THE INTESTINAL ANTIBODY RESPONSE TO BACTERIAL

GASTROENTERITIS IN HUMANS

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ABSTRACT

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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 gastro-enteritis 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

iii

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.

v

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TABLE OF CONTENTS

FIGURES

FIGURES

TABLES

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 (Sell 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 nett 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.

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

* Indicates suggested (but unproven) causes of gastroenteritis.

cholerae 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 enterotoxic 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 recticuloendothelial 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 inocculation with cholera in another study (Cash et al., 1974a) 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)   <u>Does Bacterial Gastroenteritis or Vaccination Prevent</u>
      <u>Infection?</u>

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.

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

* 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 (Hejfec, 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 unfore-
seeably 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). <u>Do Measures of Immune Function Correlate with</u>
<u>Resistance to Infection</u>?

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 agglutunin 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 gastroenteritidesare 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, β_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 immuno-globulin 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 Ferguson, 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

38

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 Brunners' 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 1.	
Gastric secretions	2.5 1	
Bile	0.5 1	
Pancreatic juice	0.7 1	
Intestinal secretions	3.0 1	

(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 immuno-
diffusion 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

immuboglobulins 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 IgA_1 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 gets into 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° 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 irrevelant 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:

No. of Cases.	A.histolytica +ve	B.dysenteriae +ve	Agglutination to B.dysenteriae
2	2	0	0
24	0	15	18

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 publiction, 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
$-20^{o}C$, the samples of fluid were heated at $56^{o}C$ 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 $-10^{O}C$ 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 immuno-diffusion 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)
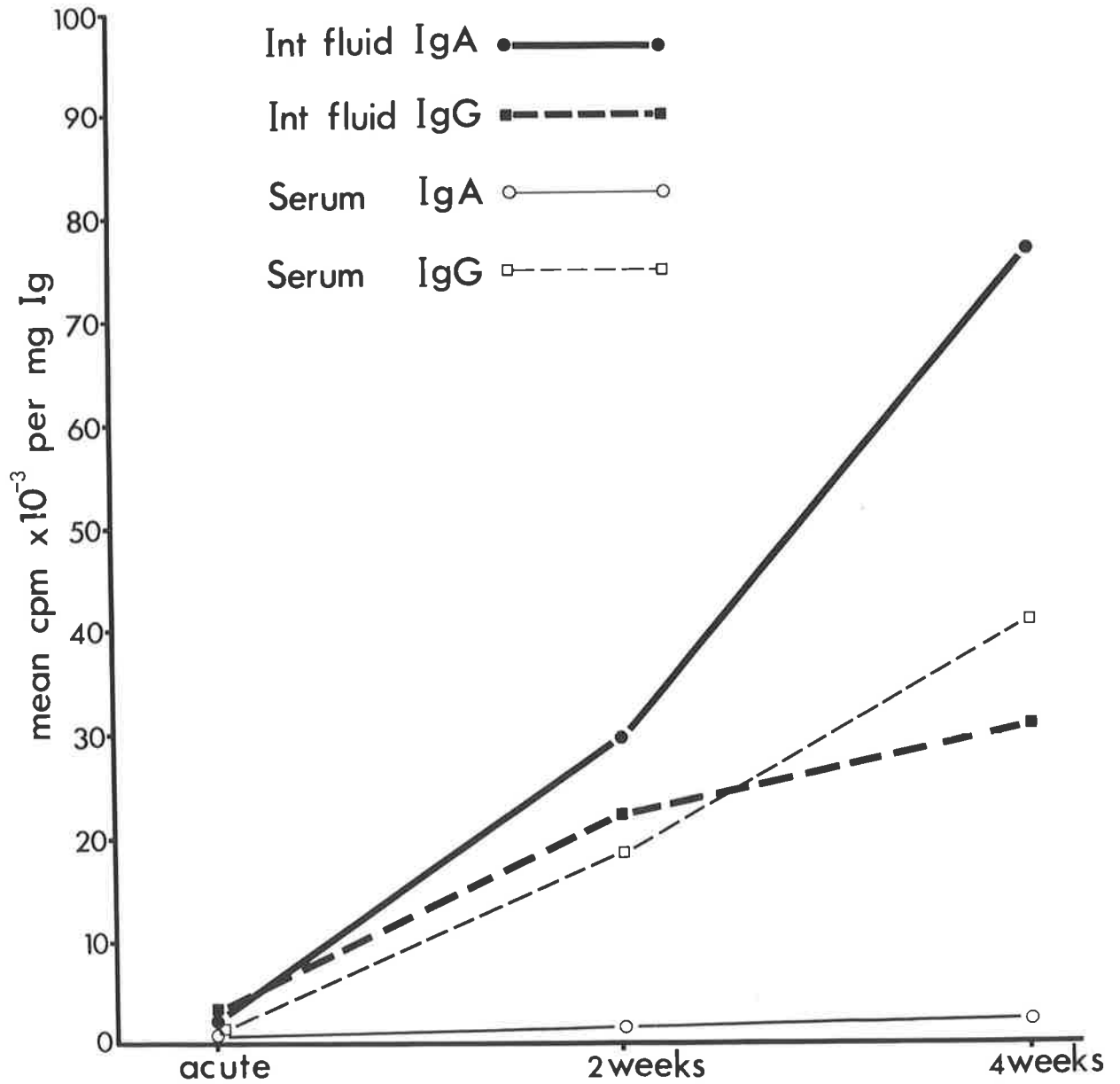
The impression given by this study was that over the
period of observation, serum antibody reflected secretory
antibody at any rate in the group as a whole, in spite of
the theory that in cholera, bacteria are confined to the
intestinal lumen.

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 $-20^{\circ}C$ and $-80^{\circ}C$. This
was done to determine whether reducing the temperature of
storage below -20° 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 $8^{O}C$. They were
grown up when required in nutrient broth (Difco Mich.)
on a shaker at $37^{O}C$.

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. Cowled 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 wave length of 280 mμ
using either a Zeiss or a Hitachi-Elman U-V spectro-photometer.

The extinction co-efficient used for calculating immuno-globulin content were $E_{280}^{1\%}$ 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 (Calbiochem Grade A) in .05 Molar Veronal pH 8.0 buffer with anti heavy-chain

specific serum at $60^{O}C$ 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 under estimated 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 $37^{O}C$ for 1 hour. After a further 48-hour incubation at $4^{O}C$, 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 precipate 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 $_{280}$ 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 $_{280}$ 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 .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 iodacetamide 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 pH8.0, 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

A   Top trough   Anti light-chain serum

    Well   IgA

             Bottom trough   Anti α serum (Hoechst)

B   Top trough   Anti μ serum (Hoechst)

    Well   IgA

             Bottom trough   Anti whole human serum
                                            (Hoechst)

C   Top trough   Anti α serum (Hoechst)

    Well   IgA

             Bottom trough   Anti γ serum (Hoechst)

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

A   Top trough   Anti α

    Middle well   IgG

             Bottom trough   Anti γ

B   Top trough   Anti μ

    Top well   IgG

             Bottom trough   Anti light-chain

C   Top trough   Anti whole human
                                            serum (Hoechst)

    Middle well   IgG

             Bottom trough   Anti whole human
                                          serum (Hoechst)
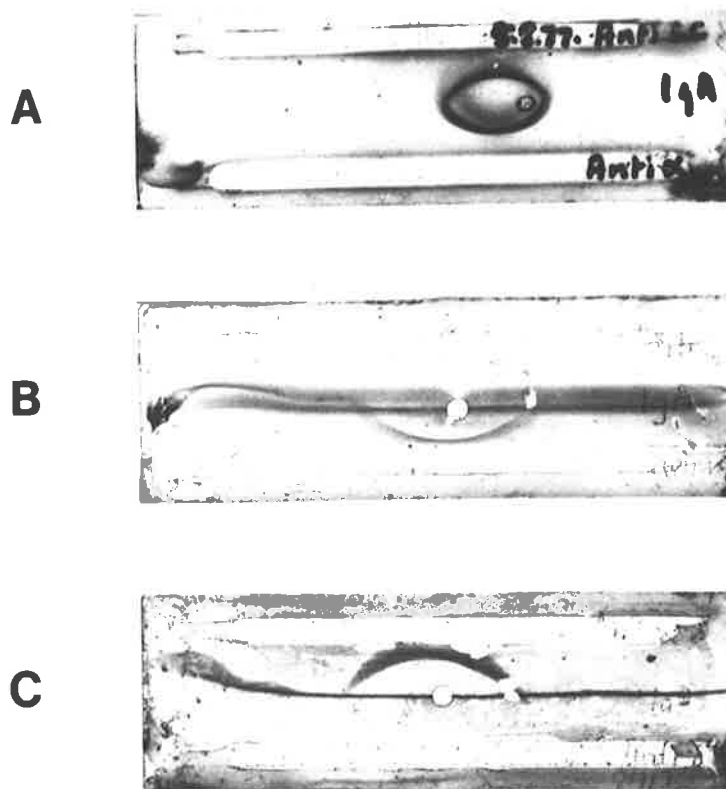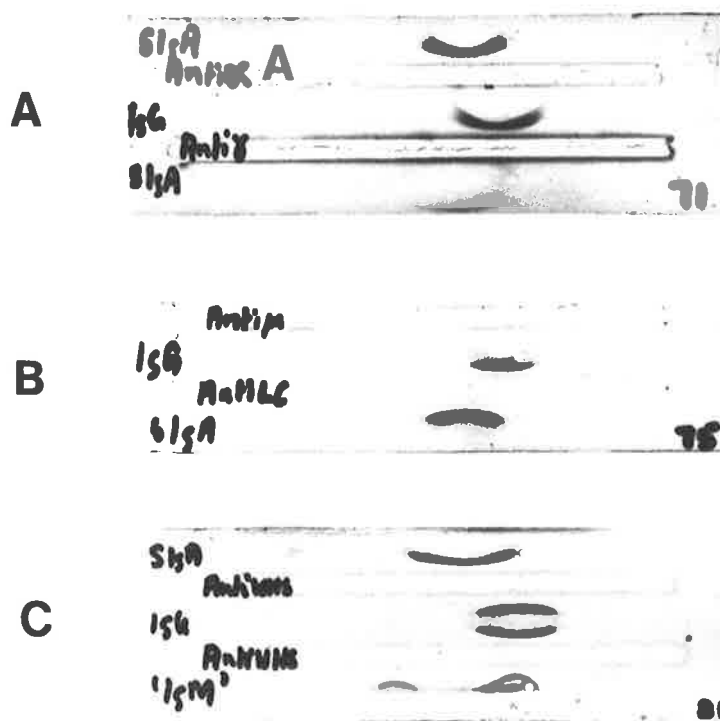
Figure Ⅲ-1

Figure Ⅲ-2

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

A   Top well   Light-chains

                        Top trough   Anti α

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)

C   Top trough   Anti whole
                                                                  human serum
                                                                  (Hoechst)

        Middle well   Secretory IgA

Figure III-3
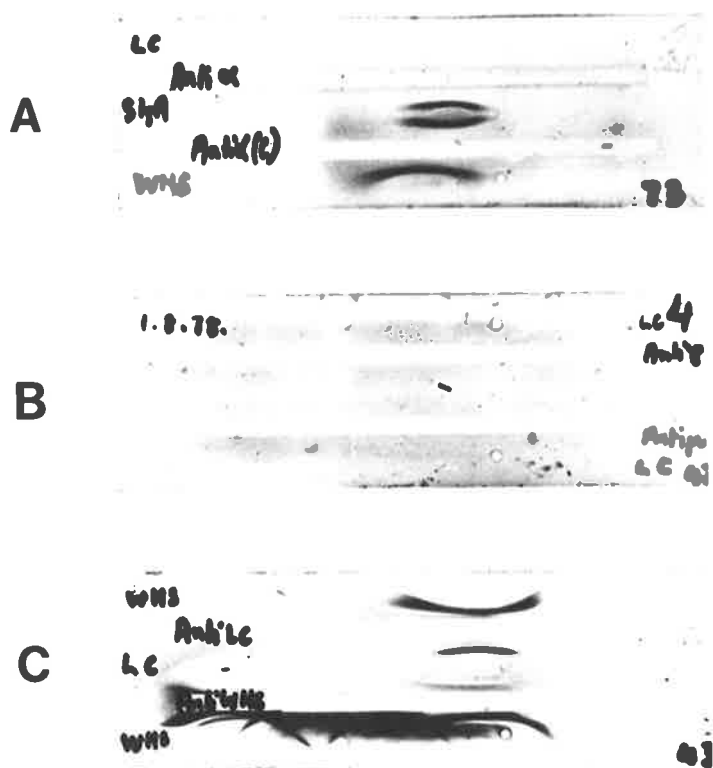
Figure III-4
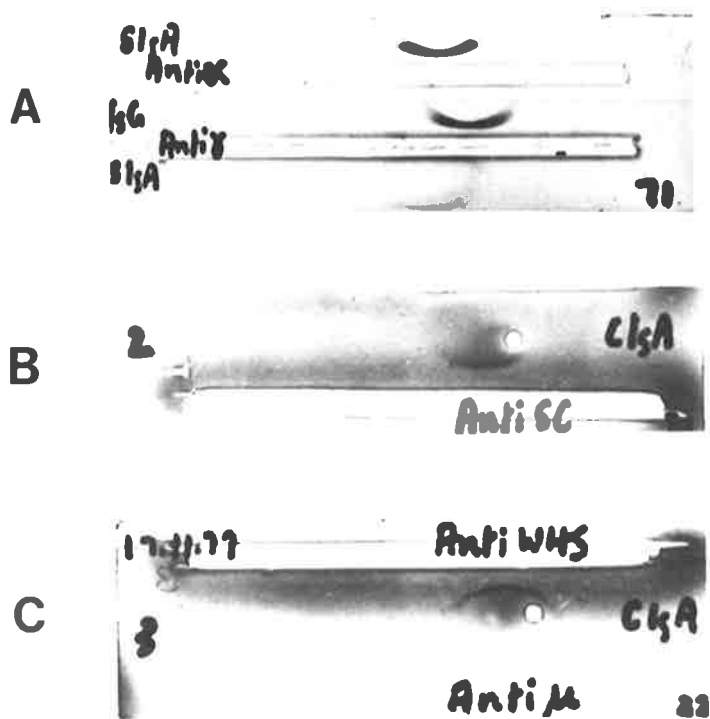
of the first protein peak containing the secretory IgA
was harvested, concentrated and re-run on the column on
two more occasions. Concentration of the second half of
the protein peak, after the third run, to 2 mgs/ml and
analysis using Ouchterlony and immuno-electrophoresis
(Fig. III - 4). with commercial 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 mgs/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 - 5

A

B

C

D

E
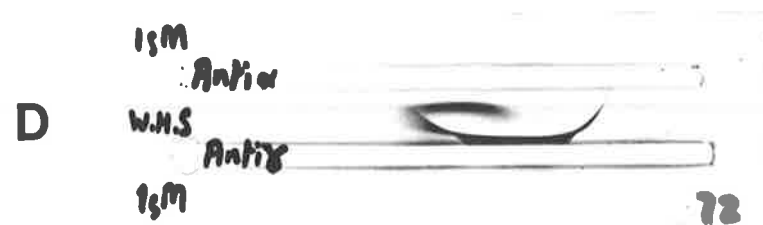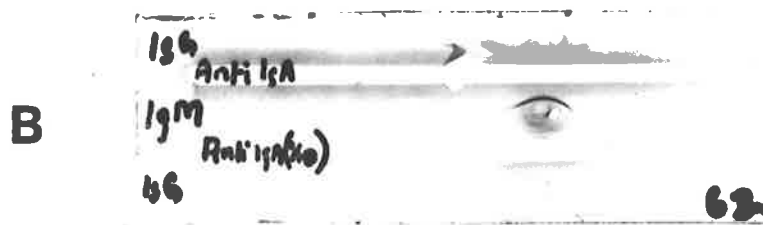
FIGURE III - 6.   ANALYSIS OF ANTI IgG SERUM AND ANTI-γ
                  ANTIBODY USING IMMUNO-ELECTROPHORESIS

A	Top well		Whole human serum
		Top trough	Crude anti IgG
	Middle well		Secretory IgA
		Bottom trough	Crude anti IgG (1/10 dilution)
	Bottom well		Whole human serum
B	Top well		IgG
		Top trough	Crude anti IgG
	Middle well		IgM
		Bottom trough	Crude anti IgG (1/10 dilution)
	Bottom well		IgG
C	Top well		Light-chains
		Top trough	Anti γ
	Middle well		IgG
		Bottom trough	Anti γ (Hoechst)
	Bottom well		Whole human serum
D	Top well		Secretory IgA
		Top trough	Anti α
	Middle well		IgG
		Bottom trough	Anti γ
E	Middle well		Whole human serum
		Bottom trough	Anti γ
	Bottom well		IgM

Figure III - 6
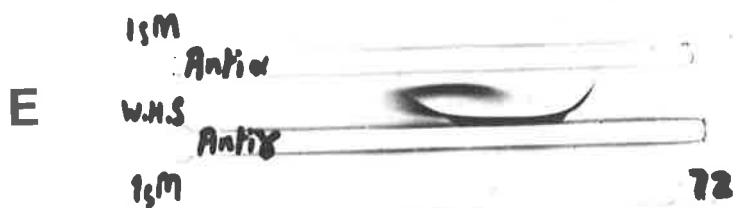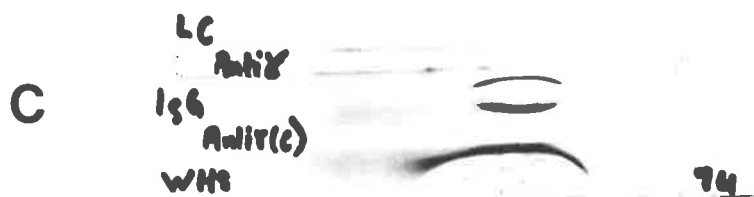
FIGURE III - 7   ANALYSIS OF ANTI LIGHT-CHAIN ANTIBODY

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 μ

         Middle well   Whole human serum

                 Bottom trough   Anti light-
                                                         chain

         Bottom well   IgM

Figure III - 7

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°-$66^{O}C$. 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 immuno-electrophoresis 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 $4^{o}C$ 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 $\pm$.

