody (in units/mg.Ig) against intestinal antibody. Some of the serum IgA antibody levels could not be quantitated as the titre of antibody in this class was less than 1/25, the lowest dilution assayed.

In the case of salivary antibody, collection of saliva was only carried out towards the end of the study. Here again the plot of salivary antibody against intestinal antibody does not suggest a clear relationship between them. (Fig. VI-18).

It would seem unlikely that measuring salivary antibody or serum IgA antibody will provide a meaningful estimate of intestinal antibody.
High responders

Two adults, (subjects 4 and 6) had intestinal antibody responses that were ten-fold higher than any of the other patients. In one of them (subject 4) the second sample still had very high levels of antibody whereas in the other (subject 6) it had dropped much closer to the levels of the other patients studied. The serum response in subject 6 was initially very high as well.

The reasons for this unusually strong antibody response are not definite. Both these patients had severe attacks of gastroenteritis and their intestinal response may merely reflect this potent antigenic stimulus. Another factor common to both of them was their low production of gastric acid. Subject 6 had previously undergone vagotomy and gastroenterostomy for peptic ulcer. Her achlorhydria and the marked hypochlorhydria of subject 4 was demonstrated using the pentagastrin test (Baron, J.H. (1970)).

The presence of antibody in gastric secretions was demonstrated on repeat sampling of subject 4, and some of this may have helped increase the concentration in the jejunum. The absences of fluxes of acid into the duodenum and upper jejunum may also have reduced degradation of immunoglobulin here.

These patients were subjected to intubation a year after their original infection and still had relatively high levels of intestinal antibody then, especially subject 4.
Persistence of Bacteria

A systematic attempt to determine whether subjects continued to carry the pathogen (at least in terms of routine stool culture) at the time of repeat sampling was not made. In five subjects (subjects 4, 5, 17, 18 and 23) however, the pathogen was found in stools obtained at the time of later sampling, in spite of the presence of intestinal antibody both at that time and also weeks earlier. All five subjects had recovered symptomatically at the time of this repeat isolation of the pathogen.
Antibody responses in serum and intestinal aspirate in adults and children expressed in units/0.1ml and units/mg.Ig as indicated in individual figures.

- Salmonella infection
- Shigella infection

All antibody estimates made using Anti-light-chain as secondary antibody.
Figure VI-1

ADULTS - ASPIRATE (units/0.1 ml)

controls 1ST sample 2ND sample

- 1900
- 850
- 560
- 53
SERUM ANTIBODY RESPONSE (ADULTS)

- 7900
- 2220

controls  1ST sample  2ND sample
Figure VI-3

ADULTS - ASPIRATE (units/0.1 ml)

weeks post infection

units/0.1 ml
Figure VI-5

ADULTS

ASPIRATE (units/mg Ig)

SERUM (units/mg Ig)
Figure VI-6

ADULTS — ASPIRATE (units/mg Ig)

units/mg Ig

weeks post infection

20000
15000
10000
5000
2000
3000
2000
1000

2 4 6 8 10 12 14 16 18 20 40 52
Figure VI-7

ADULTS — SERUM (units/mg Ig)

units/mg Ig

weeks post infection

2 4 6 8 10 12 14 16 18

20 40 52
Figure VI-8

CHILDREN — ASPIRATE (units/0.1 ml)

controls  1ST sample  2ND sample
Figure VI-9

CHILDERN — SERUM (units/0.1ml)

controls  1ST sample  2ND sample
Figure VI·10

CHILDREN – ASPIRATE

(units / 0·1ml)

units / 0·1ml

weeks post infection

2 4 6 8 10 12
Figure VI-11

CHIDREN - SERUM

(units / 0.1 ml)

weeks post infection

units / 0.1 ml
Figure VI-12

CHILDREN

SERUM (units Ab/mg Ig)

ASPIRATE (units Ab/mg Ig)

controls 1st sample 2nd sample controls 1st sample 2nd sample
Figure VI-13

CHILDREN – ASPIRATE units/mg Ig

units/mg Ig

weeks post infection

2 4 6 8 10 12
Figure VI-14

CHILDREN - SERUM (units/mg Ig)

weeks post infection

2 4 6 8 10 12
Figure VI-15

DISSOCIATION OF SERUM AND INTESTINAL ANTIBODY RESPONSES

Samples from patients - A ★, B ●, C ○

Aspirate ——— Serum ————

units Ab/mg Ig

weeks post infection
Results from subject 4 (both samples) and subject 6 (first sample) were omitted because of the scale of the graph.
Figure VI-16

Relationship of Intestinal Antibody and Serum Antibody
FIGURE VI - 17

Relationship of Intestinal Antibody and Serum IgA Antibody

Note. 1. Subjects 1,2,4 and 6 were excluded because samples were not measured using the anti-a serum or because they were off scale.

2. The lowest dilution of serum used in measuring IgA antibodies was 1/25. Samples with a lower titre are expressed as 0.
Figure VI-17

Relationship of Serum IgA Antibody and Intestinal Antibody
Relationship between Salivary and Intestinal Antibody

- Salmonella infection
- Shigella infection
Figure VI-18

Relationship Between Salivary Antibody and Intestinal Antibody
FIGURE VI - 19

Immunoglobulin class distribution of antibody in intestinal aspirate and serum of adults recovering from Bacterial Gastroenteritis expressed in units/0.1ml and units/mg. immunoglobulin.

Note. 1. These results were based on the antibody responses in subjects 7-16. (The anti-α and anti-γ secondary antibody were not available when subjects 1-6 were initially studied).

2. The reservations in attempting to compare antibody responses measured by the different antisera are discussed on pages 106 and 113-114.
ANTIBODY RESPONSE IN THE DIFFERENT IMMUNOGLOBULIN CLASSES (ADULTS)

UNITS / 0.1 ml

ASPIRATE

UNITS / mg Ig

ASPIRATE

SERUM

UNITS / mg Ig

SERUM
Comparison of total antibody (detected by anti-light-chain serum) and antibodies in the IgA and IgG classes (as measured by anti-heavy-chain sera) with the suggestion of an IgM response in the first sample as compared with the second.
Figure VI-20

ANTIBODY RESPONSE IN THE SERUM OF CHILDREN

1ST SAMPLE

2ND SAMPLE

units / 0.1 ml
The Measurement of Antibody in Intestinal Fluid.

The difficulties in studying the intestinal immune response have been discussed in Chapter 2, and the variable results in studies of this fluid mentioned. The finding of antibody uniformly in this study of the intestinal fluid of subjects with bacterial gastroenteritis vindicates Freter's belief that the fault in terms of the inconsistency of appearance of antibody after infection, lay with the techniques used for detecting intestinal antibody and not with the body's caprice in producing it.