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^OC 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^OC. The vibriocidal titre of a biological fluid was defined as the dilution which gave 50% reduction in the control levelof 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^OC, the tube was held at 4^OC 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^OC to heat-inactivate complement, the serum was stored at -20^OC.

82

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.

CHAPTER 4

STABILITY OF ANTIBODY DURING STORAGE

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 $-80^{O}C$ rather than in the more commonly available $-20^{O}C$ refrigerators.

Samson and colleagues (1973) had reported that at $-20^{O}C$ 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
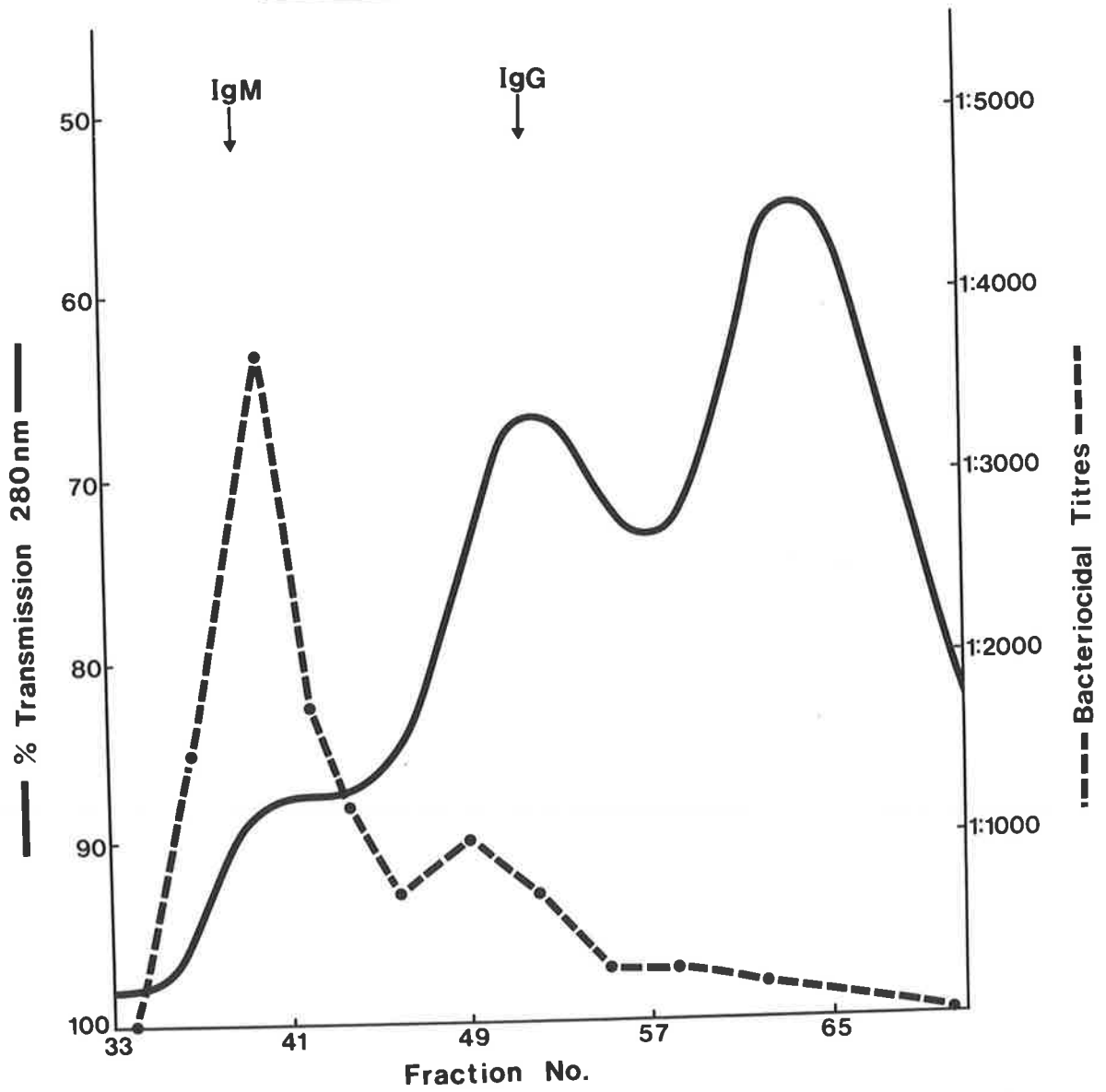
Diluted 1/100 in normal saline this gave a haemagglutination titre of 1/2048 (11 wells) to 1/8192 (13 wells) in the haemagglutination assay described in Chapter 3. (page 79). That this haemagglutination activity was in the IgG antibody class was shown by its complete resistance to 0.1M mercapto-ethanol 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 1/1000 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)

Time after seeding	Control	Intest.Aspirate $-80^{\circ}C$	Intest.Aspirate $-20^{\circ}C$	Intest.Aspirate $+4^{\circ}C$
2 days	1/1000	1/ 600	1/ 700	1/ 500
1 week	1/ 800	1/1000	1/1200	1/ 600
2 weeks	1/ 640	1/ 640	1/ 640	1/ 200
1 month	Failed	Failed	Failed	Failed
2 months	1/ 700	1/1000	1/ 900	1/ 50

TABLE IV - 2 STABILITY OF IgG ANTIBODY ON STORAGE

(Haemagglutination titres using red cells coated with tetanus toxoid)

Time after seeding	Control		Intest.Aspirate −80°C		Intest.Aspirate − 20°C	
	Wells	Titre	Wells	Titre	Wells	Titre
1 day	11	1/2048	10	1/1024	10	1/1024
4 days	12	1/4096	11	1/2048	10	1/1024
1 week	11	1/2048	10	1/1024	10	1/2048
2 weeks	12	1/4096	11	1/2048	11	1/2048
1 month	12	1/4096	11	1/2048	8	1/ 256
2 months	8	1/ 256	9	1/ 512	BG	BG

TABLE IV - 3 DECAY OF IgG ANTIBODY AT ROOM TEMPERATURE

Time at 20°C	0 hours		1 hour		3 hours		6 hours		18 hours	
	Wells	Titre	Wells	Titre	Wells	Titre	Wells	Titre	Wells	Titre
Sample(previously at −80°C)	11	1/2048	8	1/ 256	7	1/ 128	6	1/ 64	BG	BG
Control	12	1/4096	12	1/4096					11	1/2048

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° from that held at $-80^{\circ}C$ during the two months storage. There was gradual but fairly complete loss of activity of the IgM antibody held at $4^{\circ}C$ (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 $-20^{\circ}C$ and no detectable loss of activity in the sample stored at $-80^{\circ}C$. (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 $-80^{\circ}C$ has some advantage over storage at $-20^{\circ}C$ 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 immuno-globulin 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 $37^{O}C$ on a shaker. Two or three litres of this culture which usually had a density of 2×10^{9} 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 (.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 5×10^{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 ngs 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 ($4^{o}C$).

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 radio-activity 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 $\overline{V}$ - 1

Representative Binding-curve in Radio-immuno-assay
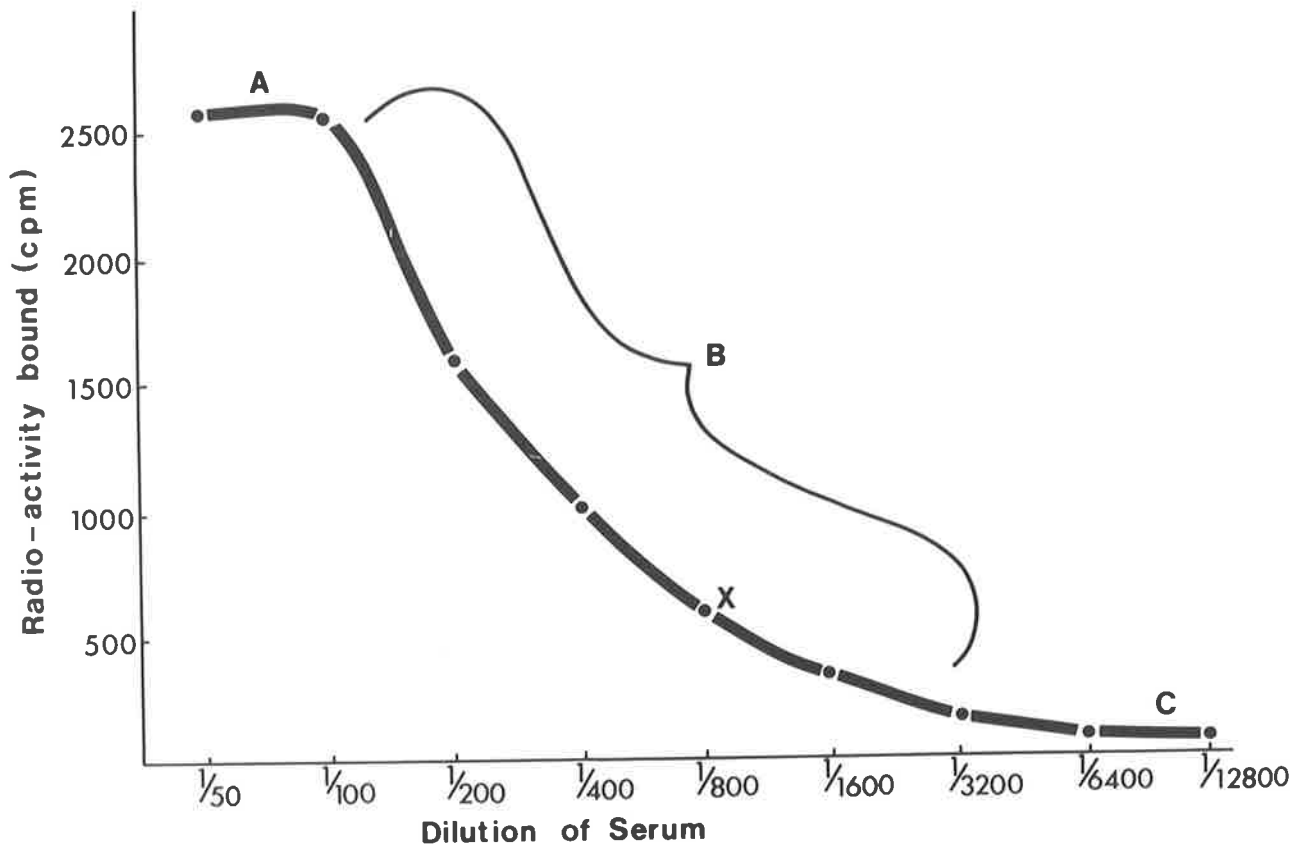
the bacteria had, prior to adding the radio-labelled antiserum (Figure V-2). The prozone probably reflects the presence of unbound immunoglobulin with no specific antibody activity remaining behind, in spite of washes to remove it. This binds some of the radio-labelled secondary antibody, and then is 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 $20^{o}C$ or at $4^{o}C$. Intestinal antibody on the other hand bound better at $4^{o}C$ than at $20^{o}C$. (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 $\overline{V}$-2

EFFECT OF WASHING BACTERIA PRIOR
TO ADDING SECONDARY ANTIBODY
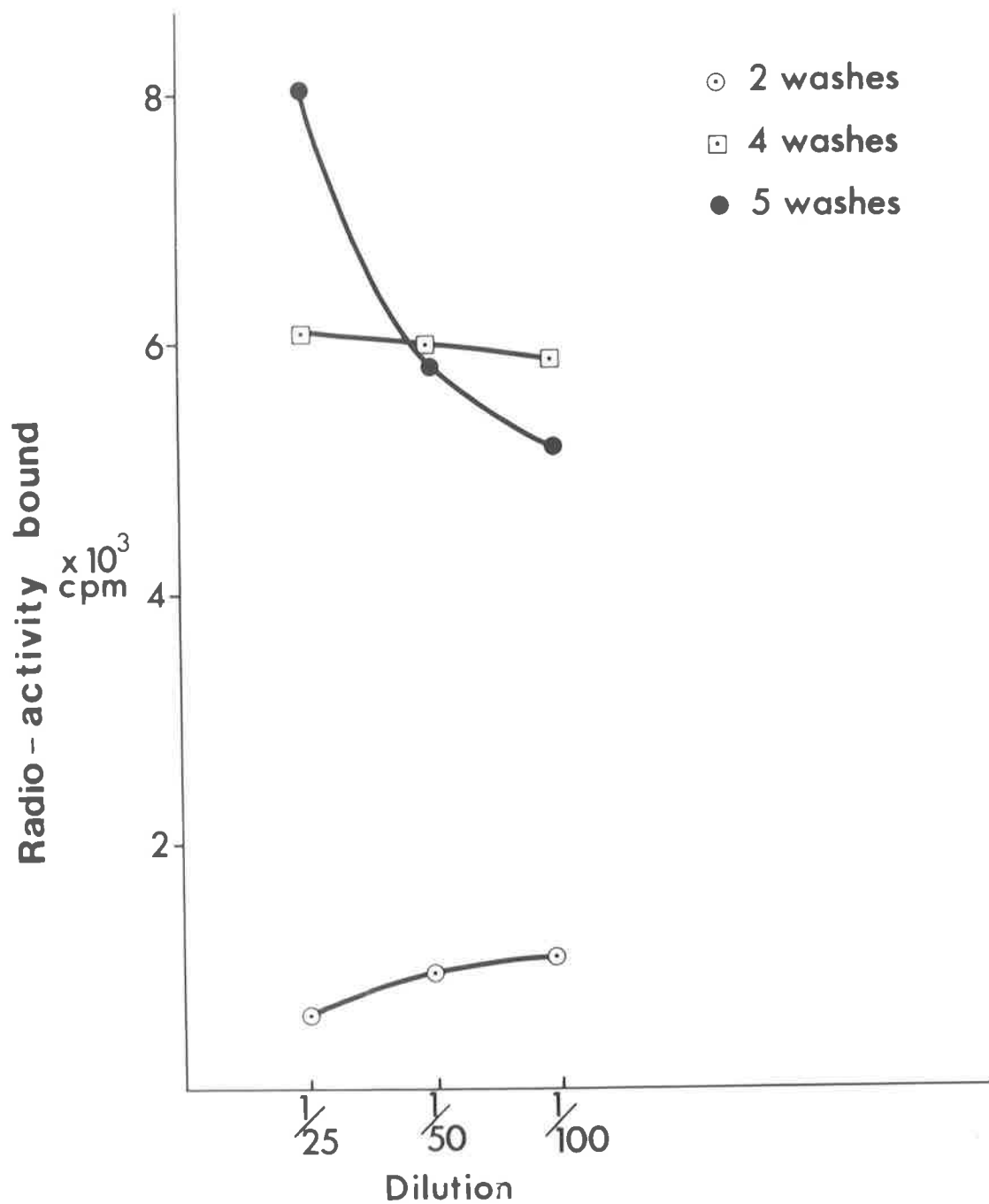
Figure $\overline{V}$-3

TIMES OF INCUBATION (PRIMARY)
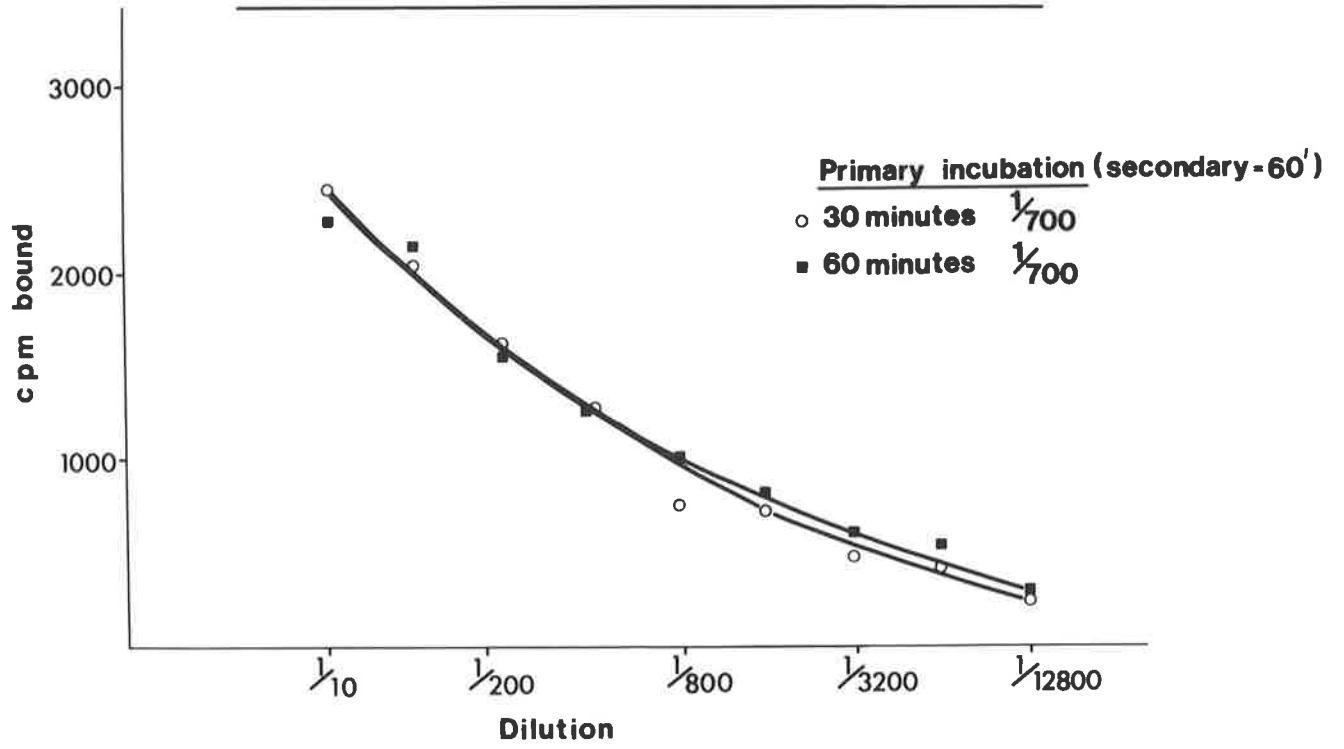
FIGURE V - 4.

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).

Time of secondary incubation.	Titre(10% binding)
● 15 minutes	1/ 260
✗ 30 minutes	1/ 480
O 60 minutes	1/ 700
◇ 4 hours	1/1400
□ 18 hours	1/7600

Figure $\overline{V}$-4

Times of Incubation (Secondary)
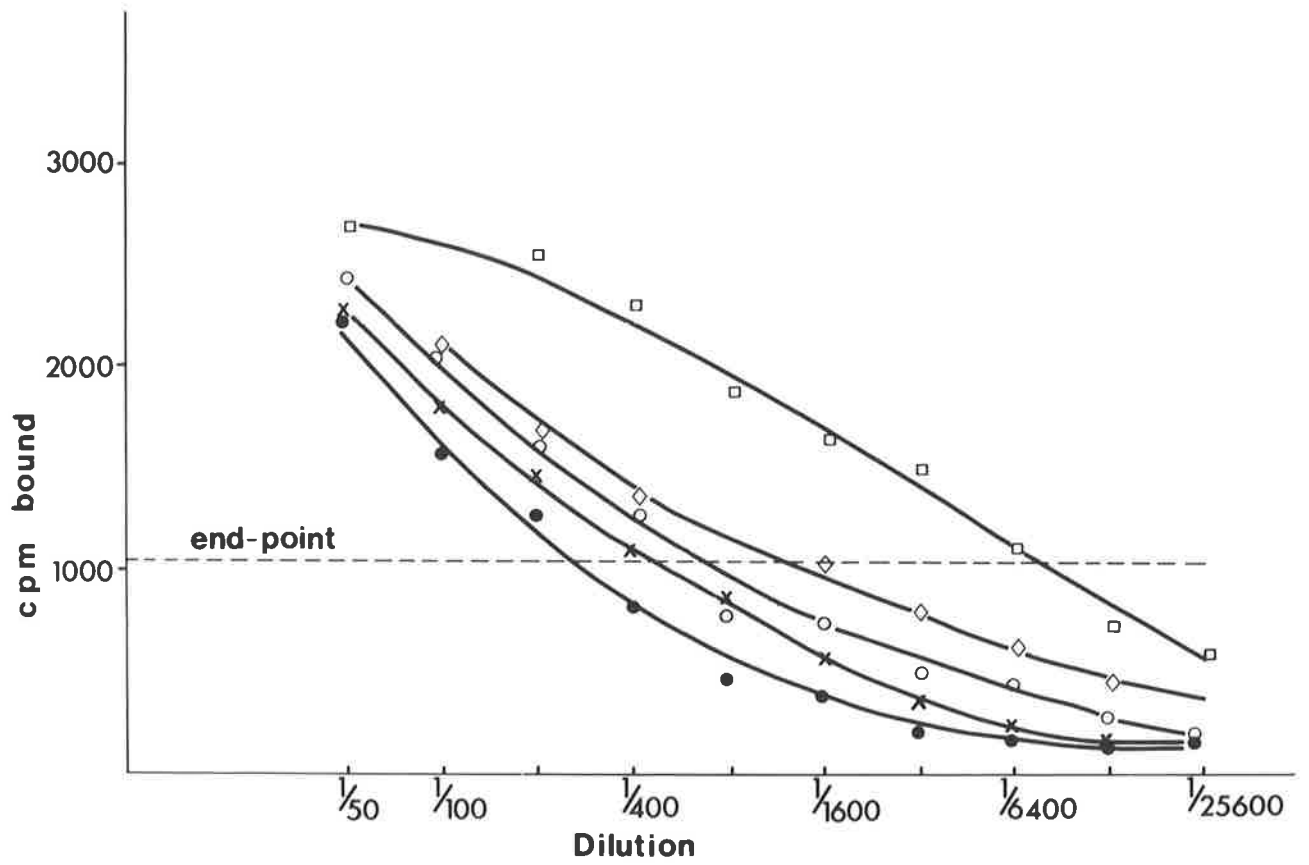
Figure $\overline{V}$-5

VARIATION IN BINDING OF INTESTINAL ANTIBODY WITH TEMPERATURE
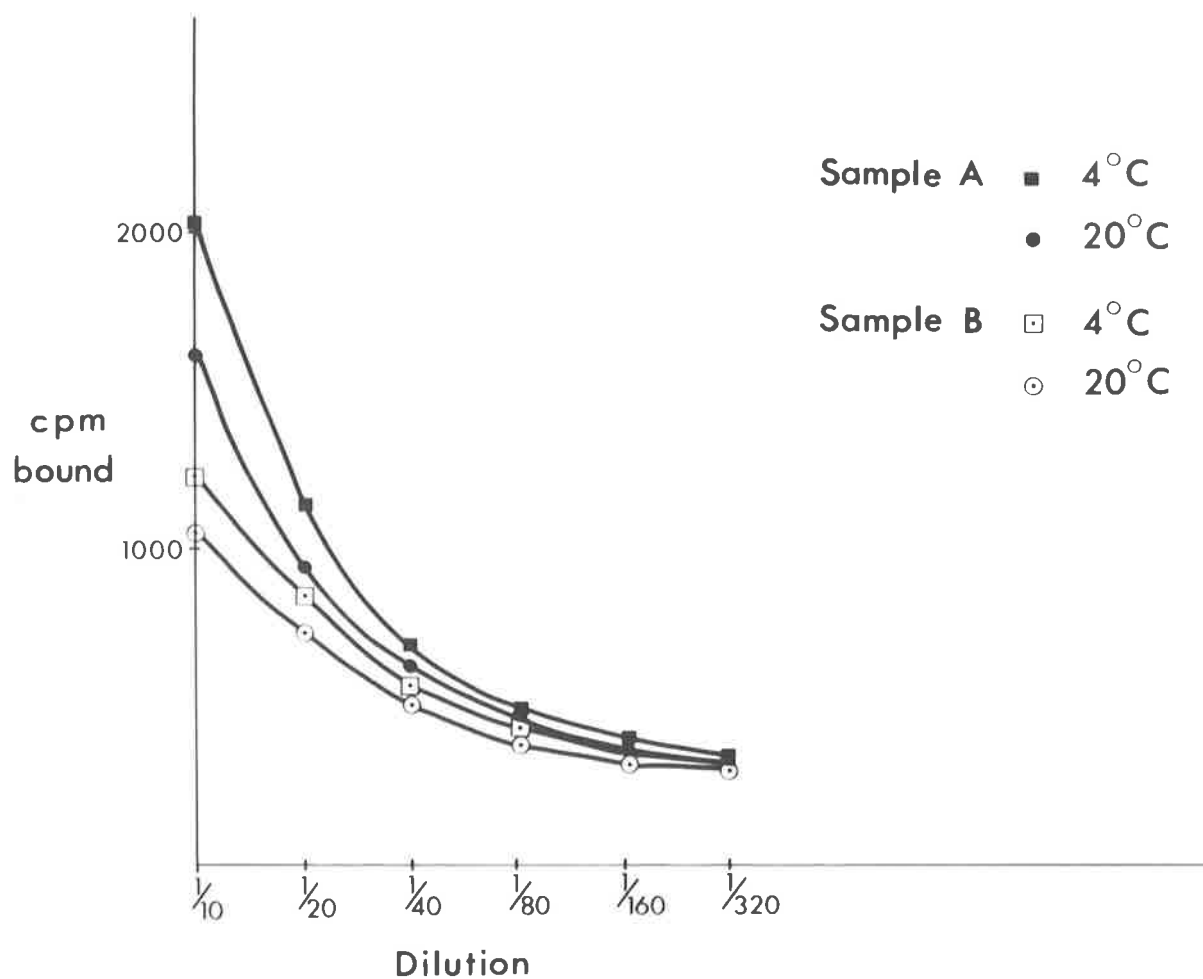
TABLE V - 1

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

Tube	Added Radio-activity (c.p.m.)	Coated tube		Uncoated tube	
		cpm bound	% bound	cpm bound	% bound
1	200	30	15%	28	14%
2	400	36	9%	39	10%
3	800	85	11%	74	9%
4	1000	107	11%	136	14%
5	2000	310	15%	339	17%
6	4000	795	20%	403	10%
7	10000	2729	27%	7982	71%
8	20000	5789	29%	10738	59%

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 $\underline{V}$-6

VOLUMES OF ANTIBODY CONTAINING FLUID

Volumes incubated		Titres
● 0·05 ml	----	1/600
■ 0·1 ml	----	1/1100
○ 0·5 ml	----	1/5600
□ 1·0 ml	----	1/11500

cpm bound

6000

4000

2000

Serum Dilution

1/50   1/200   1/800   1/3200   1/12800
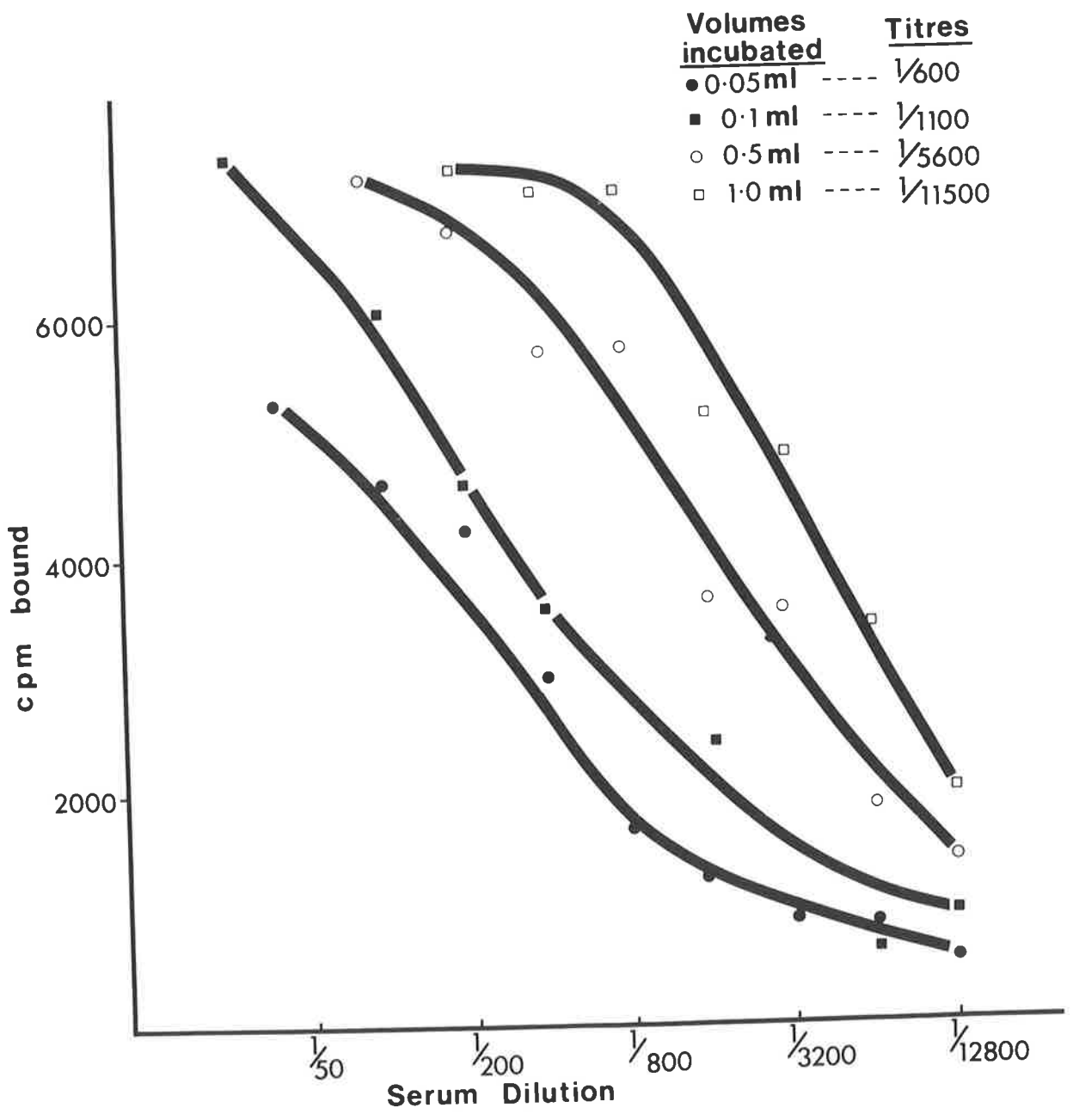
This is clearly dependent on the bacteria providing a great excess of antigen binding sites relative to primary antibody in zone B (Fig. V-1) of the binding curve.

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

FIGURE V - 7

Excess of bacteria used as an immuno-adsorbent.

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 $\underline{V}$ - 7

ABSORPTIVE SURFACE PROVIDED BY 10^8 BACTERIA IS NOT LIMITING

Bacteria (10^{10}/ml added)
- ● 10 μls
- △ 25 μls
- ⊙ 100 μls

cpm bound

2000

1000

1000   5000   20000

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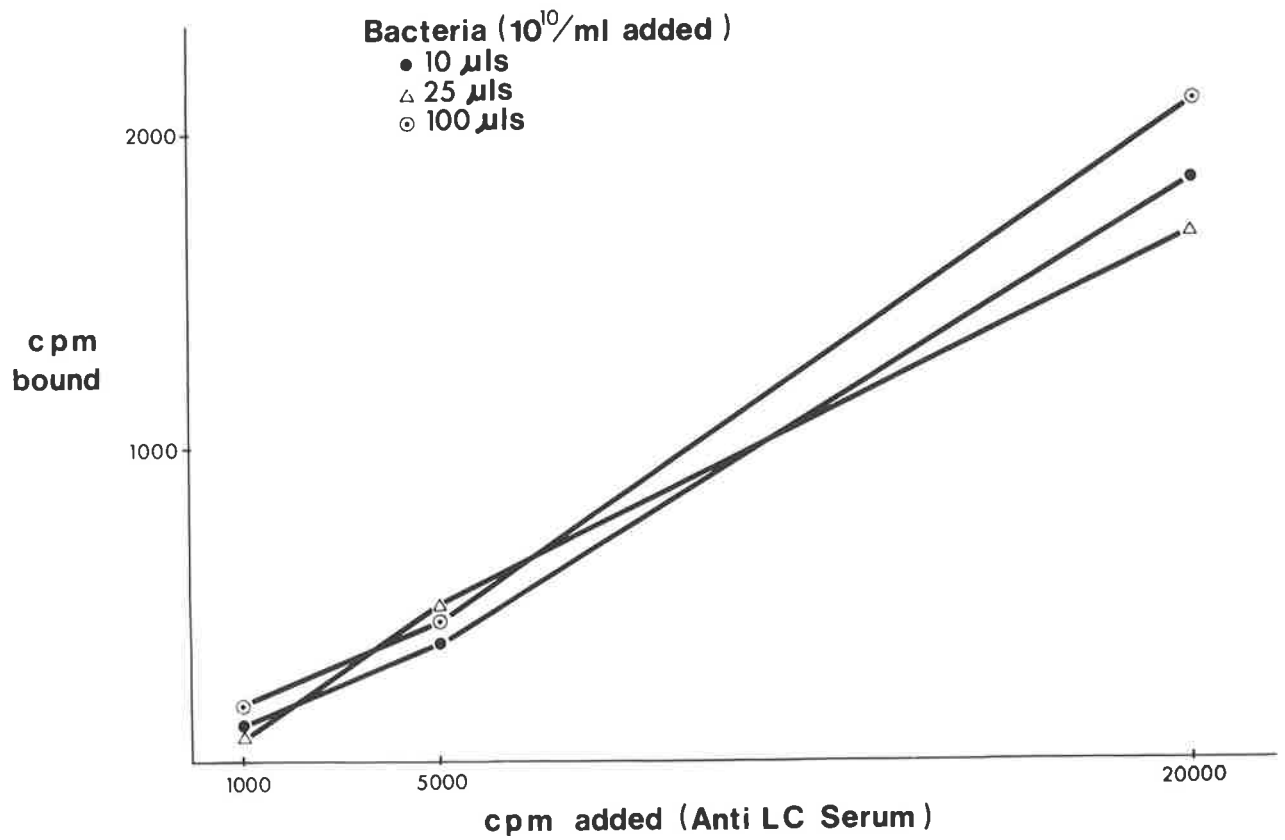
Glutaraldehyde fixation, boiling and alcohol fixation.
Experiments were done using these methods, and also
with live organisms to determine how the different
methods of fixing affected the binding of antibody
from an immune serum.