This demonstration of intestinal antibody was possible because of the development of a radio-immuno assay as described in Chapter 5. Its limitations will be discussed in more detail in this Chapter but it fulfilled its main purpose in being able to detect antibody regularly in patients after infection. Among the premises on which the choice of this form of assay was based were that monomeric IgA, IgG and IgM were broken down in jejunal fluid to fragments that retained their ability to bind and that antibody fragments that bind to bacteria are still able to perform their antibacterial function in the gut effectively. Most of the conclusions reached in this study were based on estimates of total antibody measured with radio-labelled anti light-chain antibody. Apart from rapid freezing of the samples obtained and storing them at -80°C, no steps were taken to decrease proteolysis.
Though there was scarcely any overlap of intestinal antibody levels in patients compared with controls, the actual degree of antibody stimulation seen in the patients varied quite markedly. Two patients with the highest responses are discussed later in this Chapter. Some of the factors that may be relevant to the wide spectrum of response bear mentioning. The intensity of the attack is one obvious factor: the first group of patients studied were all patients who entered hospital as did the children; most of the adults studied later did not get ill enough to require admission. A second factor that may be relevant to antibody levels in the intestinal fluid is that acid fluxes in the upper jejunum are well recognised and this may affect patients differently depending on gut motility, acid production and relative distribution of antibody among the immunoglobulin classes. The possibility that antibody secretion may be maximal at the site of antigen stimulation (Husband and Gowans, 1978) has been mentioned. The area of intestine subject to maximal barrage by the relevant pathogen may vary in different patients, and thus samples from the high jejunum may underestimate the response. Ideally, samples of intestinal fluid from various levels of the intestine should be sampled to determine just how uniform the antibody response is. This was not carried out on any of the patients studied.

While the results obtained raise further questions of interest, they do provide the basic information sought at the outset concerning regularity, temporal pattern and immunoglobulin pattern of the intestinal antibody response.
Temporal Pattern of Intestinal Antibody Response.

The intestinal antibody response to bacterial gastroenteritis is clearly an early response. The length of time spent in tracing patients with bacterial gastroenteritis meant that by the time the first sample was taken the patients were already recovering from their disease. The consistent presence of antibody at this time is compatible with the hypothesis that intestinal antibody plays a part in recovery from the disease. This theory can only be put to the test by determining whether in some patients symptoms persisted in spite of high antibody levels in the intestine and whether recovery occurred in others (e.g. patients with hypogammaglobulin-aemia) without the production of antibody. Obtaining such evidence was not a primary aim of this study.

At the time of second sampling there was no marked reduction of intestinal antibody in the adults. In the two high responders (subjects 4 and 6), a year after infection there were still moderate amounts of antibody in the jejunal aspirates, well above control levels. An important continuation of this study will be to sample the other subjects again months to years after the infection to determine whether the antibody response persists for that length of time. The intestinal antibody response of the children even though it was slightly greater than that of the adults in this study, seemed to decline more rapidly. This impression will require more data to confirm it. The reason for such a difference is not obvious, though a not unreasonable speculation is that the difference is a consequence of the adult immune system's previous experience of the relevant bacterial antigens; the suggestion of a primary IgM antibody response in the children's serum at the time of
first sampling contrasted with the later IgG response, and the IgG response "ab initio" in the adults would fit this.

The persistence over 1 - 2 months of intestinal antibody in the adults in this study is in keeping with Waldman's findings in the study his group made of cholera (1972) (see figure 2-1). If the premise which stimulated these studies is correct, namely that intestinal antibody plays a fundamental role in protection against gut infection, it is clearly of major importance to obtain more information about the duration of the intestinal response to infection and also to any candidate vaccines both in children and in adults.

**Relationship of Intestinal Antibody to Other Measures of Humoral Immunity**

As discussed in the first two Chapters, much of the work done on immunity to gastrointestinal infections has used serum antibody as an index of the immune response, mainly because it is an easy item to measure. It has been observed in certain experimental models of gastrointestinal infection that serum antibody does not correlate with immunity to rechallenge with infection, and that serum and secretory antibody do not always correlate with each other. The lack of clear evidence in gastroenteritis in humans as to correlates of immunity and correlates of intestinal antibody has been discussed.

In this study the presence of similar serum and intestinal antibody responses to the infections when considering groups of individuals together was striking. Equally striking was the
dissociation of intestinal antibody from serum antibody in individuals as compared to the whole group. This finding provides an explanation for the paradox of the findings of Mosley and colleagues that serum antibody relates well to the immunity of population groups in the field (Mosley et al., 1968a & b) and their apparent contradiction by the challenge studies of Cash and colleagues (1974b) where serum antibody did not predict the level of immunity of individuals. That the patterns of antibody response in different forms of gastroenteritis may vary significantly was mentioned in Chapter 1. This may have particular importance for the antibody response to Salmonella which may vary in the degree of systemic invasion it sets up and this may in part explain the dissociation seen in antibody response between serum and intestinal fluid. On the other hand the quite marked serum antibody response seen in cholera which is the classic form of 'non-invasive' gastroenteritis and its poor predictive value of immunity in individuals, justifies this speculation on the serum antibody response in groups as against individuals, and its relationship to intestinal antibody.

This dissociation of secretory from serum antibody becomes even more important on considering the efficacy of vaccination. Different routes of vaccination have been clearly demonstrated to have varying effects on antibody levels in serum and at secretory surfaces (Ogra et al., 1968; Perkins et al., 1969; Smith et al., 1966). If the hypothesis that the intestinal antibody response is what determines resistance to bacterial gastroenteritis proves true, it is clearly of paramount importance to measure intestinal antibody in developing and trying out new vaccines.
Apart from the difficulty of measuring intestinal antibody, a major limitation to using it in studies of intestinal immunity is the difficulty in obtaining samples of intestinal fluid. Although total serum antibody did not indicate the intestinal antibody response in individuals, it was hoped that other measures of the humoral response would prove useful in this regard. The antibody response in IgA in the serum was one such index examined but this did not appear to be any more useful than all classes of serum antibody. The reason for hoping that serum IgA antibody would be of use was the suggestion that a significant amount of serum IgA is gut derived but the evidence that this is so in humans is not strong (see Chapter 2). It is still possible that the small amount of dimeric IgA antibody in the serum may reflect intestinal antibody production, but measuring specific dimeric IgA antibody in human serum is beyond the capability of currently available antibody techniques.

From first principles (see Chapter 1), the possibility that IgA antibody produced at other secretory surfaces after bacterial sensitisation in the gut would reflect the local secretory response seemed worth studying. The evidence provided by the studies of Allardyce and colleagues (1974) and Goldblum and colleagues (1975) hinted at the possible value of measuring the secretory antibody response in the special case of breast secretions in the pregnant female. Mestecky's description (1978) of a consistent salivary antibody response after oral immunisation with Streptococcus sanguis indicated a more generally available source of secretory antibody for measurement. It was disappointing
to find that parotid saliva, in which secretory IgA is the predominant immunoglobulin, did not correlate well with intestinal antibody. This finding is in keeping with the observations of Ogra in studies of vaccination locally at secretory surfaces with polio vaccines and the animal studies of Husband and Gowans (1978) which suggest that not merely is the predominant response to local vaccination at the secretory surface but that it is in close relation to the site of antigenic stimulation.

In summary then, it appears that after bacterial gastroenteritis, serum antibody, IgA antibody in the serum and secretory antibody in parotid saliva are not indices that can be used to gain insight into the intestinal immune response of any individual, though in a group of individuals, the serum antibody response is not dissimilar to that in the intestine. Hence if intestinal antibody is taken to be of critical importance in the immune response to infection or vaccination, there does not seem to be any alternative to obtaining samples of intestinal fluid and determining antibody levels in it.