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 $100^{O}C$ on a water bath for one
hour, cooled, and held at $100^{O}C$ 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 $4^{O}C$ 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 $\underline{V}$-8

Different Preparations of Bacteria

Series	Bacterial Preparation	Titre
● A	Live	1/5000
■ B	Glutaradehyde fixed	1/6400
○ C	Boiled	1/5000
□ D	Ethanol fixed	1/2400
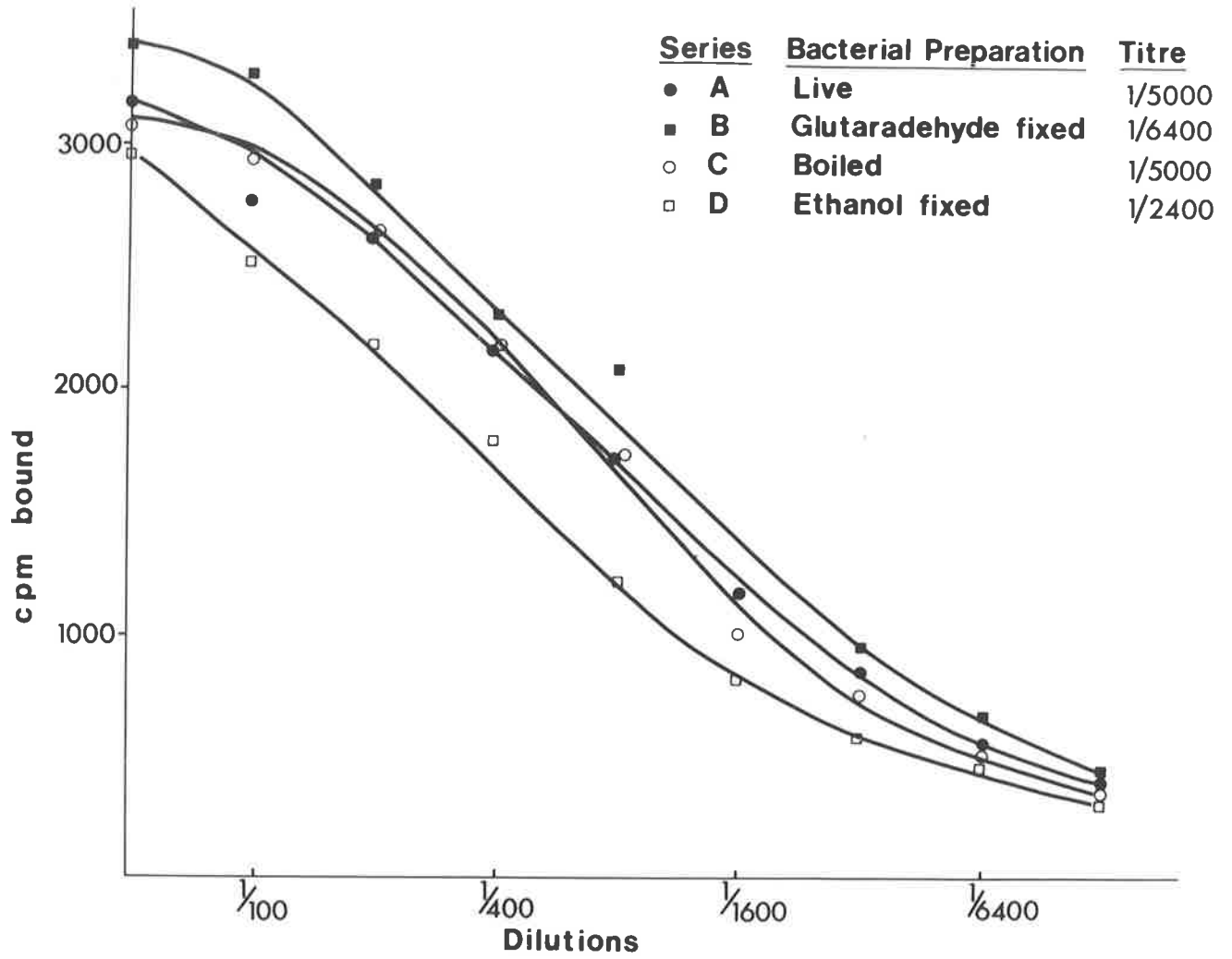
In the original description of the assay (Nielsen, Parratt and White 1973) the need to wash off antigen that went into solution before using the micro-organisms each time was emphasised. Though preliminary experiments did not confirm that this was necessary when using glutaraldehyde fixed bacteria in 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 Citro-bacter, 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-MURIUM INFECTION.

	Absorbed with S.typhi-murium	Absorbed with E.coli	Absorbed with V.cholerae	Unabsorbed
Titrated vs.S.typhi-murium	110	1100	1800	1100
Titrated vs.E.coli	170	120	200	240
Titrated vs.V.chol.	110	120	160	120

TABLE V - 3

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

Organism	O Antigens	H Antigens
Salmonella typhi-murium	1,4,5,12	i: 1,2
Salmonella senftenberg	1,3,19	g [s]t
Salmonella newport	35	f,g
Salmonella adelaide	6,8	eh:1,2
Citrobacter	5,12	

Figure $\underline{V}$- 9

Serum from a patient with Salmonella - typhi - murium gastro - enteritis
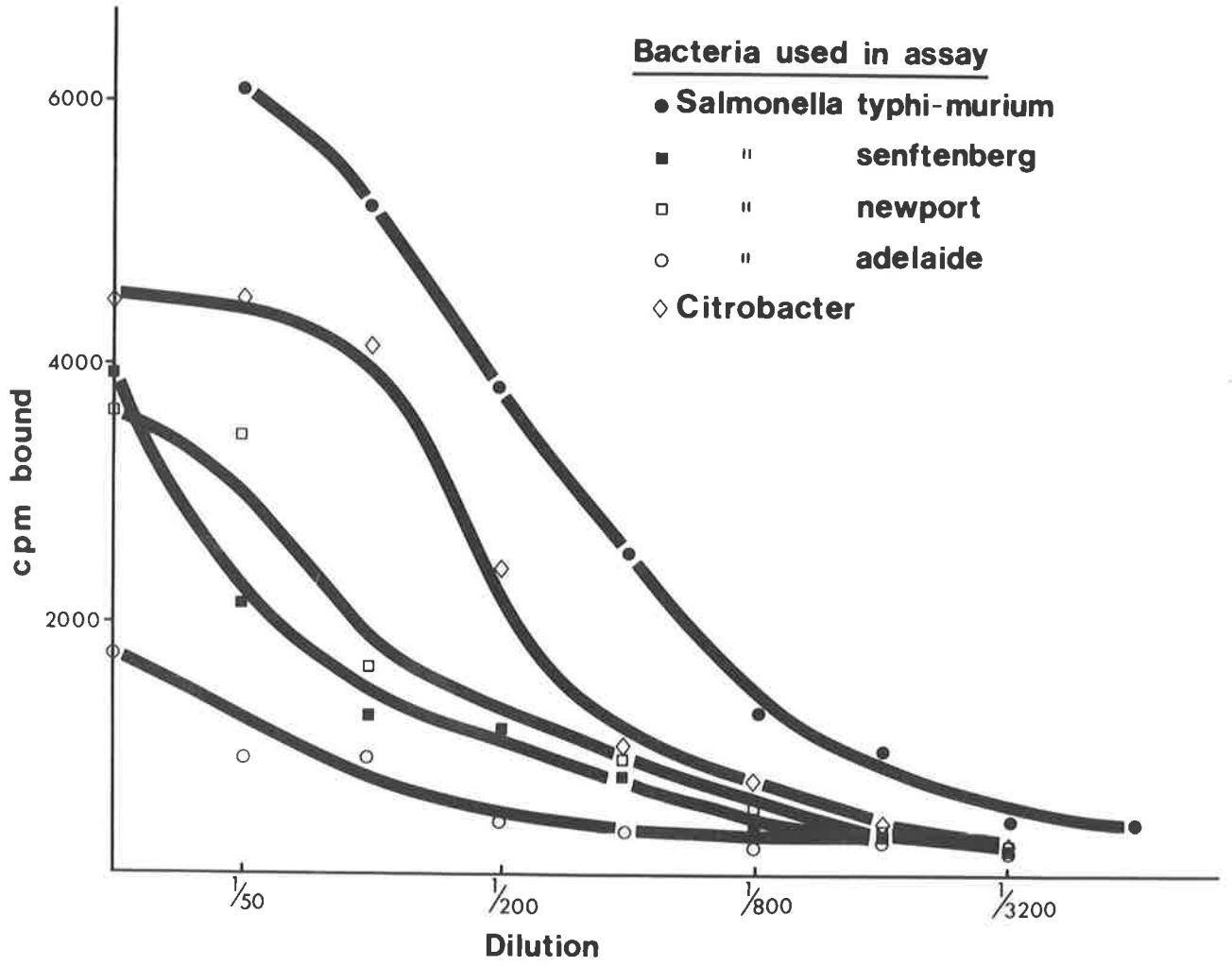
Figure $\overline{V}$-10

Serum from a patient with Salmonella newport gastro-enteritis
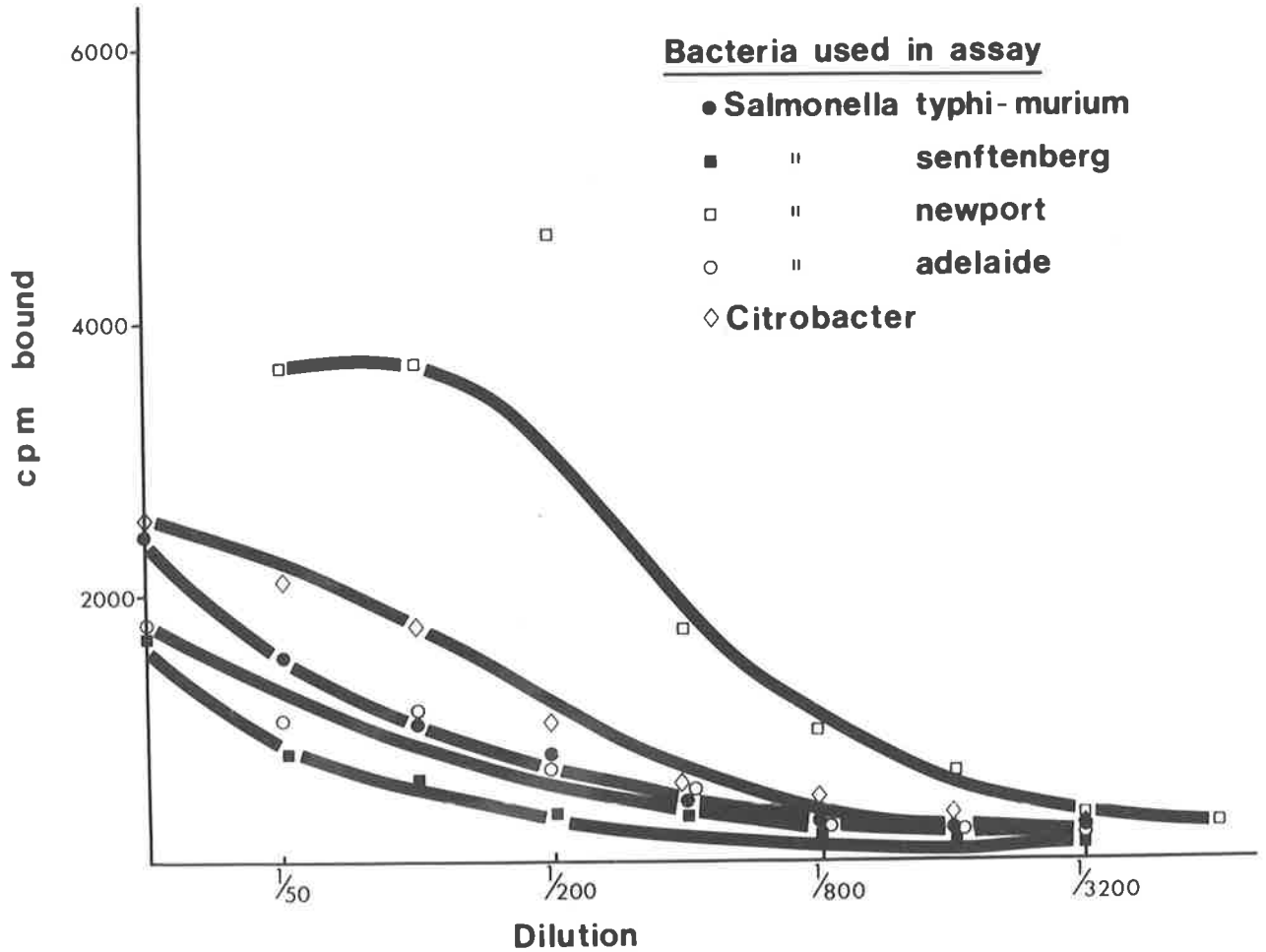
(Rowley -1977). As seen from the figures the titres of
antibody demonstrated by the different organisms closely
reflect the degree with which the antigens of the
infecting strain are carried by the organisms used in
the assay.

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 $\overline{V}$-11
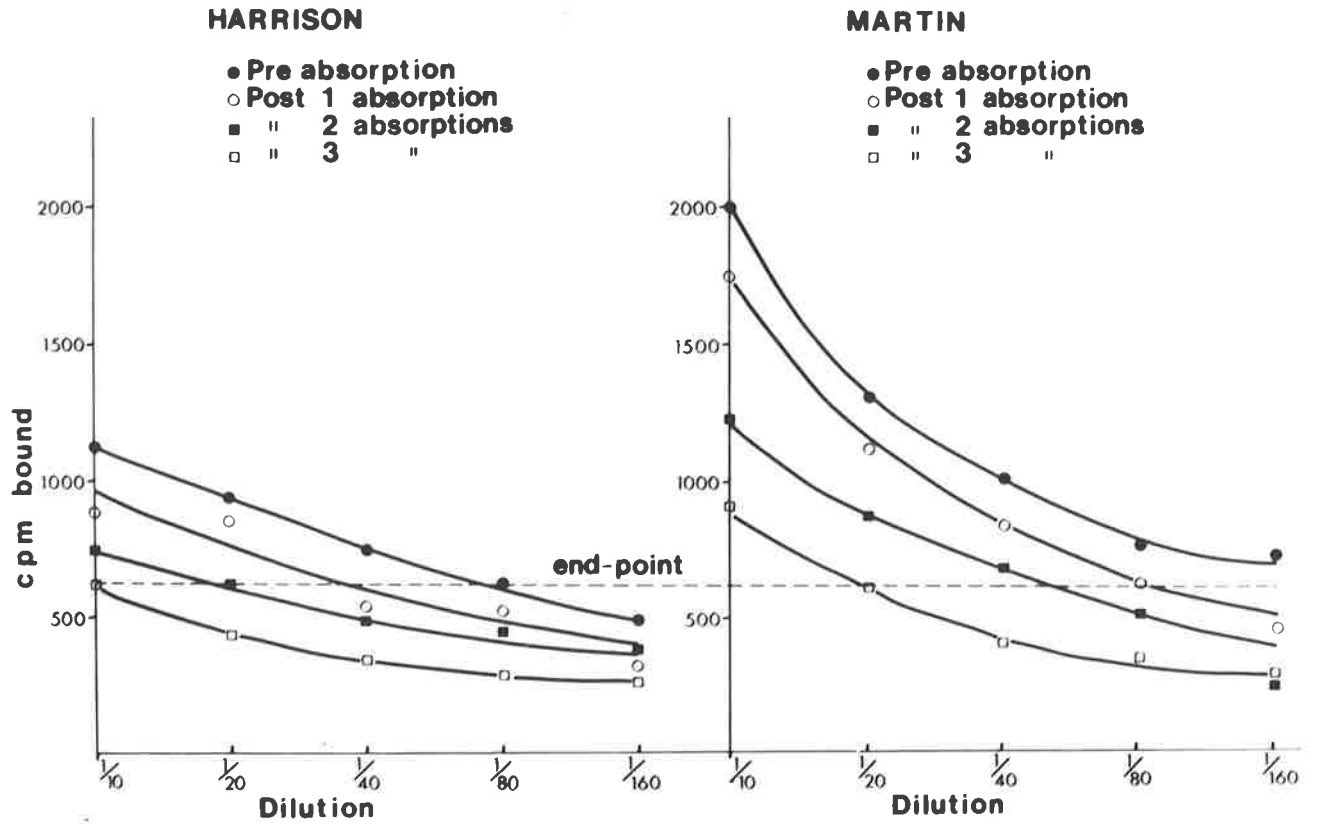
concentrations of serum. If it was due to antibody, the antibody could be either 'natural antibody' (see page 2) or antibody from a previous mild infection.

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 µl 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 I_{125} 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_{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 $\underline{V}$-12

Maximum binding of Secondary Antibody

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

⊙
•) Controls
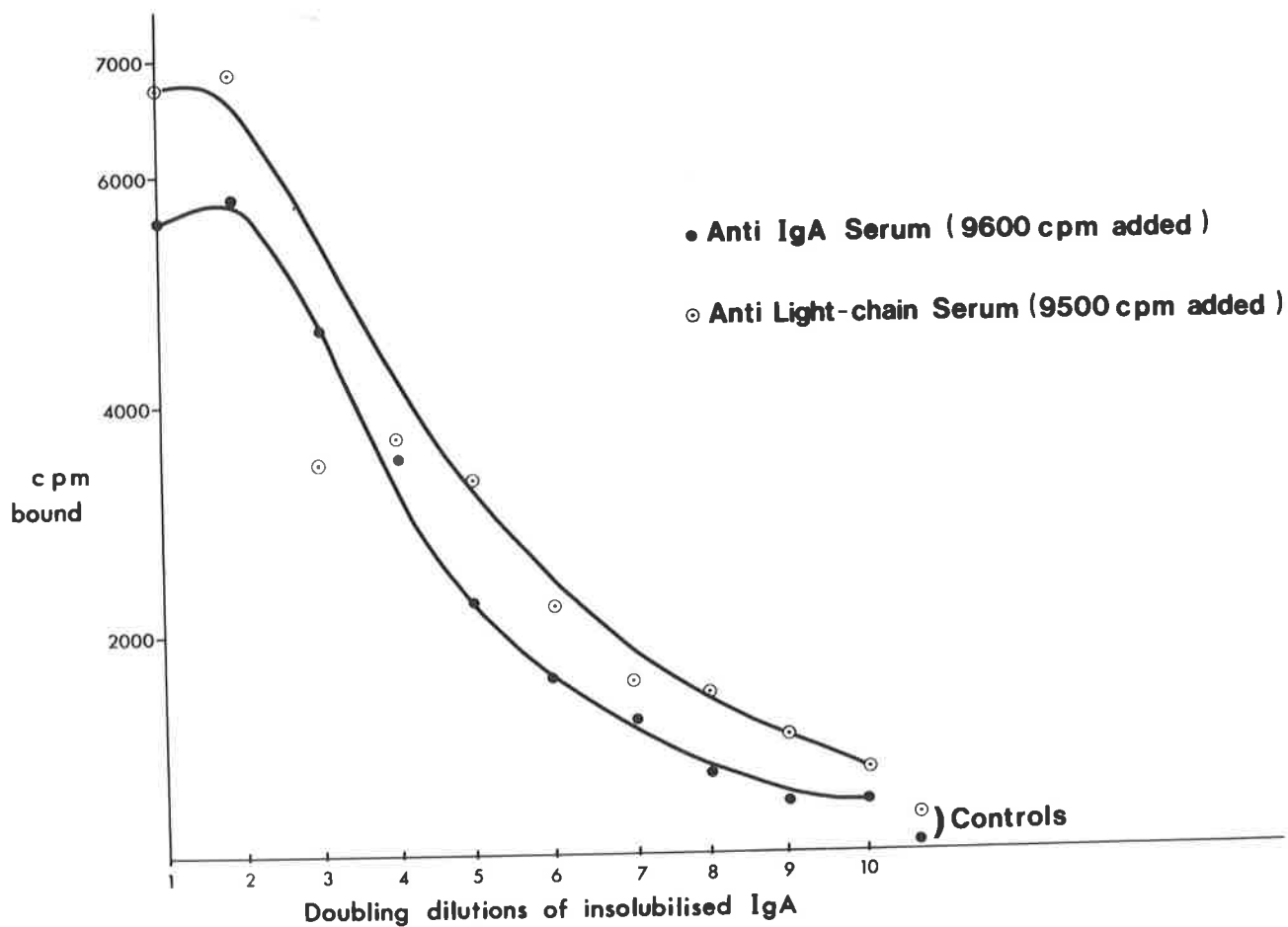
Saturation of Antigenic Surface of Primary Antibody by
Secondary Antibody.