Class of Antibody

The pattern of distribution of antibody in the IgA and IgG classes was much less variable than in the reports of Northrup and Hossain on cholera, Reed and Williams with Shigella and McNeish on E.coli gastroenteritis. The response in the serum was mainly in the IgG class except possibly for the first sample in the children where IgM antibody may have been predominant. In the intestinal fluid IgA antibody predominated over IgG antibody in both age groups.
However, the possibility exists that under the conditions of this study, antibodies in intestinal fluid were partially degraded and though antigen binding fragments derived from them may have been picked up by the anti light-chain antibody, they may not have been detected by the anti heavy-chain antiserum.

Definitive statements in this area await further work on inhibition of proteolysis. As discussed in Chapter 2, though many authors mention steps taken to avoid proteolysis, only Waldman and his group published data to suggest such measures were effective. In their study relating to cholera, the pattern seen in the studies described here was seen with IgA antibody predominating in intestinal secretions and IgG in serum. It is arguable whether the ability to prevent proteolysis will yield more useful information with regard to antibacterial immunity than is obtainable at present except perhaps in states of immunodeficiency such as selective IgA deficiency. This follows from the observations that secretory IgA is fairly resistant to digestion in the upper small bowel and is the predominant immunoglobulin produced here as far as can be judged from counts of plasma cells in the lamina propria.

**Persistence of Bacteria in the Presence of Antibody**

The serendipitous observation was made in five patients (subjects 4, 5, 17, 18 and 23) in this study, of bacteria which persisted in the gut long after recovery from gastro-enteritis caused by the bacteria and in the presence of high levels of secretory antibody. This raises questions of the interaction between antibody and bacteria in the gut. Though
there is evidence from other sources that intestinal antibody may reduce the chance of colonisation by bacteria (Knop and Rowley, 1975) it nevertheless can occur. It may be that in these patients the intestinal antibody is maximally active at the epithelial border of the intestine and plays a role in preventing adherence, bacterial invasion of the tissues and hence diarrhoea. These observations may provide insight into the carrier state where subjects harbour pathogenic organisms such as Salmonella in their intestines and infect others while being asymptomatic themselves.

**High Antibody Responders.**

Two patients (subjects 4 and 6) both of whom were relatively old, low on gastric acid, and severely ill with gastroenteritis, responded with very high intestinal antibody output to the infection. They suggest possibilities that are worth pursuing in further work in this area.

Firstly, it opens the possibility that the level of immune stimulation shown by them may be attained in other individuals. In the case of these two patients, it occurred after bacterial gastroenteritis but it is not impossible that repetitive stimulation by a vaccine may achieve this. Whether a very high antibody response will result in a prolonged one is another question: in one of these patients (subject 6) the antibody levels in the gut quickly returned to more normal levels, while in the other (subject 4) they persisted much higher for a long period of time.
The very height of these antibody responses may indicate an abnormal immune response. The normal immune response is a fine balance between humoral and cellular immune mechanisms (see Chapter 1 and 2) with a controlled feed-back by helper and suppressor T cells, antibody and antigen fitting the response to the stimulus. For reasons touched on in the introduction, these studies have focussed on the humoral response to infection. It may be that the severity of these patients' illnesses, in spite of their antibody response, was a result of failure of other parts of their immunological apparatus. The need for studying other aspects of the immune response to bacterial gastroenteritis must not be underestimated.

**Improvement in Methodology – The Assay**

Though the assay used to detect intestinal antibody in these studies was adequate for the purpose, it has limitations that would hamper further work in the area.

While it is clearly a sensitive assay, the inability to measure antibody in adult control subjects underlines the limits of its powers. The presence of antibody in the serum of normal controls against organisms such as Salmonella at titres of 75-100 units/0.1 ml suggests that trace amounts of antibody should be found in intestinal secretions where presumably the initial stimulus had been. Particularly, if the antibody response to infection or to vaccines is to be studied over a period of time, a more sensitive assay would be of great advantage.

As discussed in Chapter 4, the end point of this assay was taken at a point where saturation of available binding sites for the radio-labelled secondary antibody had not
occurred and the incubation times did not allow for equilibrium to be reached. For this reason results had to be standardised by running internal controls in each assay. It would be advantageous to use an assay that did not have these limitations.

An assay for measuring antibacterial antibody described by Zollinger, Dalrymple and Artenstein (1976) may overcome the inadequacies of the assay used in these studies. It is very similar in conception to the assay described here except that extracted bacterial antigen is fixed on the wells of plastic plates, and used for absorbing antibody. The antibody is quantitated with radio-labelled antiserum as in this study. The end point is defined in terms of binding of a known quantity of radio-labelled antibody. Preliminary experiments we have done with this suggest a marked increase in sensitivity over the assay used in the present studies. The most likely reason for this is that centrifuging which may physically disrupt antibody binding is omitted. This assay has the added potential of possibly increasing the specificity of antibody being looked for by better definition of the antigens against which the antibody is directed. With the development of vaccines that stimulate antibody against specific "protective antigens", this has the potential of separating relevant from irrelevant antibody in a way that the assay described in this thesis is incapable of doing.
The use of anti-\(\mu\) serum would obviously complement the use of the anti-alpha and anti-gamma sera and this serum has now been developed.

**Improvement in Methodology - Inhibitors of Proteolysis**

The possible advantages of being able to inhibit proteolysis in gastrointestinal secretions particularly if there is any cogent reason to study non IgA antibodies high in the intestine or any class of antibody lower down have been discussed. There has been no published systematic examination of the methods to prevent proteolysis in intestinal fluid in particular with regard to newer protease inhibitors.

**Improvement in Methodology - Controls.**

The controls used in this study both for adults and children were drawn from young adults working mainly in the laboratory environment. It could be argued that these controls are if anything more likely to have been exposed to the infections against which the antibody response is being examined, so that any bias would be against the experiment showing a significant difference between infected patients and the controls used. Ideally in addition to these controls, groups of both adults and children with gastroenteritis caused by an agent such as E. coli or rota-virus would have removed any doubts as to whether the antibody rises demonstrated were an anamnestic response or non-specific response to gut inflammation.
Further Studies Arising Out of This Thesis

The obvious application of the work described in this thesis, the study of the humoral immune response to bacterial gastroenteritis, lies in the prophylaxis and treatment of this condition.

The interplay between humoral and cellular immunity particularly in the invasive diarrhoeas was discussed in Chapters 1 and 2. The methodological difficulties likely to be encountered in any meaningful study of specific cellular immunity are great. The potential benefits though, of being able to follow changes both systemically and in the gut in relation to increased cell interaction with other cells and with the relevant bacteria, would greatly enhance understanding of recovery from disease and improve the laboratory scientist's ability to evaluate candidate vaccines. Meaningful progress in this area depends on developing effective measures of cellular immunity and also on harvesting cells from intestinal tissues.