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.

FIGURE V - 13

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 $\overline{V}$-13

SATURATION OF PRIMARY ANTIBODY BY SECONDARY ANTIBODY
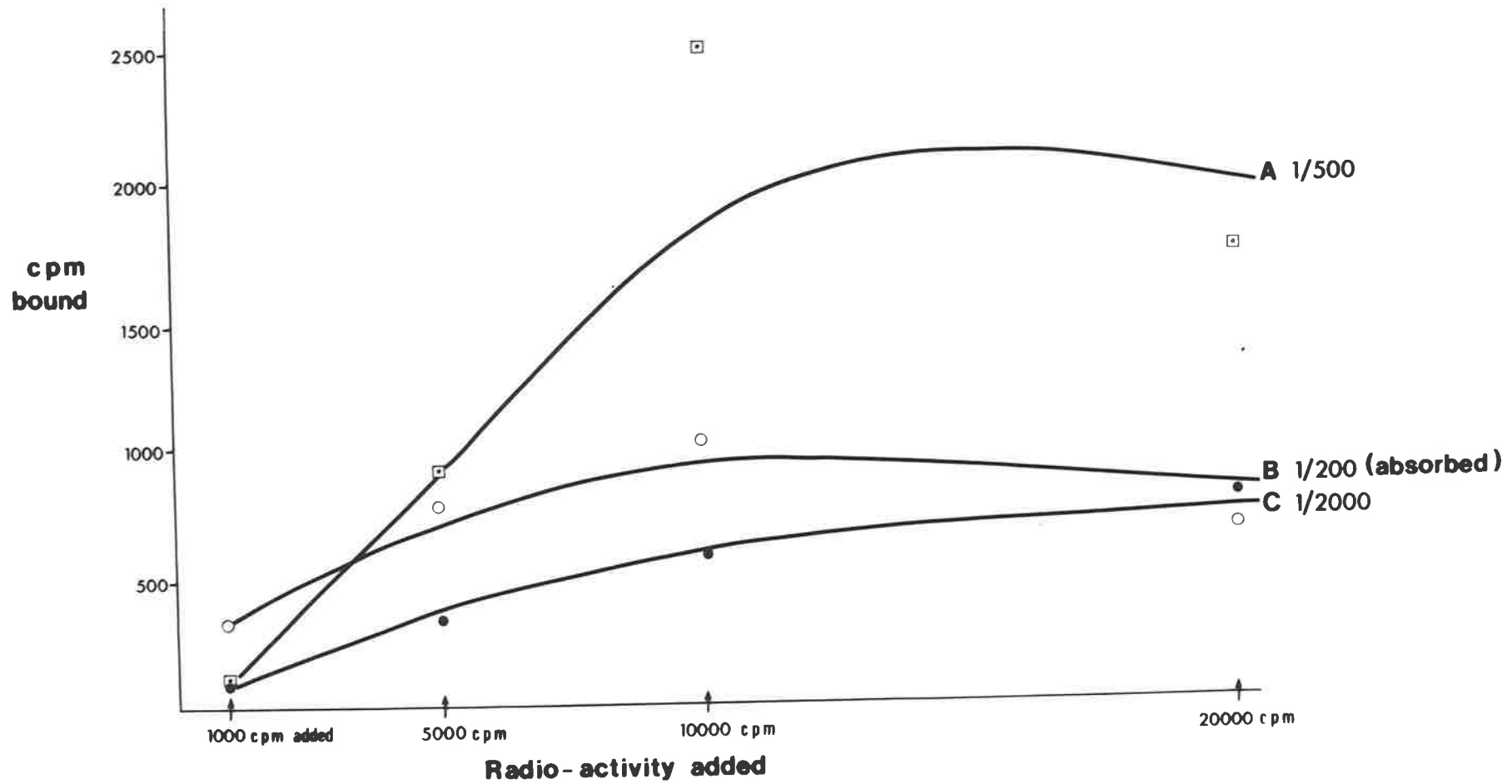
A similar result was obtained with another serum in which series A was diluted 1/100, B 1/200, C 1/400, D 1/800 and E 1/1600. Each series had 6 tubes. Anti LC antibody was added in increasing amounts to each tube (from 18.7 ngs and 4350 cpm in tube 1 to 1233 ngs carrying 286800 cpm in tube 6). As shown 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
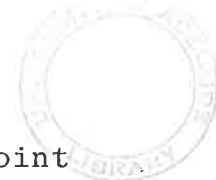
when the background was low (0.5%). Also as this end-point tended to be on the flattened part of the sigmoid curve (zone C), this led to slight variations in counts giving great apparent differences in 'titre'.

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 $\overline{V}$-14

SATURATION OF PRIMARY ANTIBODY BY
SECONDARY ANTIBOBY
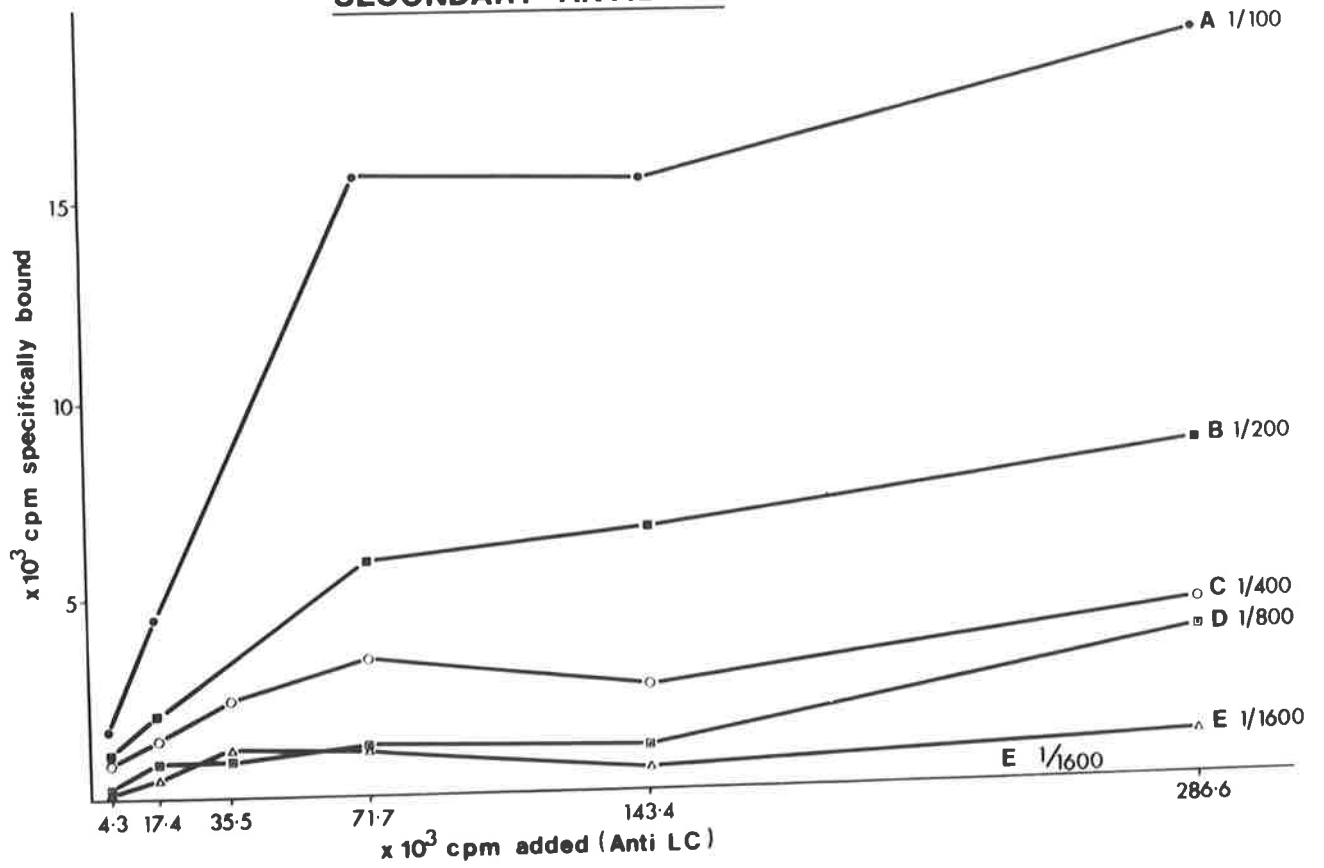
Figure $\overline{V}$ - 15

Identical Series of a Serum with

A] 10ngs (2000 cpm) secondary antibody added

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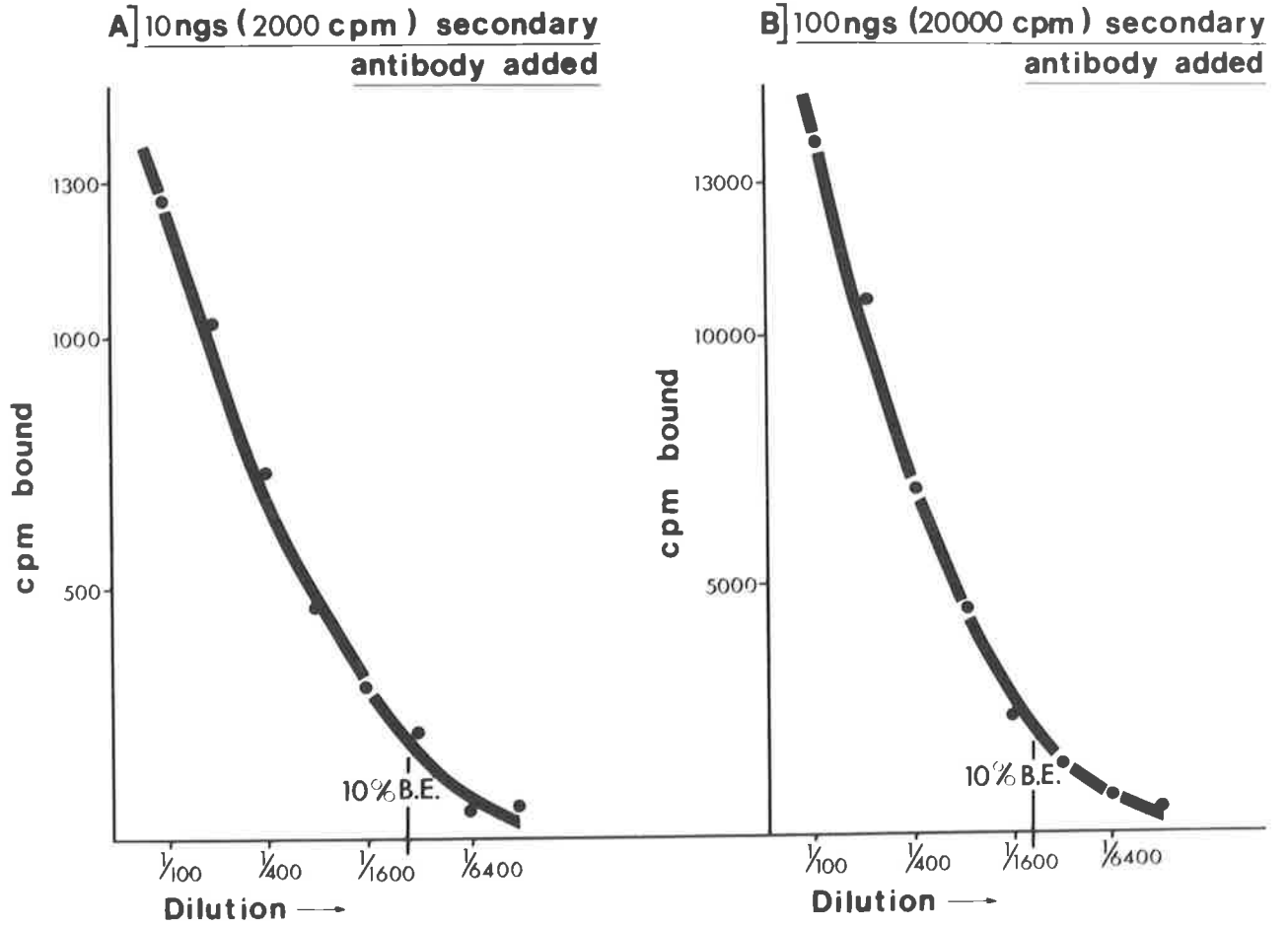
FIGURE V - 16

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 $\overline{V}$-16

Increasing Binding with Increasing Secondary Antibody
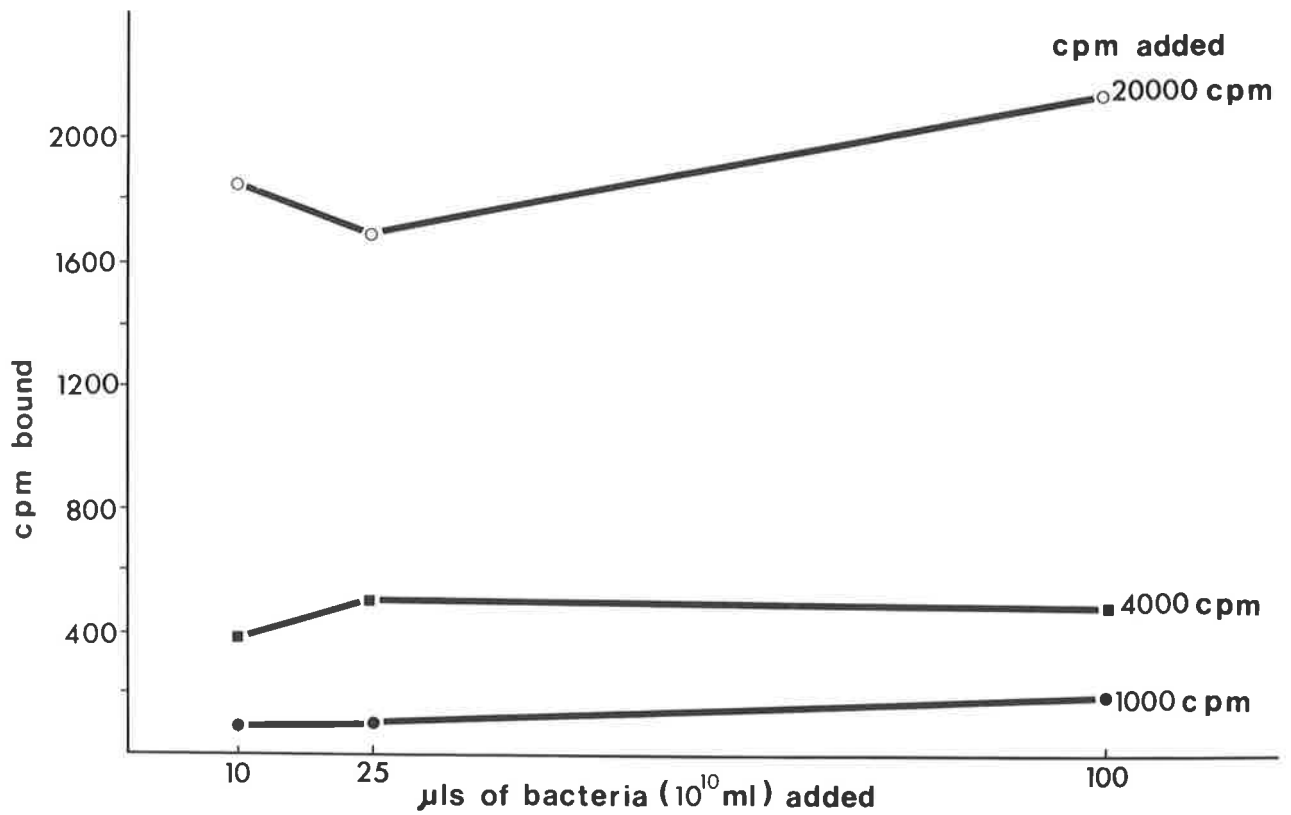
antibody with secondary antibody, a similar increase in binding of secondary antibody proportional to increasing amounts of secondary antibody added (Figs.V-7 & V-16) was seen. This was at a time when the amount of secondary antibody added was far short of that required to saturate the primary antibody 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 V - 4. VARIATION ON TITRE WITH VARYING END-POINTS IN 3 SERA

(Fig. V-17)

End-point	Titre(reciprocal)			Ratio of Titres		
% age of added radio-activity	A	B	C	A	B	C
10	1450	470	2450	.592	.192	1
15	650	280	1560	.436	.179	1
20	470	150	1250	.376	. 12	1
25	320	88	1000	. 32	.088	1
30	220	72	820	.268	.088	1
35	50	59	560	.089	.105	1

Figure $\overline{\underline{V}}$-17

Selection of a suitable end-point for assay
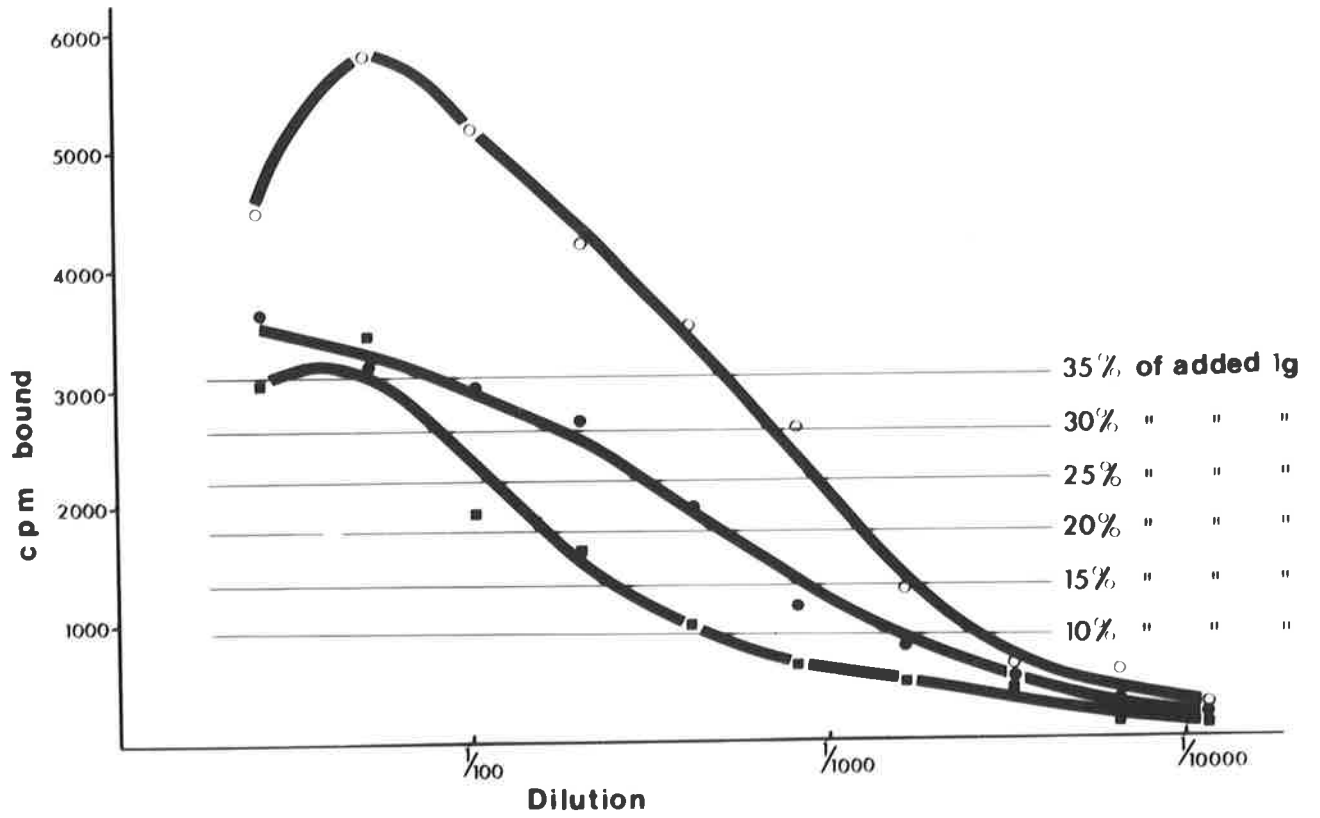
Variation Intra-Assay and Inter-Assay

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 $-20^{O}C$, 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_{280} 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 $4^{O}C$ 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

4 mls of Immune Serum

Ultra-centrifuge (65 rotor)
40,000 rpm - 1 hr.

2 mls serum	2 mls serum
+ 2 mgs L.P.S.	+ 4 mgs L.P.S.

48 hrs at 4°C
occasional mixing

made up to 8 mls
with normal saline

Ultra-centrifuge, 40,000 rpm-hr

→ supernatant assayed

Pellet

Dissolved in 0.5mls

IN NaOH

Micro-Folin Assay for protein

as opposite

Protein in pellet	=	2950	μgs	3200 μgs
Protein in L.P.S.	=	20	μgs	40 μgs
∴ Antibody in pellet	=	2930	μgs	3160 μgs

∴ Serum contains 1.5 mgs/ml Antibody

1500 ugs/ml precipitated antibody) or 15 ngs (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 immuno-
electrophoretic analysis. The specificity of these anti-
sera 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 ngs down
to 0.4 ngs) were added to one series, and pure secretory
IgA (20,000 ngs down to 40 ngs) 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 ngs of IgG. 20,000 ngs 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 quantitavely 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 $4^{O}C$ 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.

CHAPTER 6

STUDY AND RESULTS

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 gastro-enteritis 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 KOLMOGROV- SMIRNOV TEST OF EQUALITY OF DISTRIBUTIONS.

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

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.

FIGURES VI - 1 to VI - 14

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)

Figure VI-2

SERUM ANTIBODY RESPONSE (ADULTS)

Figure $\overline{VI}$-3
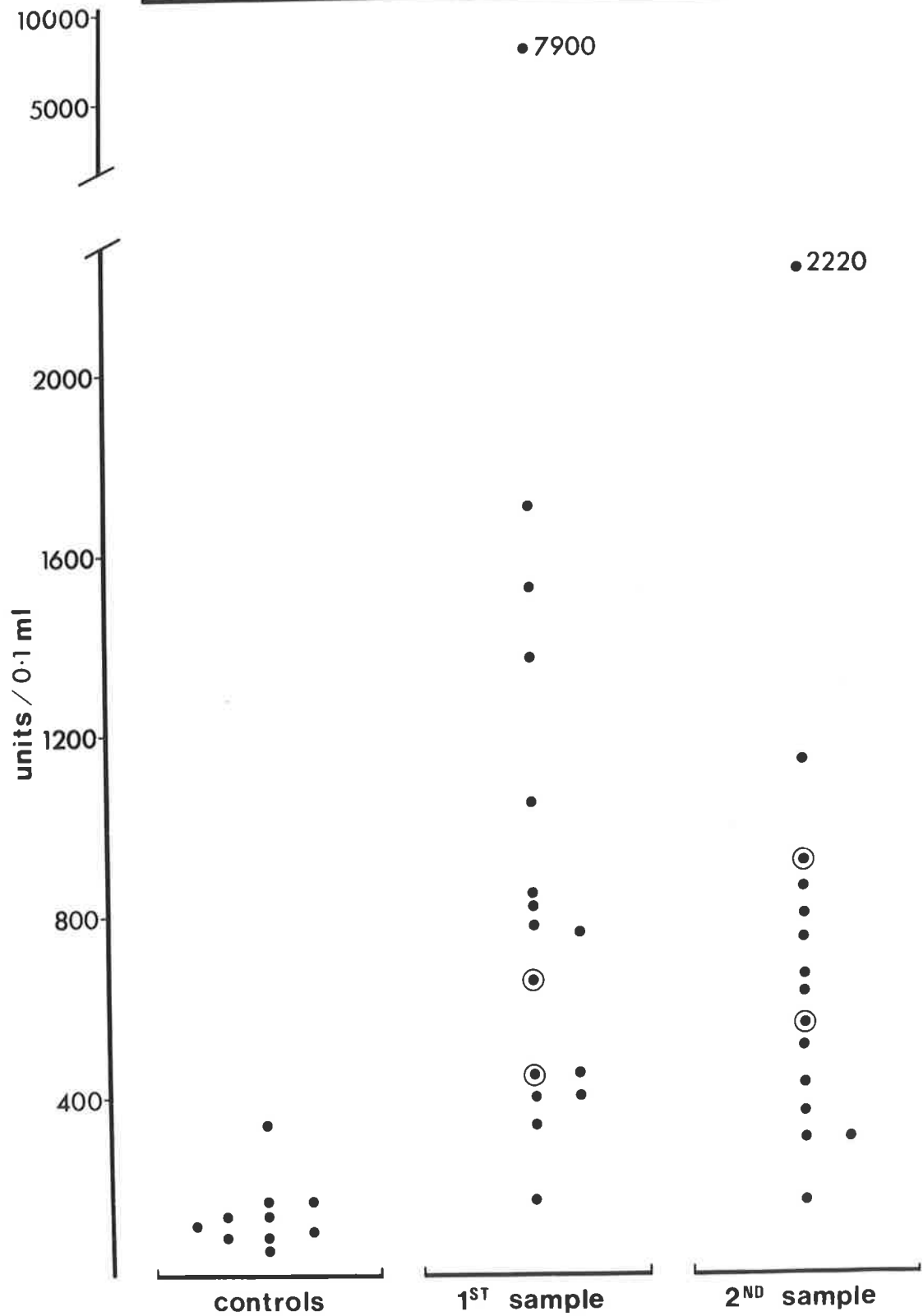
ADULTS — ASPIRATE (units/0·1 ml)
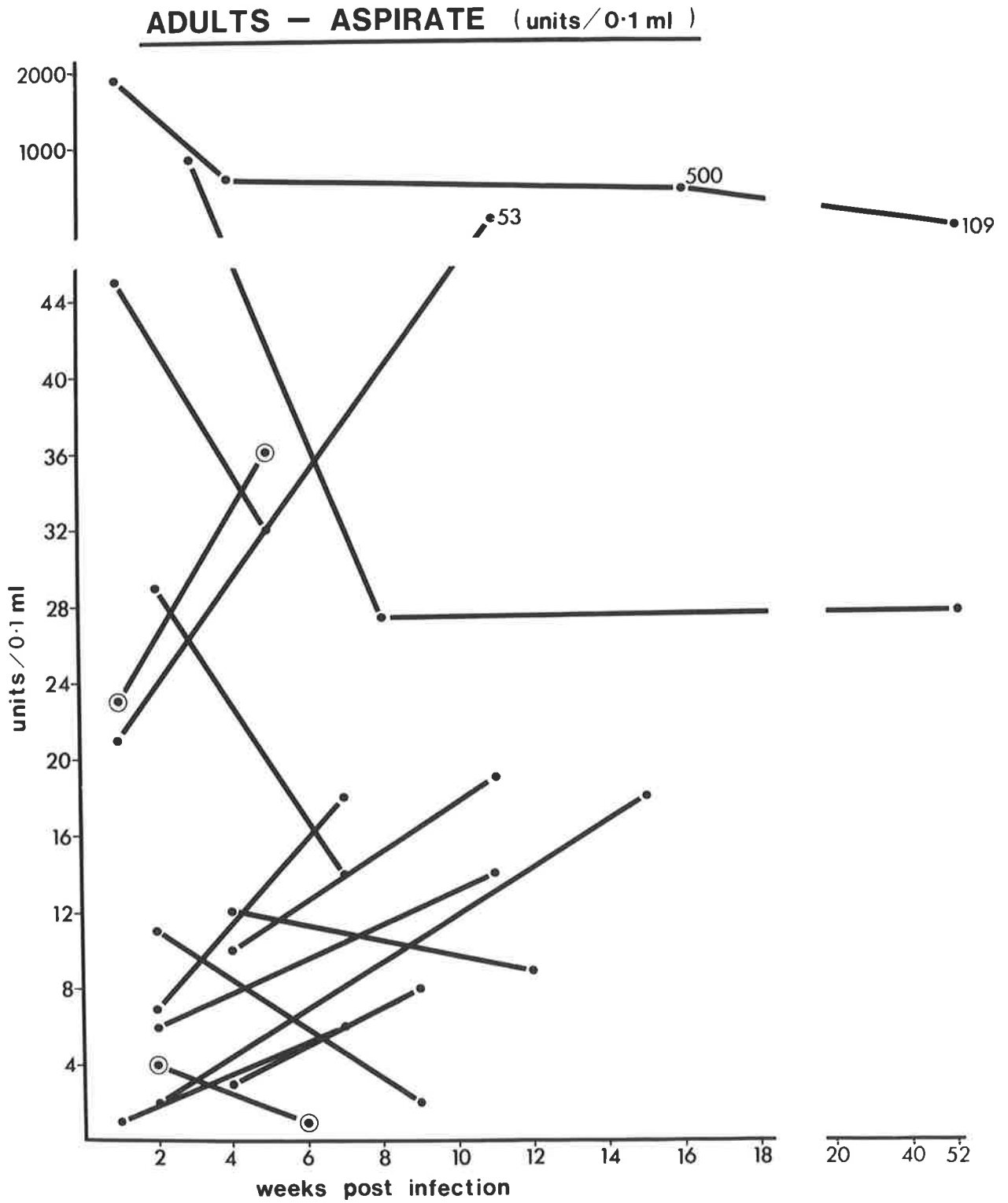
Figure $\overline{VI}$-4

ADULTS — SERUM (units／0·1 ml)

Figure $\overline{VI}$- 5

ADULTS
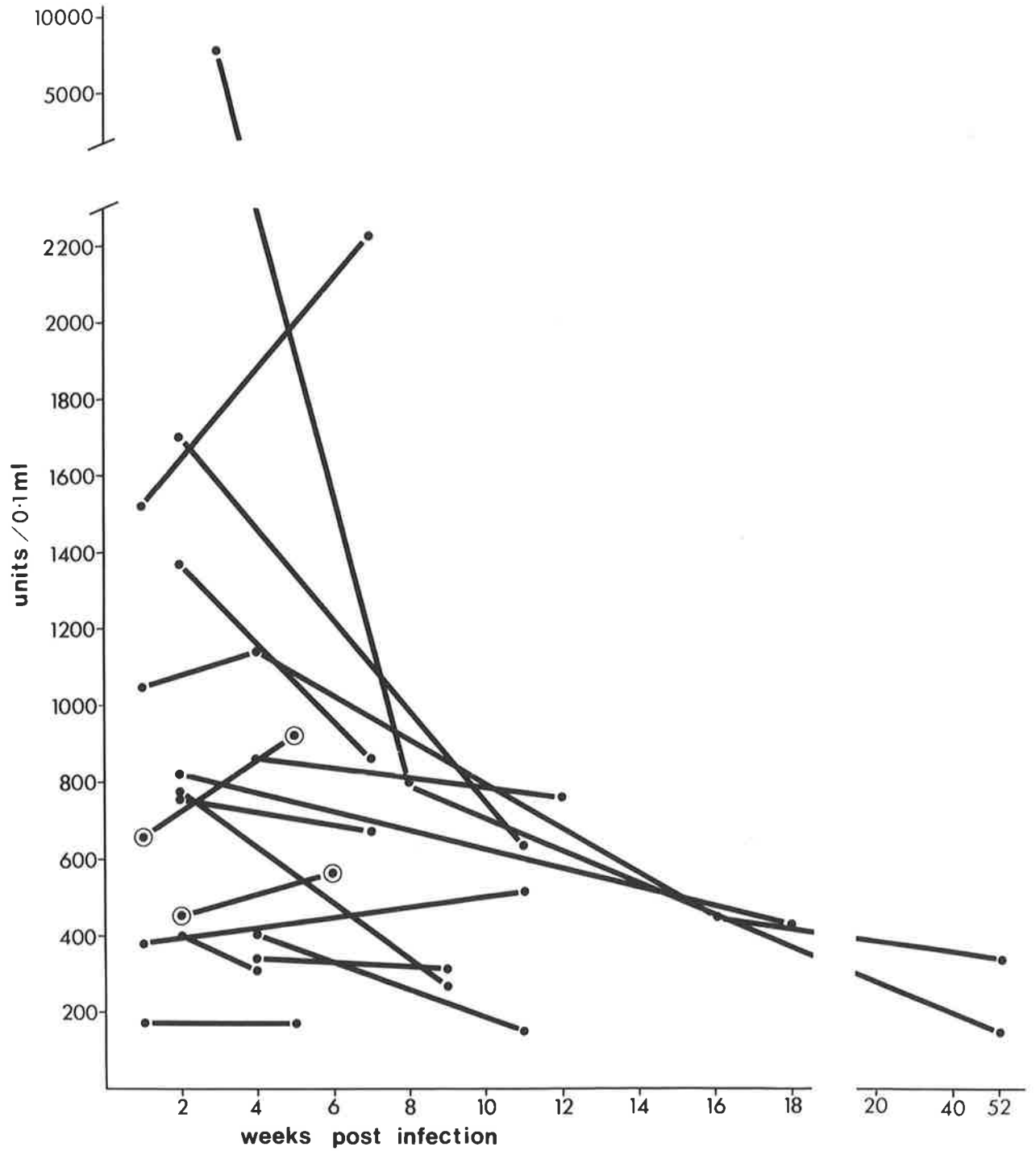
ASPIRATE （units／mg Ig ）　　　SERUM （units／mg Ig ）
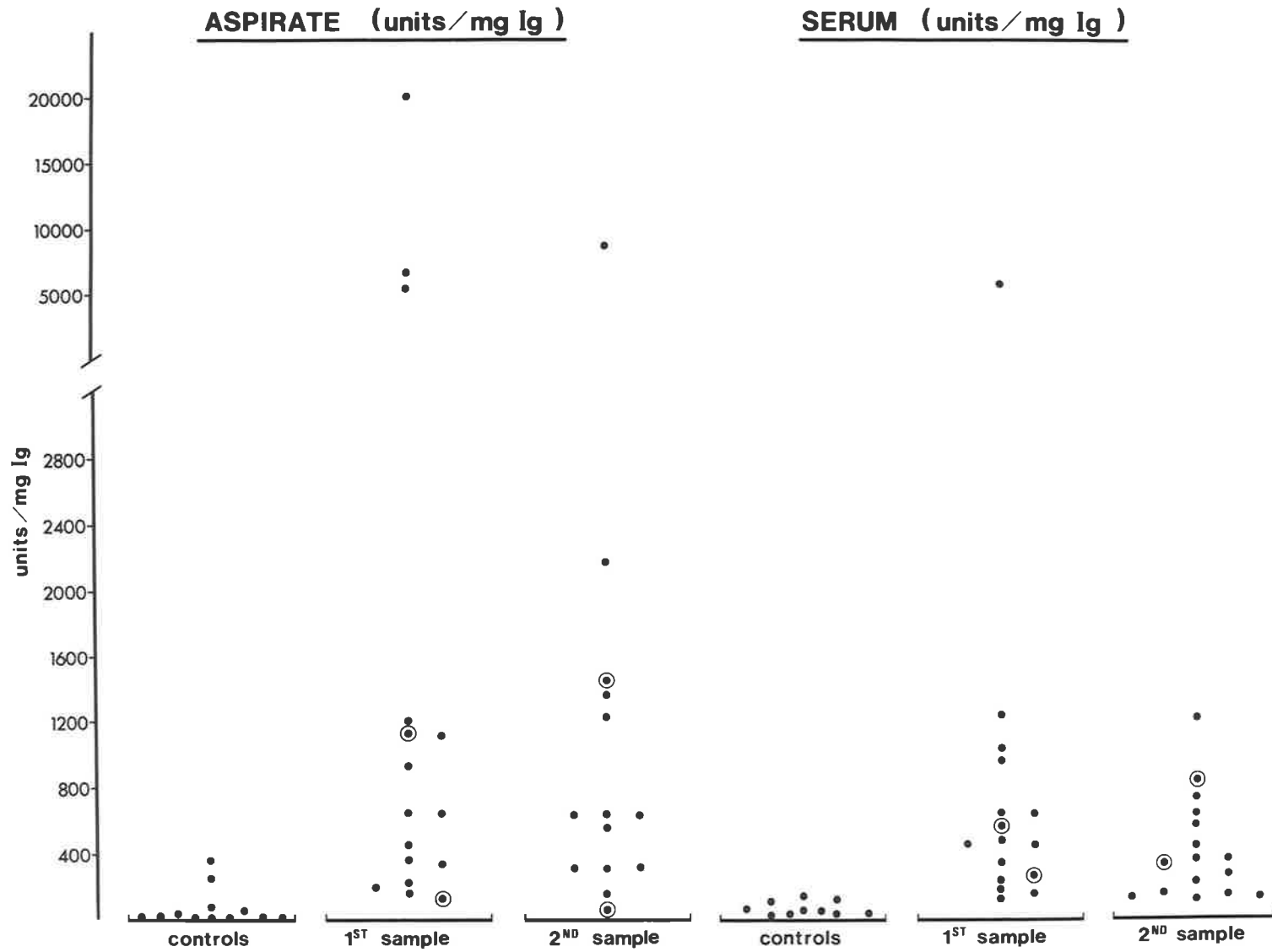
Figure $\overline{VI}$-6