The ability to measure intestinal antibody as a measure of the intestinal immune response and the lack of any suitable alternative to this at present, are important messages of this thesis. The ultimate question as to whether the antigenic stimulus of a vaccine is the appropriate one and as to whether it protects against disease can only be answered by either large scale vaccine trials in areas where the disease is found or by challenge with virulent
organisms after vaccination; the amount of energy expanded in the one case and the risks taken in the other could be more fruitfully rationed by using intestinal antibody measurements as an assessment of the likely value of such a vaccine.
APPENDIX

The pathogenic organism isolated from stools before the first sampling is given under "Bacteriology" without a date. Any subsequent isolations are given along with the date and site of isolation e.g. stools or jejunal aspirate.
Mrs Phyllis ______

D.O.B. Age 58 years
Past Medical History Nil of note
Presenting Illness Sudden onset of diarrhoea with abdominal colic and vomiting.
Diarrhoea lasted a week.
Bacteriology Salmonella typhi-murium
Onset of illness 7/10/76

<table>
<thead>
<tr>
<th>SAMPLING</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
</tr>
<tr>
<td>------</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>
Miss Devalynn

D.O.B.  
Past Medical History  
Presenting Illness  
Bacteriology  
Onset of Illness

Age 14 years  
Nil of note  
Diarrhoea and abdominal colic that lasted twelve days  
Salmonella newport  
3.11.76

### SAMPLING

<table>
<thead>
<tr>
<th>No.</th>
<th>Date</th>
<th>Wks after Infection</th>
<th>Sample</th>
<th>Immunoglobulin (mg%)</th>
<th>Antibody (u/0.1ml)</th>
<th>Antibody (u/mg Ig)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A</td>
<td>G</td>
<td>M</td>
</tr>
<tr>
<td>1</td>
<td>17.11.76</td>
<td>2</td>
<td>Serum</td>
<td>130</td>
<td>1620</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int. Asp.</td>
<td>5</td>
<td>1</td>
<td>95</td>
</tr>
<tr>
<td>2</td>
<td>1.12.76</td>
<td>4</td>
<td>Serum</td>
<td>107</td>
<td>1800</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int. Asp.</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
MR DEREK

D.O.B.: 8

Past Medical History
Nil of note

Presenting Illness
Diarrhoea with blood and slime that lasted ten days

Bacteriology
Shigella sonnei

Onset of Illness
25.4.77

**SUBJECT 3**

---

**SAMPLING**

<table>
<thead>
<tr>
<th>No.</th>
<th>Date</th>
<th>Wks after Infection</th>
<th>Sample</th>
<th>Immunoglobulin (mg%)</th>
<th>Antibody (u/0.1ml)</th>
<th>Antibody (u/mg Ig)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A</td>
<td>G</td>
<td>M</td>
</tr>
<tr>
<td>1</td>
<td>2.5.77</td>
<td>1</td>
<td>Serum</td>
<td>197</td>
<td>900</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int. Asp.</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>27.5.77</td>
<td>5</td>
<td>Serum</td>
<td>167</td>
<td>900</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int. Asp.</td>
<td>25</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Mr. John

D.O.B.
Past Medical History
Presenting Illness
Bacteriology
Onset of Illness

Age 65 years
Nil of note
Profuse watery diarrhoea that precipitated renal failure.
Symptoms eased after 1 week.
Salmonella havana
Salmonella havana (stool) 6.3.1978
9.2.77

<table>
<thead>
<tr>
<th>No.</th>
<th>Date</th>
<th>Wks after Infection</th>
<th>Sample</th>
<th>Immunoglobulin (mg%)</th>
<th>Antibody (u/0.1ml)</th>
<th>Antibody (u/mg Ig)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A</td>
<td>G</td>
<td>M</td>
</tr>
<tr>
<td>1</td>
<td>17.2.77</td>
<td>1</td>
<td>Serum</td>
<td>255</td>
<td>1320</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int. Asp.</td>
<td>68</td>
<td>9</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>7.3.77</td>
<td>4</td>
<td>Serum</td>
<td>255</td>
<td>1520</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int. Asp.</td>
<td>47</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>6.6.77</td>
<td>16</td>
<td>Serum</td>
<td>267</td>
<td>1520</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int. Asp.</td>
<td>71</td>
<td>16</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>1.3.78</td>
<td>54</td>
<td>Serum</td>
<td>267</td>
<td>1400</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int. Asp.</td>
<td>44.5</td>
<td>8.7</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Gastr. Asp.</td>
<td>29</td>
<td>21</td>
<td>3</td>
</tr>
</tbody>
</table>

SUBJECT 4
Mr. Francis

D.O.B. Age 56 years
Past Medical History Poliomyelitis as a child. Ureteric stone.
Presenting Illness Frequency of bowel actions late in June culminating in watery diarrhoea and vomiting over 2 days in early July. Loose bowel actions from then till early September.

Bacteriology Salmonella bredeni
Onset of illness Salmonella bredeni (stool) 14.9.77
25.6.77

SAMPLING

<table>
<thead>
<tr>
<th>No.</th>
<th>Date</th>
<th>Wks after Infection</th>
<th>Sample</th>
<th>Immunoglobulin(mg%)</th>
<th>Antibody(u/0.1ml)</th>
<th>Antibody(u/mg.Ig)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>19.8.77</td>
<td>7</td>
<td>Serum</td>
<td>198 1800 60 390 50 3120</td>
<td>189.5 252.5 1733.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int.Asp.</td>
<td>18 4 0 21 15 10</td>
<td>954.5 833.3 2500</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>21.9.77</td>
<td>11</td>
<td>Serum</td>
<td>220 2170 75 510 80 3930</td>
<td>206.9 363.6 1811.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int.Asp.</td>
<td>24 11 3 53 43 23</td>
<td>1394.7 1791.7 2090.9</td>
<td></td>
</tr>
</tbody>
</table>
**SUBJECT 6**

Mrs. Anna-Liese

**D.O.B.**

Age 52 years

**Past Medical History**

Gastric Surgery (Polya Gastrectomy in 1971)

**Presenting illness**

Severe mucoid diarrhoea 5-10 times/day. Vomiting for 2 days, 10 days after start of diarrhoea. Symptoms easing at time of study.

**Bacteriology**

Salmonella bovis-morbificans

**Date of illness**

1.5.77

**SAMPLING**

<table>
<thead>
<tr>
<th>No.</th>
<th>Date</th>
<th>Wks after Infection</th>
<th>Sample</th>
<th>Immunoglobulin (mg%)</th>
<th>Antibody (u/0.1ml)</th>
<th>Antibody (u/mg.Ig)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A</td>
<td>G</td>
<td>M</td>
</tr>
<tr>
<td>1</td>
<td>25.5.77</td>
<td>3</td>
<td>Serum</td>
<td>202</td>
<td>1120</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int. Asp.</td>
<td>71</td>
<td>11</td>
<td>32</td>
</tr>
<tr>
<td>2</td>
<td>29.6.77</td>
<td>8</td>
<td>Serum</td>
<td>155</td>
<td>1000</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int. Asp.</td>
<td>18</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>9.5.78</td>
<td>50</td>
<td>Serum</td>
<td>180</td>
<td>1170</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int. Asp.</td>
<td>44</td>
<td>3</td>
<td>6</td>
</tr>
</tbody>
</table>
Mr. Robert D.O.B.

Past Medical History

Presenting illness

Bacteriology

Onset of Illness

**SAMPLING**

<table>
<thead>
<tr>
<th>No.</th>
<th>Date</th>
<th>Wks after Infection</th>
<th>Sample</th>
<th>Immunoglobulin(mg%)</th>
<th>Antibody(u/0.1ml)</th>
<th>Antibody(u/mg.Ig)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A</td>
<td>G</td>
<td>M</td>
</tr>
<tr>
<td>1</td>
<td>22.6.77</td>
<td>2</td>
<td>Serum</td>
<td>68</td>
<td>112</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int.Asp.</td>
<td>5</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Saliva</td>
<td>2.5</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>12.10.77</td>
<td>18</td>
<td>Serum</td>
<td>63</td>
<td>142</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int.Asp.</td>
<td>5</td>
<td>9</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Saliva</td>
<td>6</td>
<td>1.9</td>
<td>0</td>
</tr>
</tbody>
</table>

Age 34 years
Nil of note

Urgency of bowel actions three days followed by explosive diarrhoea and rigors for 24 hours. Diarrhoea settled after 10 days.

Salmonella heffarek
10.6.77
Mrs. Judy  
D.O.B.  
Age 36 years  
Past Medical History  
Ruptured appendix (19 years)  
Presenting Illness  
Explosive diarrhoea, 20 bowel actions a day and fever for 24 hours. Diarrhoea ceased after 3 days (with Diphenoxylate.)  
Bacteriology  
Salmonella typhi-murium  
Onset of illness  
15.8.77

<table>
<thead>
<tr>
<th>No.</th>
<th>Date</th>
<th>Wks after Infection</th>
<th>Sample</th>
<th>Immunoglobulin(mg%)</th>
<th>Antibody(u/0.1ml)</th>
<th>Antibody(u/mg.Ig)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A</td>
<td>G</td>
<td>M</td>
</tr>
<tr>
<td>1</td>
<td>31.8.77</td>
<td>2</td>
<td>Serum</td>
<td>195</td>
<td>1325</td>
<td>205</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int.Asp.</td>
<td>7</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Saliva</td>
<td>2.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>2.11.77</td>
<td>11</td>
<td>Serum</td>
<td>175</td>
<td>260</td>
<td>195</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int.Asp.</td>
<td>17</td>
<td>17</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Saliva</td>
<td>21.5</td>
<td>2.2</td>
<td>0</td>
</tr>
</tbody>
</table>
Mrs Joan
Age 33 years
Nil of note
Diarrhoea with hourly bowel actions and rigors with vomiting after 48 hours. Diarrhoea stopped after a week.
Salmonella typhi-murium 6.12.76

<table>
<thead>
<tr>
<th>No.</th>
<th>Date</th>
<th>Wks after Infection</th>
<th>Sample</th>
<th>Immunoglobulin(mg%)</th>
<th>Antibody(u/0.1ml)</th>
<th>Antibody(u/mg.Ig)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>21.12.77</td>
<td>2</td>
<td>Serum</td>
<td>175 1300 125 780 80</td>
<td>487.5 457.1 600</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int.Asp.</td>
<td>23 0 5 11 5 0</td>
<td>392.9 217.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Saliva</td>
<td>8.5 0 0 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>24.2.78</td>
<td>9</td>
<td>Serum</td>
<td>175 1540 150 270 20</td>
<td>144.8 114.3 246.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int.Asp.</td>
<td>6 0 0 2 0 0</td>
<td>333.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Saliva</td>
<td>8.5 0 0 1 1</td>
<td>111.1 111.1</td>
<td></td>
</tr>
</tbody>
</table>
**SUBJECT 10**

Miss Lisa

D.O.B.
Past Medical History
Presenting Illness

Age 16 years
Appendicitis - 14 years.
Headache, myalgia, and abdominal pain followed within twenty four hours by watery diarrhoea with some blood in it. Normal bowel pattern returned in 2 weeks.
Salmonella typhi-murium
28th December, 1977.

**SAMPLING**

<table>
<thead>
<tr>
<th>No.</th>
<th>Date</th>
<th>Wks after</th>
<th>Sample</th>
<th>Immunoglobulin (mg%)</th>
<th>Antibody (u/0.1ml)</th>
<th>Antibody (u/mg.Ig)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A</td>
<td>G</td>
<td>M</td>
</tr>
<tr>
<td>1</td>
<td>24.1.78</td>
<td>4</td>
<td>Serum</td>
<td>213</td>
<td>1540</td>
<td>175</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int. Asp.</td>
<td>6</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Saliva</td>
<td>11</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>20.3.78</td>
<td>12</td>
<td>Serum</td>
<td>213</td>
<td>1640</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int. Asp.</td>
<td>10</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Saliva</td>
<td>4</td>
<td>1.9</td>
<td>0</td>
</tr>
</tbody>
</table>
Mrs Theresa

D.O.B.: Age 40 years
Past Medical History: Nil of note
Presenting Illness: Profuse diarrhoea with colic and fever was followed by vomiting. Symptoms persisted for 2 weeks.

Bacteriology: Salmonella typhi-murium
Onset of illness: 23.1.78

**SAMPLING**

<table>
<thead>
<tr>
<th>No.</th>
<th>Date</th>
<th>Wks after Infection</th>
<th>Sample</th>
<th>Immunoglobulin (mg%)</th>
<th>Antibody (u/0.1ml)</th>
<th>Antibody (u/mg.lg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.2.78</td>
<td>2</td>
<td>Serum</td>
<td>187 860 60 1370 320 1910</td>
<td>1237.6 1711.2 2220.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int. Asp.</td>
<td>21 2 3 29 17 1</td>
<td>1115.4 809.5 500</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Saliva</td>
<td>6.5 0 0 2 1</td>
<td>285.7 142.9</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>15.3.78</td>
<td>7</td>
<td>Serum</td>
<td>175 890 80 860 80 1000</td>
<td>751.1 457.1 1123.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int. Asp.</td>
<td>18 2 2 14 9 1</td>
<td>636.4 500 500</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Saliva</td>
<td>5 0 0 4 3</td>
<td>800 600</td>
<td></td>
</tr>
</tbody>
</table>
Mrs Alma

D.O.B. Past Medical History

Age 54 years
Ovarian Cystectomy - 31 years; Asian 'flu and Pneumonia - 36 years.
Cholecystectomy - 51 years.

Presenting Illness

Diarrhoea two days after a course of antibiotics for cystitis.
Diarrhoea settled after three weeks.

Bacteriology
Salmonella typhi-murium

Onset of Illness
11.1.78

SAMPLING

<table>
<thead>
<tr>
<th>No.</th>
<th>Date</th>
<th>Wks after Infection</th>
<th>Sample</th>
<th>Immunoglobulin(mg%)</th>
<th>Antibody(u/0.1ml)</th>
<th>Antibody(u/mg.Ig)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>10.2.78</td>
<td>4</td>
<td>Serum</td>
<td>215</td>
<td>60</td>
<td>359.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int. Asp.</td>
<td>15</td>
<td>4</td>
<td>434.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Saliva</td>
<td>6</td>
<td>0</td>
<td>266.7</td>
</tr>
<tr>
<td>2</td>
<td>29.3.78</td>
<td>11</td>
<td>Serum</td>
<td>225</td>
<td>0</td>
<td>111.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int. Asp.</td>
<td>32</td>
<td>10</td>
<td>365.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Saliva</td>
<td>4.5</td>
<td>0</td>
<td>312.5</td>
</tr>
</tbody>
</table>
Mr. Robert

D.O.B.        Age 38 years
Past Medical History  Nil of note
Presenting Illness  Diarrhoea accompanied by myalgia. Diarrhoea eased after 12 days.
Bacteriology  Salmonella typhi-murium
Onset of illness  30.1.78