ADULTS — ASPIRATE (units∕mg Ig)
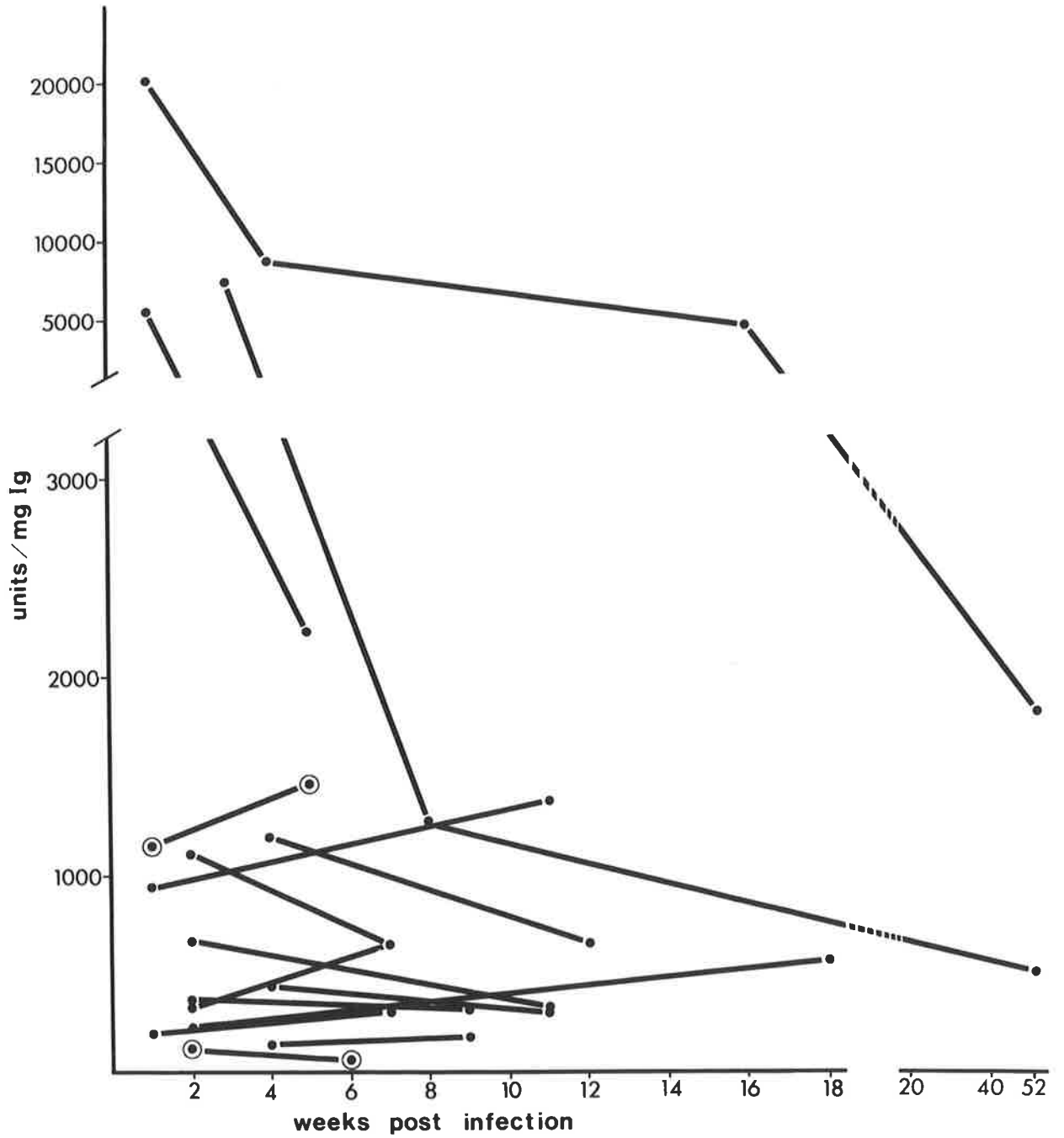
Figure $\overline{VI}$-7

ADULTS — SERUM (units ⁄ mg Ig)
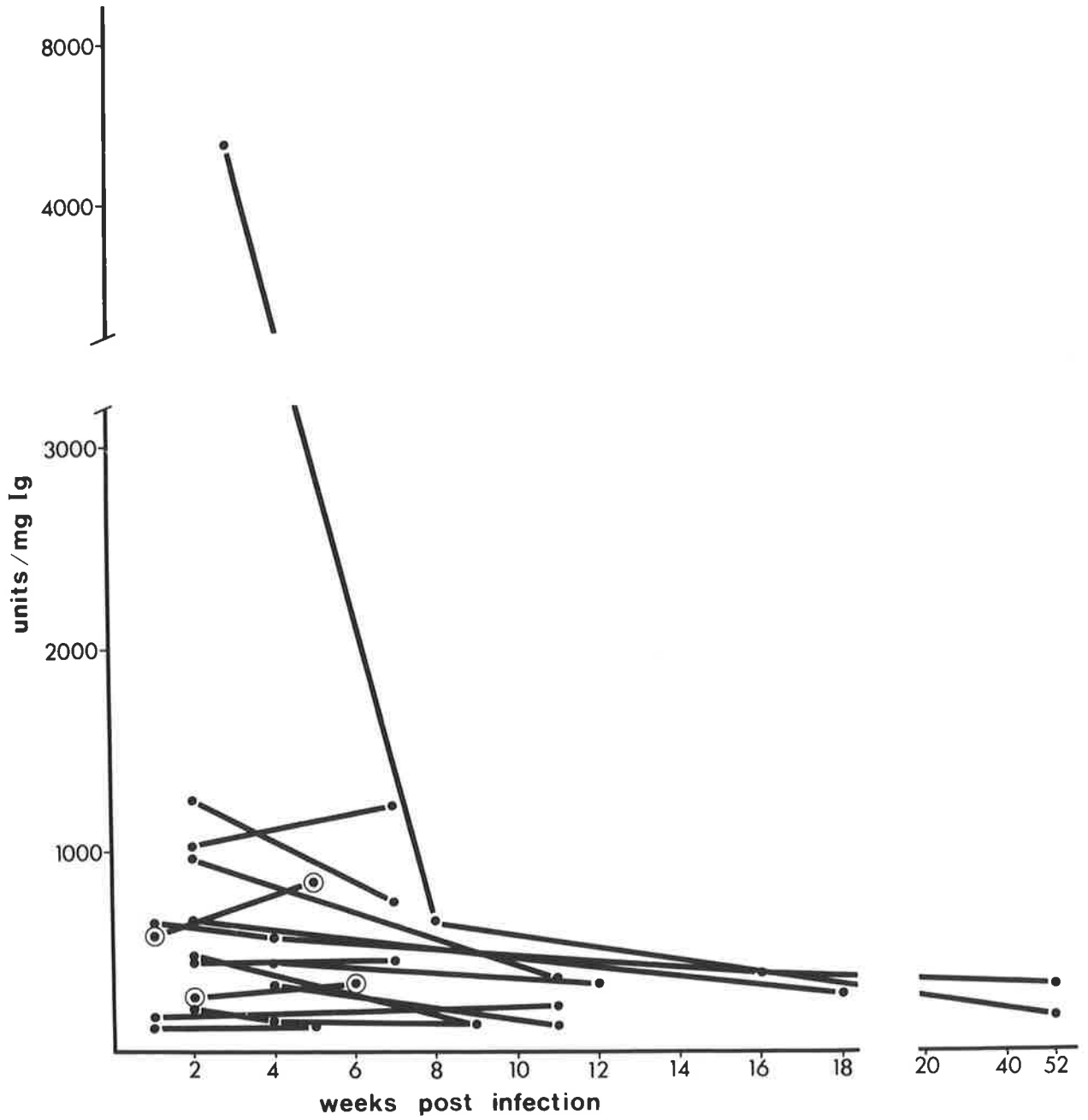
Figure VI-8

CHILDREN — ASPIRATE (units/0·1 ml)
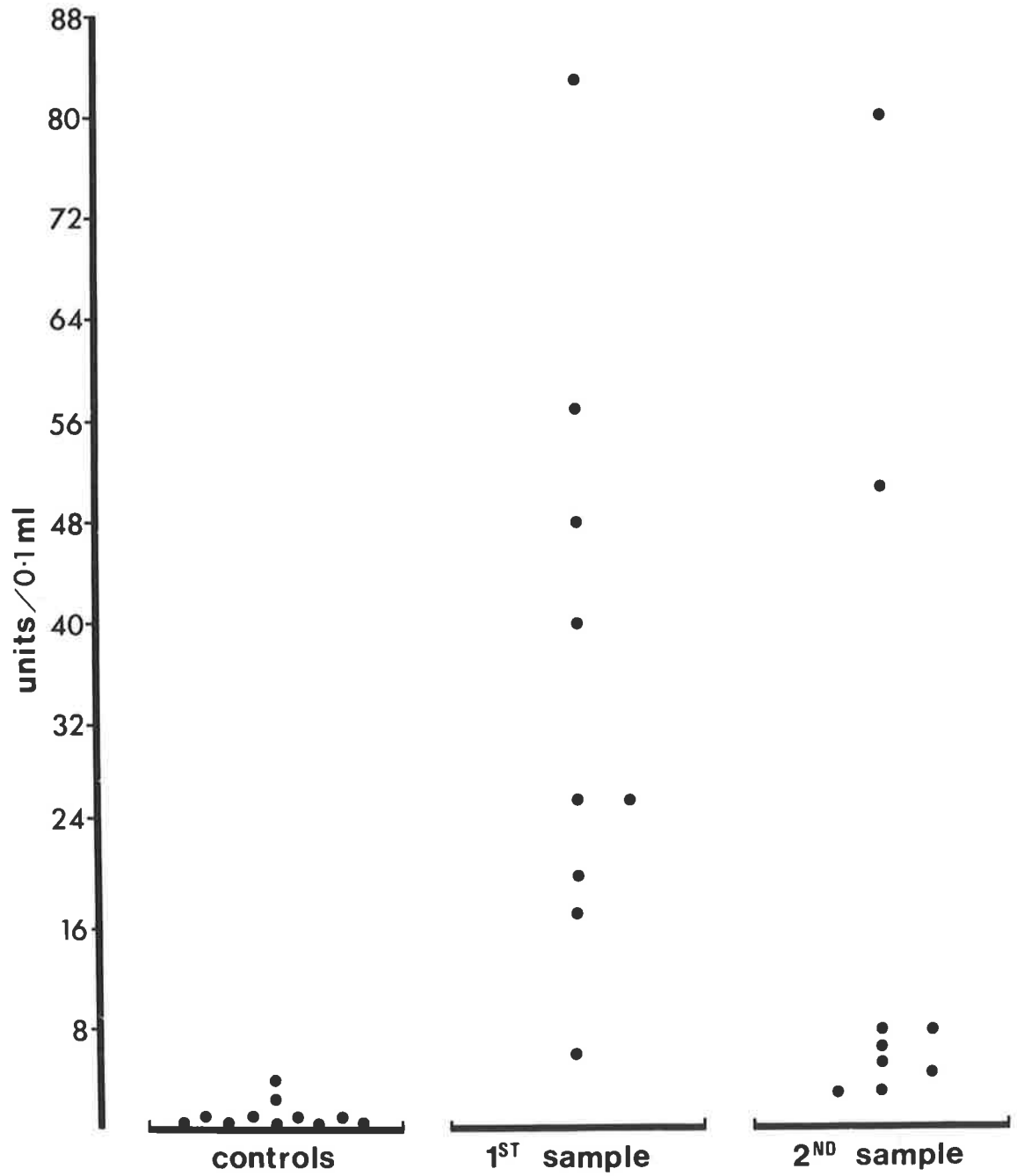
Figure $\overline{VI}$-9

CHILDREN — SERUM (units ∕ 0·1ml)
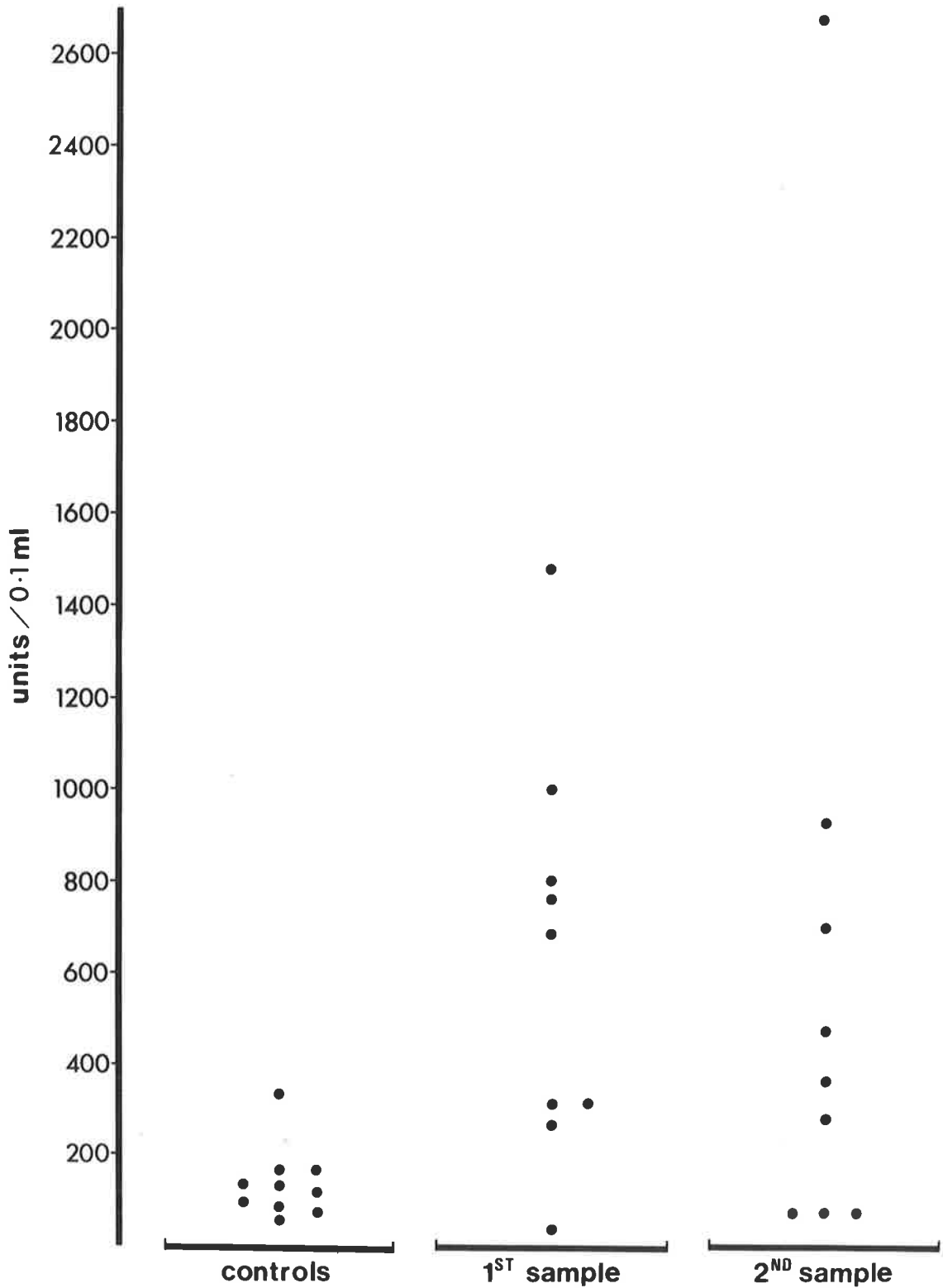
Figure $\overline{VI}$-10

CHILDREN — ASPIRATE

(units / 0·1 ml)

Figure $\overline{VI}$-11

CHIDREN — SERUM

(units / 0·1 ml)

Figure $\overline{VI}$-12

CHILDREN

SERUM (units Ab/mg Ig)