**SUBJECT 13**

<table>
<thead>
<tr>
<th>No.</th>
<th>Date</th>
<th>Wks after Infection</th>
<th>Sample</th>
<th>Immunoglobulin(mg%)</th>
<th>Antibody(u/0.1ml)</th>
<th>Antibody(u/mg.Ig)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Serum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.3.78</td>
<td>4</td>
<td></td>
<td>250</td>
<td>1600</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int. Asp.</td>
<td>13</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Saliva</td>
<td>8.5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>431.3</td>
</tr>
<tr>
<td>2</td>
<td>4.4.78</td>
<td>9</td>
<td></td>
<td>268</td>
<td>1700</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int. Asp.</td>
<td>12</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Saliva</td>
<td>8.5</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>341.2</td>
</tr>
</tbody>
</table>
Mrs Ursula

D.O.B.
Past Medical History
Presenting illness
Bacteriology
Onset of Illness

Age 43 years
Nil of note
Colicky right sided abdominal pain and diarrhoea which lasted 9 days.
Shigella sonnei
9.11.77

SAMPLING

<table>
<thead>
<tr>
<th>No.</th>
<th>Date</th>
<th>Wks after Infection</th>
<th>Sample</th>
<th>Immunoglobulin(mg%)</th>
<th>Antibody(u/0.1ml)</th>
<th>Antibody(u/mg.Ig)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>24.11.77</td>
<td>2</td>
<td>Serum</td>
<td>155</td>
<td>450</td>
<td>720</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int.As.</td>
<td>11</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Saliva</td>
<td>10</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>20.12.77</td>
<td>6</td>
<td>Serum</td>
<td>110</td>
<td>560</td>
<td>1520</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int.As.</td>
<td>8</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Saliva</td>
<td>6</td>
<td>4</td>
<td>3</td>
</tr>
</tbody>
</table>
Mrs Denise

D.O.B. Age 29 years
Past Medical History Mild hay fever
Presenting Illness Diarrhoea with vomiting and rigors at the onset. Diarrhoea settled after three days.
Bacteriology Salmonella senftenberg
Onset of Illness 19.6.78

SAMPLE

<table>
<thead>
<tr>
<th>No.</th>
<th>Date Wks after Infection</th>
<th>Sample</th>
<th>Immunoglobulin (mg%)</th>
<th>Antibody (u/0.1ml)</th>
<th>Antibody (u/mg Ig)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30.6.78 2</td>
<td>Serum</td>
<td>213</td>
<td>540</td>
<td>431</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Int.Asp.</td>
<td>8</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Saliva</td>
<td>5.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>7.8.78 7</td>
<td>Serum</td>
<td>188</td>
<td>480</td>
<td>428</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Int.Asp.</td>
<td>13</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Saliva</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Mr. Ronald

D.O.B. Past medical history Presenting Illness Bacteriology Onset of Illness

Age 32 years Nil of note Diarrhoea and vomiting lasting 72 hours. Salmonella typhi-murium 19.6.78

<table>
<thead>
<tr>
<th>No.</th>
<th>Date</th>
<th>Wks after Infection</th>
<th>Sample</th>
<th>Immunoglobulin(mg%)</th>
<th>Antibody(u/0.1ml)</th>
<th>Antibody(u/mg.Ig)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A</td>
<td>G</td>
<td>M</td>
</tr>
<tr>
<td>1</td>
<td>29.6.78</td>
<td>1</td>
<td>Serum</td>
<td>390</td>
<td>960</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int.Asp.</td>
<td>2</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Saliva</td>
<td>6.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>8.8.78</td>
<td>7</td>
<td>Serum</td>
<td>390</td>
<td>1280</td>
<td>175</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int.Asp.</td>
<td>13</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Saliva</td>
<td>5.5</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
**SUBJECT 17**

**Tony**

**D.O.B.**
Past Medical History
Presenting Illness

**Bacteriology**
Onset of Illness

**SAMPLING**

<table>
<thead>
<tr>
<th>No.</th>
<th>Date</th>
<th>Wks after Infection</th>
<th>Sample</th>
<th>Immunoglobulin (mg%)</th>
<th>Antibody (u/0.1ml)</th>
<th>Antibody (u/mg.Ig)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A</td>
<td>G</td>
<td>M</td>
</tr>
<tr>
<td>1</td>
<td>21.2.78</td>
<td>1</td>
<td>Serum</td>
<td>105</td>
<td>1370</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int.Asp.</td>
<td>20</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>2</td>
<td>22.3.78</td>
<td>4</td>
<td>Serum</td>
<td>80</td>
<td>1470</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int.Asp.</td>
<td>6</td>
<td>8</td>
<td>3</td>
</tr>
</tbody>
</table>
Samantha D. O.B. Past Medical History Presenting Illness Bacteriology

Presenting Illness

Bacteriology

Onset of Illness 19.2.78

SAMPLING

<table>
<thead>
<tr>
<th>No.</th>
<th>Date</th>
<th>Wks after Infection</th>
<th>Sample</th>
<th>Immunoglobulin (mg%)</th>
<th>Antibody (u/0.1ml)</th>
<th>Antibody (u/mg.Ig)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>28.2.78</td>
<td>1</td>
<td>Serum</td>
<td>30</td>
<td>750</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int. Asp.</td>
<td>7</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1000</td>
<td>80</td>
<td>1210</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
<td>1111.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2666.7</td>
<td>1613.3</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>29.3.78</td>
<td>4</td>
<td>Serum</td>
<td>5</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int. Asp.</td>
<td>1</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1333.3</td>
<td>1200</td>
<td></td>
</tr>
</tbody>
</table>
Mandy
D.O.B.: 
Past Medical History
Presenting Illness
Bacteriology
Onset of Illness

SAMPLING

<table>
<thead>
<tr>
<th>No.</th>
<th>Date</th>
<th>Wks after Infection</th>
<th>Sample</th>
<th>Immunoglobulin(mg%)</th>
<th>Antibody(u/0.1ml)</th>
<th>Antibody(u/mg.Ig)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>23.1.78</td>
<td>1</td>
<td>Serum</td>
<td>185</td>
<td>860</td>
<td>310</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int. Asp.</td>
<td>13</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>8.3.78</td>
<td>7</td>
<td>Serum</td>
<td>105</td>
<td>1070</td>
<td>230</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int. Asp.</td>
<td>6</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

Age 2yrs 8 months
Diarrhoea and tonsillitis
Salmonella typhi-murium (stool negative on 8.2.78)
14.1.78
**SUBJECT 20**

Kimberley

D.O.B.  
Past Medical History  
Presenting Illness  
Bacteriology  
Onset of Illness  

Age 2 yrs 2 months.  
Fever and diarrhoea  
Salmonella typhi-murium (stools and aspirate negative on 19.4.78)  
14.3.78  

**SAMPLING**

<table>
<thead>
<tr>
<th>No.</th>
<th>Date</th>
<th>Wks after Infection</th>
<th>Sample</th>
<th>Immunoglobulin(mg%)</th>
<th>Antibody(u/0.1ml)</th>
<th>Antibody(u/mg.Ig)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A</td>
<td>G</td>
<td>M</td>
</tr>
<tr>
<td>1</td>
<td>23.3.78</td>
<td>1</td>
<td>Serum</td>
<td>50</td>
<td>750</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int.Asp.</td>
<td>13</td>
<td>6</td>
<td>13</td>
</tr>
<tr>
<td>2</td>
<td>19.4.78</td>
<td>4</td>
<td>Serum</td>
<td>43</td>
<td>900</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int.Asp.</td>
<td>8</td>
<td>23</td>
<td>5</td>
</tr>
</tbody>
</table>
SUBJECT 21

Jason

D.O.B.
Past Medical History - 
Presenting Illness
Vomiting, diarrhoea, fever, 20 bowel actions per day.
Bacteriology
Salmonella typhi-murium (stools negative on 24.5.78)
Onset of Illness
9.4.78

SAMPLING

<table>
<thead>
<tr>
<th>No.</th>
<th>Date</th>
<th>Wks after</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Infection</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample</th>
<th>Immunoglobulin(mg%)</th>
<th>Antibody(u/0.1ml)</th>
<th>Antibody(u/mg.Ig)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>G</td>
<td>M</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>G</td>
<td>M</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Serum</th>
<th>100</th>
<th>720</th>
<th>300</th>
<th>1480</th>
<th>140</th>
<th>960</th>
<th>1321.4</th>
<th>1400</th>
<th>1333.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Int.Asp.</td>
<td>6</td>
<td>4</td>
<td>4</td>
<td>19</td>
<td>4</td>
<td>5</td>
<td>1357.1</td>
<td>666.7</td>
<td>1250</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>4</td>
<td>4</td>
<td>19</td>
<td>4</td>
<td>5</td>
<td>1357.1</td>
<td>666.7</td>
<td>1250</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Serum</th>
<th>45</th>
<th>680</th>
<th>155</th>
<th>700</th>
<th>≤25</th>
<th>1330</th>
<th>795.5</th>
<th>1955.9</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Int.Asp.</td>
<td>6</td>
<td>5</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>545.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>5</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>545.5</td>
<td></td>
</tr>
</tbody>
</table>
Leah

D.O.B.: 1

Past Medical History

Presenting Illness

Bacteriology

Onset of Illness

4.4.78

Salmonella typhi-murium (aspirate negative on 1.5.78 and 6.6.78)

SAMPLING

<table>
<thead>
<tr>
<th>No.</th>
<th>Date</th>
<th>Wks after Infection</th>
<th>Sample</th>
<th>Immunoglobulin(mg%)</th>
<th>Antibody(u/0.1ml)</th>
<th>Antibody(u/mg.Ig)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Serum</td>
<td>A  13 G  300 M  15 LC  40 α  10 γ  80</td>
<td>LC  122 A  769.2 G  266.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int.Asp.</td>
<td>8  6 G  6 M  6 LC  26 α  7 γ  2</td>
<td>LC  1300 A  875 G  333.3</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.5.78</td>
<td>4</td>
<td>Serum</td>
<td>25  560 G  90 M  80 LC  10 α  120</td>
<td>LC  118.5 A  400 G  214.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.6.78</td>
<td>8</td>
<td>Int.Asp.</td>
<td>10  6 G  2 M  5 LC  5 α  3 γ  0</td>
<td>LC  277.8 A  300 G  300</td>
<td></td>
</tr>
</tbody>
</table>
SUBJECT 23

Habib

D.O.B. : Age 6 months
Past Medical History -
Presenting Illness: Fever and diarrhoea
Bacteriology: Salmonella typhi-murium
Onset of Illness: Salmonella typhi-murium (stool) 26.6.78 (aspirate negative 26.6.78)

13.5.78

SAMPLING.

<table>
<thead>
<tr>
<th>No.</th>
<th>Date</th>
<th>Wks after Infection</th>
<th>Sample</th>
<th>Immunoglobulin (mg%)</th>
<th>Antibody (u/0.1ml)</th>
<th>Antibody (u/mg.Ig)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A G M LC α γ</td>
<td>LC α γ</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>22.5.78</td>
<td>1</td>
<td>Serum</td>
<td>30 300 170 320 30 200</td>
<td>640 1000 666.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int. Asp.</td>
<td>5 3 5 40 5 1</td>
<td>3076.9 1000 333.3</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>26.6.78</td>
<td>5</td>
<td>Serum</td>
<td>25 340 90 80 30 220</td>
<td>175.8 1200 647.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int. Asp.</td>
<td>8 2 17 51 29 1</td>
<td>1888.9 3625 500</td>
<td></td>
</tr>
</tbody>
</table>
### SUBJECT 24

**David**

**D.O.B.**:  
Age 2 years 5 months

**Past Medical History**:  
- Diarrhoea, fever and vomiting

**Presenting Illness**:  
Salmonella typhi-murium (aspirate negative 26.6.78)

**Onset of Illness**:  
11.5.78

### SAMPLING

<table>
<thead>
<tr>
<th>No.</th>
<th>Date</th>
<th>Wks after Infection</th>
<th>Sample</th>
<th>Immunoglobulin(mg%)</th>
<th>Antibody(u/0.1ml)</th>
<th>Antibody(u/mg.Ig)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22.5.78</td>
<td>1</td>
<td>Serum</td>
<td>83 1000 260 800 30 380</td>
<td>595.7 361.4 380</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int.Asp.</td>
<td>8 16 9 57 12 2</td>
<td>1727.3 1500 125</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>26.6.78</td>
<td>5</td>
<td>Serum</td>
<td>50 860 170 480 30 380</td>
<td>444.4 600 441.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int.Asp.</td>
<td>6 2 3 3 1 1</td>
<td>272.7 166.7 500</td>
<td></td>
</tr>
</tbody>
</table>
**SUBJECT 25**

Troy

D.O.B. Age 2 years 1 month
Past Medical History -
Presenting Illness Fever and diarrhoea
Bacteriology *Salmonella newport* (stool negative 17.5.78)
Onset of Illness 1.4.78

**SAMPLING**

<table>
<thead>
<tr>
<th>No.</th>
<th>Date.</th>
<th>Wks after Infection</th>
<th>Sample</th>
<th>Immunoglobulin (mg%)</th>
<th>Antibody (u/0.1ml)</th>
<th>Antibody (u/mg. Ig)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A</td>
<td>G</td>
<td>M</td>
</tr>
<tr>
<td>1</td>
<td>7.4.78</td>
<td>1</td>
<td>Serum</td>
<td>135</td>
<td>1000</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int. Asp.</td>
<td>10</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>25.5.78</td>
<td>8</td>
<td>Serum</td>
<td>105</td>
<td>1000</td>
<td>230</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int. Asp.</td>
<td>10</td>
<td>13</td>
<td>4</td>
</tr>
</tbody>
</table>
SUBJECT 26

Terence

D.O.B.
Past Medical History
Nil of note
Presenting Illness
Diarrhoea which lasted 3 days and one episode of vomiting
Bacteriology
Negative for bacterial pathogens

SAMPLING

<table>
<thead>
<tr>
<th>No.</th>
<th>Date</th>
<th>Wks after Infection</th>
<th>Sample</th>
<th>Immunoglobulin(mg%)</th>
<th>Antibody(u/0.1ml)</th>
<th>Antibody(u/mg.Ig)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22.4.77</td>
<td></td>
<td>Serum</td>
<td>275 1170 200 60 0 440</td>
<td>36.5 376.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int. Asp.</td>
<td>10 2 5 1 0 0</td>
<td>58.8</td>
<td></td>
</tr>
</tbody>
</table>
**SUBJECT 27**

Rowan

**D.O.B.**

**Past Medical History**

**Presenting Illness**

**Bacteriology**

**SAMPLING**

<table>
<thead>
<tr>
<th>No.</th>
<th>Date</th>
<th>Wks after Infection</th>
<th>Sample</th>
<th>Immunoglobulin(mg%)</th>
<th>Antibody(u/0.1ml)</th>
<th>Antibody(u/mg.Ig)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.6.77</td>
<td></td>
<td>Serum</td>
<td>288 1060 105 100</td>
<td></td>
<td>68.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int.Asp.</td>
<td>3 0 0 0</td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>
SUBJECT 28

Justin

D.O.B.  
Past Medical History  
Healthy control

Age 31 years  
Nil of note

SAMPLING

<table>
<thead>
<tr>
<th>No.</th>
<th>Date</th>
<th>Wks after Infection</th>
<th>Sample</th>
<th>Immunoglobulin (mg%)</th>
<th>Antibody (u/0.1ml)</th>
<th>Antibody (u/mg.Ig)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.11.77</td>
<td></td>
<td>Serum, Int. Asp.</td>
<td>158, 1970, 170, 170, 10, &gt;160</td>
<td>74, 63.3</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>G</th>
<th>M</th>
<th>LC</th>
<th>α</th>
<th>γ</th>
<th>LC</th>
<th>α</th>
<th>γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>158</td>
<td>1970</td>
<td>170</td>
<td>170</td>
<td>10</td>
<td>&gt;160</td>
<td>74</td>
<td>63.3</td>
<td></td>
</tr>
</tbody>
</table>
John
D.O.B.: Age 27 years
Healthy control.

### SAMPLING

<table>
<thead>
<tr>
<th>No.</th>
<th>Date</th>
<th>Wks after Infection</th>
<th>Sample</th>
<th>Immunoglobulin (mg%)</th>
<th>Antibody (u/0.1ml)</th>
<th>Antibody (u/mg Ig)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14.6.78</td>
<td></td>
<td>Int. Asp.</td>
<td>A: 10 G: 1 M: 0</td>
<td>LC: 4 α: 0 γ: 0</td>
<td>LC: 393.6</td>
</tr>
</tbody>
</table>

SUBJECT 29
<table>
<thead>
<tr>
<th>No.</th>
<th>Date</th>
<th>Wks after Infection</th>
<th>Sample</th>
<th>Immunoglobulin (mg%)</th>
<th>Antibody (u/0.1ml)</th>
<th>Antibody (u/mg.Ig)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.7.78</td>
<td></td>
<td>Serum</td>
<td>75 920 330 170</td>
<td></td>
<td>128.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int. Asp.</td>
<td>5 0 0 0</td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>
**SUBJECT 31**

Robert

D.O.B. Age 30 years

Healthy control

**SAMPLING**

<table>
<thead>
<tr>
<th>No.</th>
<th>Date</th>
<th>Wks after Infection</th>
<th>Sample</th>
<th>Immunoglobulin(mg%)</th>
<th>Antibody(u/0.1ml)</th>
<th>Antibody(u/mg.Ig)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15.6.77</td>
<td></td>
<td>Serum</td>
<td>75  920  330  140</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int. Asp.</td>
<td>6  1  0  0</td>
<td></td>
<td>119.1</td>
</tr>
</tbody>
</table>
Jill

D.O.B. Age 23 years
Healthy Control

<table>
<thead>
<tr>
<th>SAMPLING</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
</tr>
<tr>
<td>------</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>
### SUBJECT 33

Paul

D.O.B. Age 30 years

Healthy control

#### SAMPLING

<table>
<thead>
<tr>
<th>No.</th>
<th>Date</th>
<th>Wks After Infection</th>
<th>Sample</th>
<th>Immunoglobulin (mg%)</th>
<th>Antibody (u/0.1ml)</th>
<th>Antibody (u/mg.Ig)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15.6.77</td>
<td></td>
<td>Serum Int. Asp.</td>
<td>5</td>
<td>1</td>
<td>0 0</td>
</tr>
</tbody>
</table>
### SUBJECT 34

Alan

D.O.B. Age 26 years

Healthy control

### SAMPLING

<table>
<thead>
<tr>
<th>No.</th>
<th>Date</th>
<th>Wks after Infection</th>
<th>Sample</th>
<th>Immunoglobulin (mg%)</th>
<th>Antibody (u/0.1ml)</th>
<th>Antibody (u/mg. Ig)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15.6.77</td>
<td></td>
<td>Serum</td>
<td>8.3</td>
<td>2.5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int. Asp.</td>
<td>3</td>
<td>272.7</td>
<td></td>
</tr>
</tbody>
</table>
David
D.O.B.: Age 31 years
Healthy control

**SAMPLING**

<table>
<thead>
<tr>
<th>No.</th>
<th>Date</th>
<th>Wks after Infection</th>
<th>Sample</th>
<th>Immunoglobulin(mg%)</th>
<th>Antibody(u/0.1ml)</th>
<th>Antibody(u/mg.Ig)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Serum</td>
<td>212.5</td>
<td>1120</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int. Asp.</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>12.7.78</td>
<td></td>
<td></td>
<td>140</td>
<td></td>
<td>96</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>
**SUBJECT 36**

Deborah Healthy control

**SAMPLING**

<table>
<thead>
<tr>
<th>No.</th>
<th>Date</th>
<th>Wks after Infection</th>
<th>Sample</th>
<th>Immunoglobulin (mg%)</th>
<th>Antibody (u/0.1ml)</th>
<th>Antibody (u/mg. Ig)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11.7.78</td>
<td></td>
<td>Serum</td>
<td>187.5 1320 115 70</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int. Asp.</td>
<td>3.5 1.0 1.5 0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
**SUBJECT 37**

Neville

Age 30 years

Healthy control

**SAMPLING**

<table>
<thead>
<tr>
<th>No.</th>
<th>Date</th>
<th>Wks after Infection</th>
<th>Sample</th>
<th>Immunoglobulin(mg%)</th>
<th>Antibody(u/0.1ml)</th>
<th>Antibody(u/mg.Ig)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>11.7.78</td>
<td></td>
<td>Serum</td>
<td>230.5</td>
<td>1220</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Int.As.</td>
<td>7</td>
<td>3.8</td>
<td>3.0</td>
</tr>
</tbody>
</table>

167


Grybowski, J.D. (1977) in Clinics in Gastroenterology Vol.6, No. 2, ed. M. Davidson, publ. W.B. Saunders, "Gastrointestinal function in the infant and young child".


Miller, C.E., Wong, K.H., Feeley, J.C. and Forlines, M.E. 
(1972). Infection and Immunity 6, 739-742. Immunologic 
conversion of Vibrio cholerae in gnotobiotic mice.

Evidence for a primary association between immunoblasts 
and small gut.

supp. 227-241. Vaccines and Somatic Antigens. The 
role of immunity in Cholera. A review of epidemiological 
and serological studies.

vibriocidal antibody titre to susceptibility to cholera 
in family contacts of cholera patients.

47, 229-238. Report of the 1966-67 Cholera vaccine trial 
in rural East Pakistan.

Mosley, W.H., Aziz, K., Rahman, A.M., Chowdhury, A. and 
Field trials of monovalent Ogawa and Inaba cholera 
vaccines in rural Bangla Desh - three years of observation.

cholera antibodies in rural East Pakistan.


Abnormalities in IgA containing mononuclear cells in the gastric lesion of pernicious anaemia.


Properties of antibody fragments.