ASPIRATE (units Ab/mg Ig)
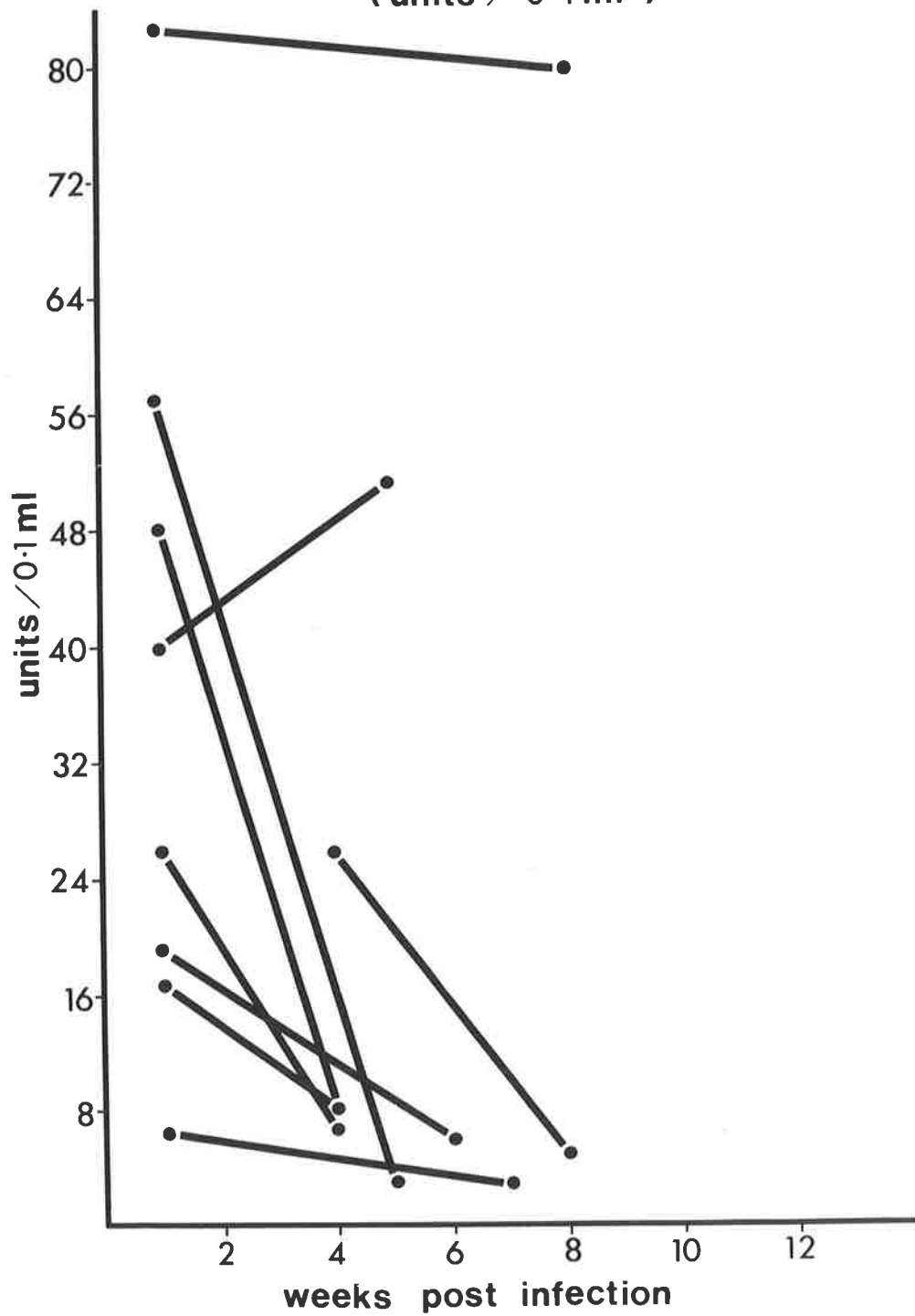
Figure $\overline{VI}$-13

CHILDREN — ASPIRATE   units/mg Ig
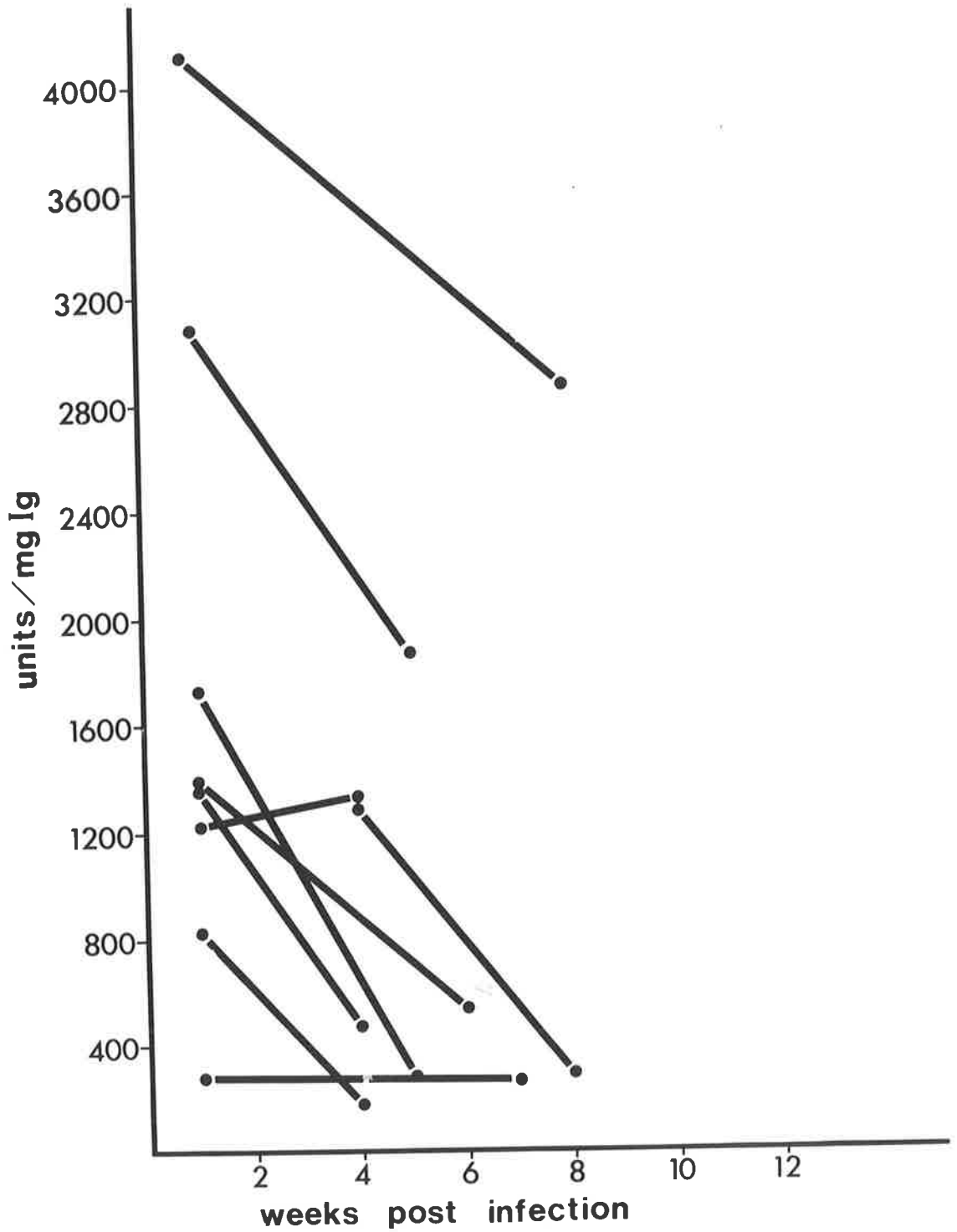
Figure $\overline{VI}$-14

CHILDREN — SERUM (units/mg Ig)

weeks post infection
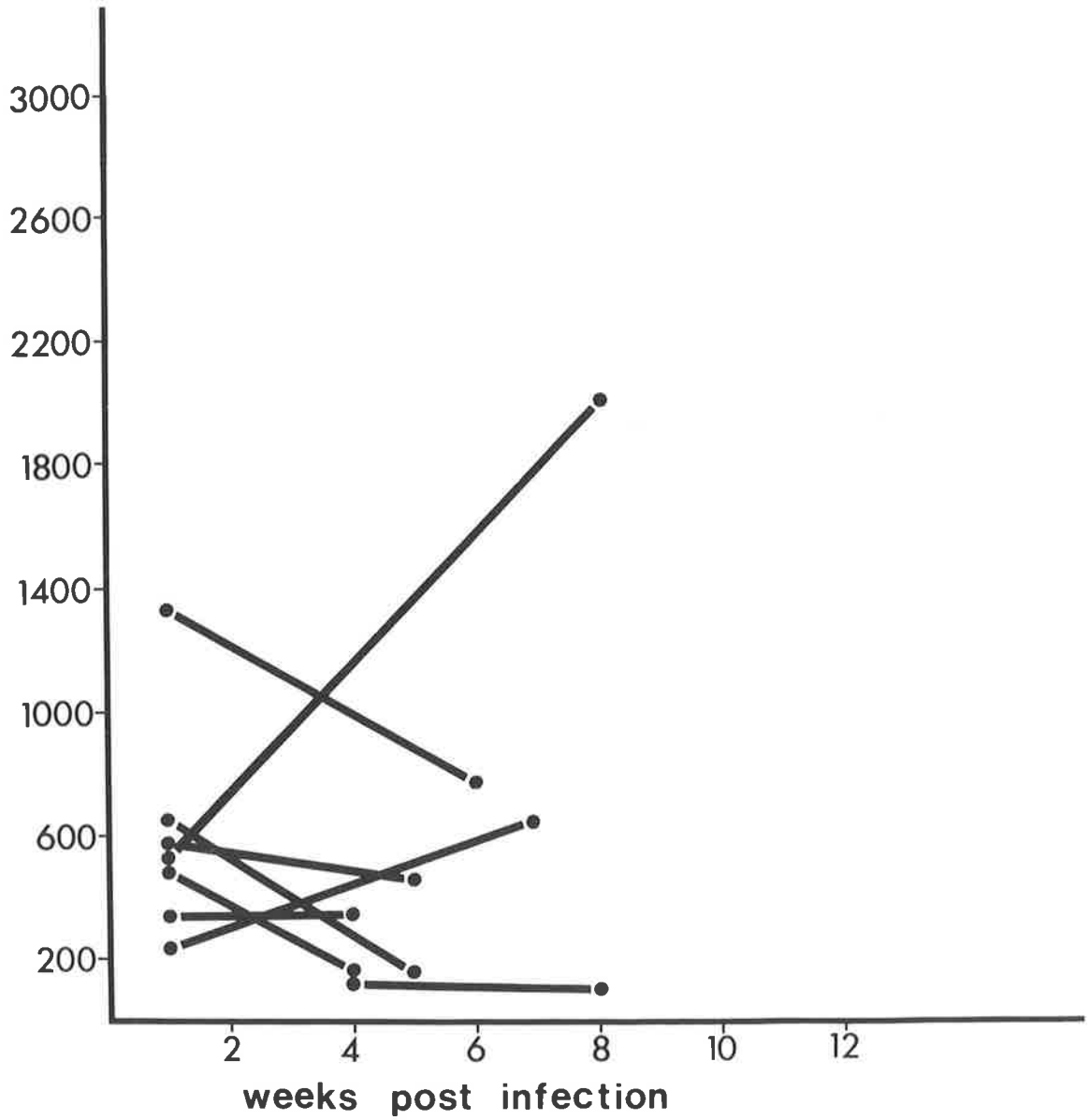
Figure $\overline{VI}$-15

DISSOCIATION OF SERUM AND INTESTINAL
ANTIBODY RESPONSES

Samples from patients — A ★ , B ● , C ⊙

Aspirate ——————   Serum ------
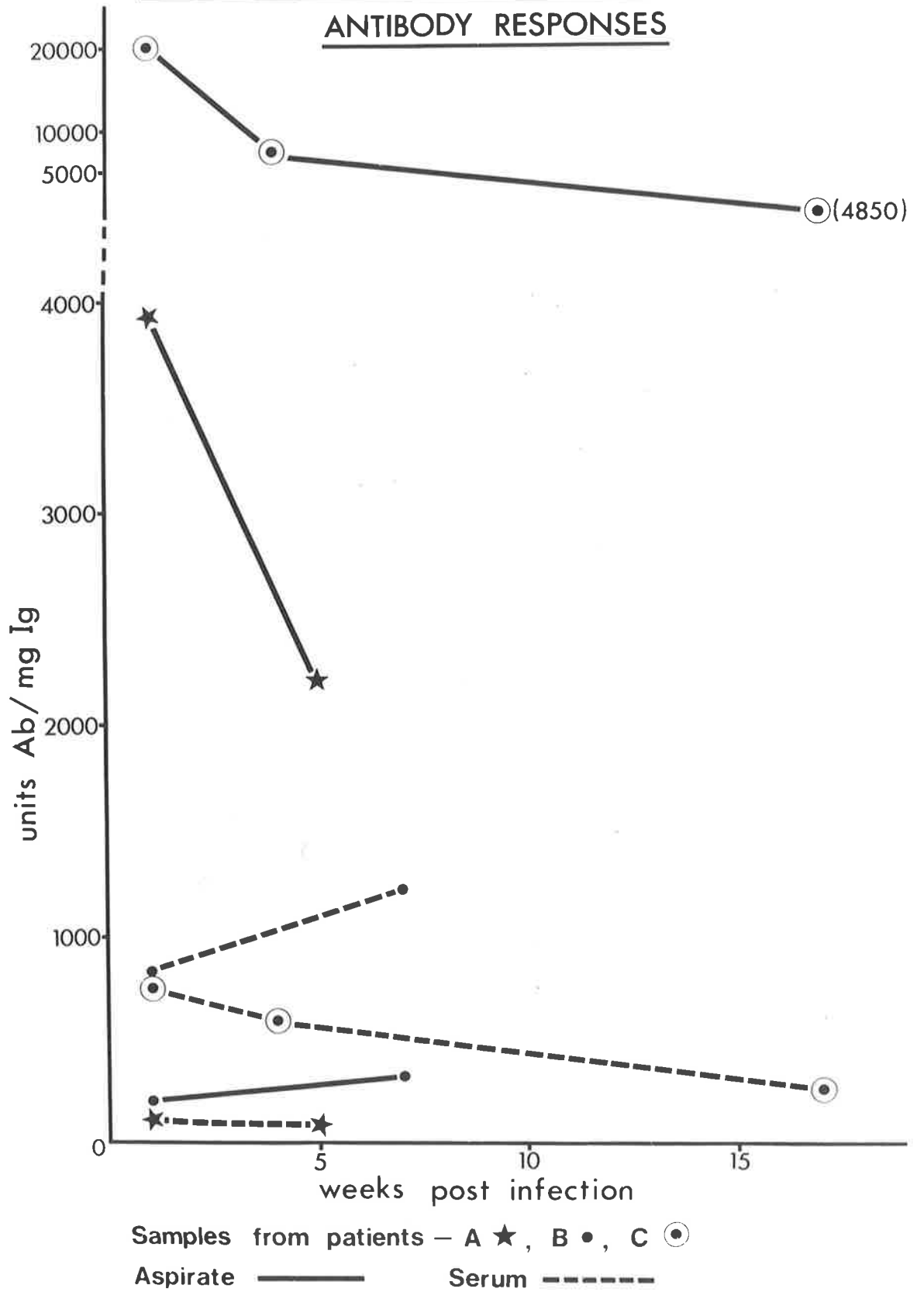
FIGURE VI - 16

Relationship of Intestinal and Serum Antibody

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
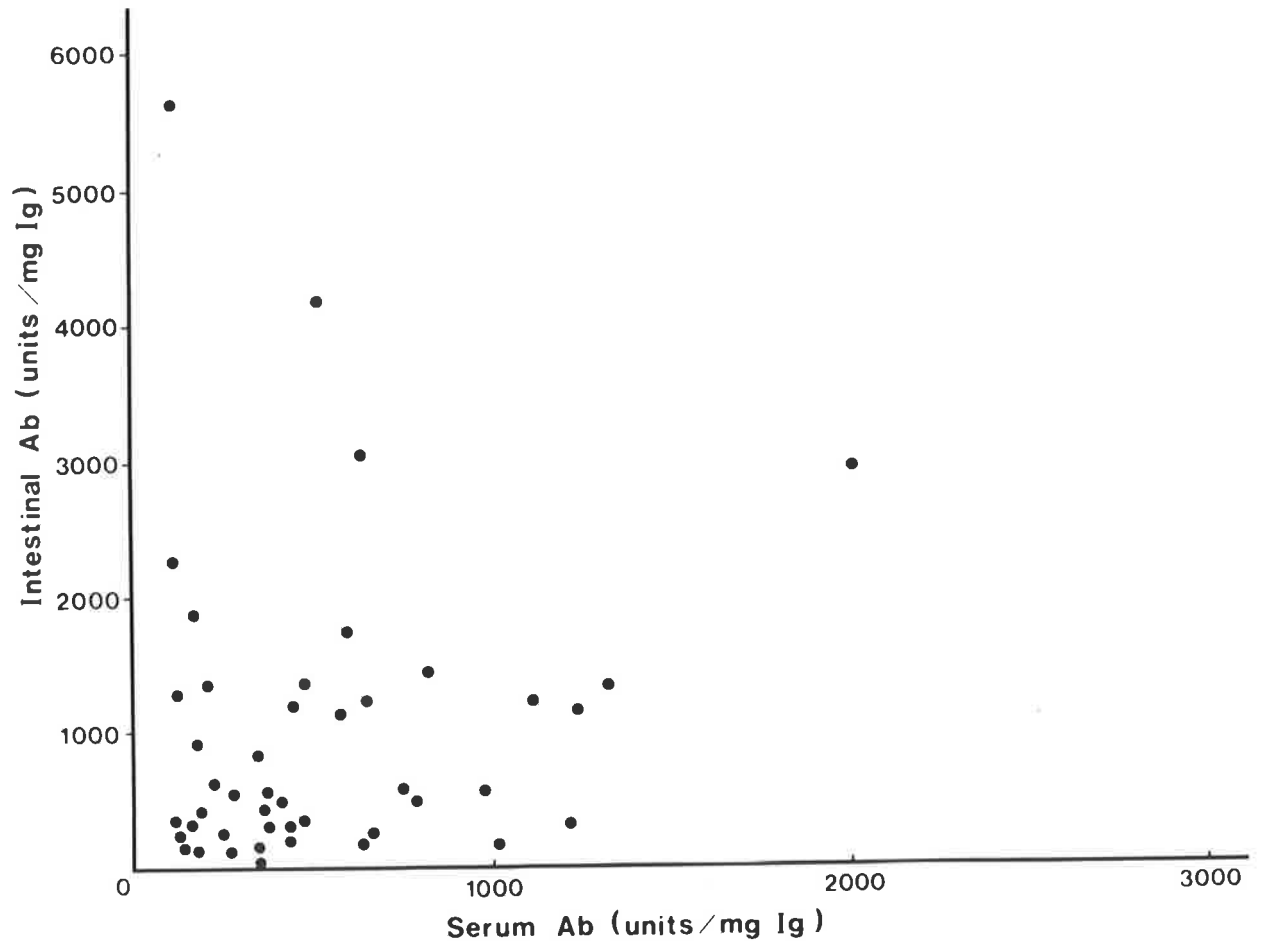
FIGURE VI - 17

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-α 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 O.

Figure VI-17

Relationship of Serum IgA Antibody and Intestinal Antibody
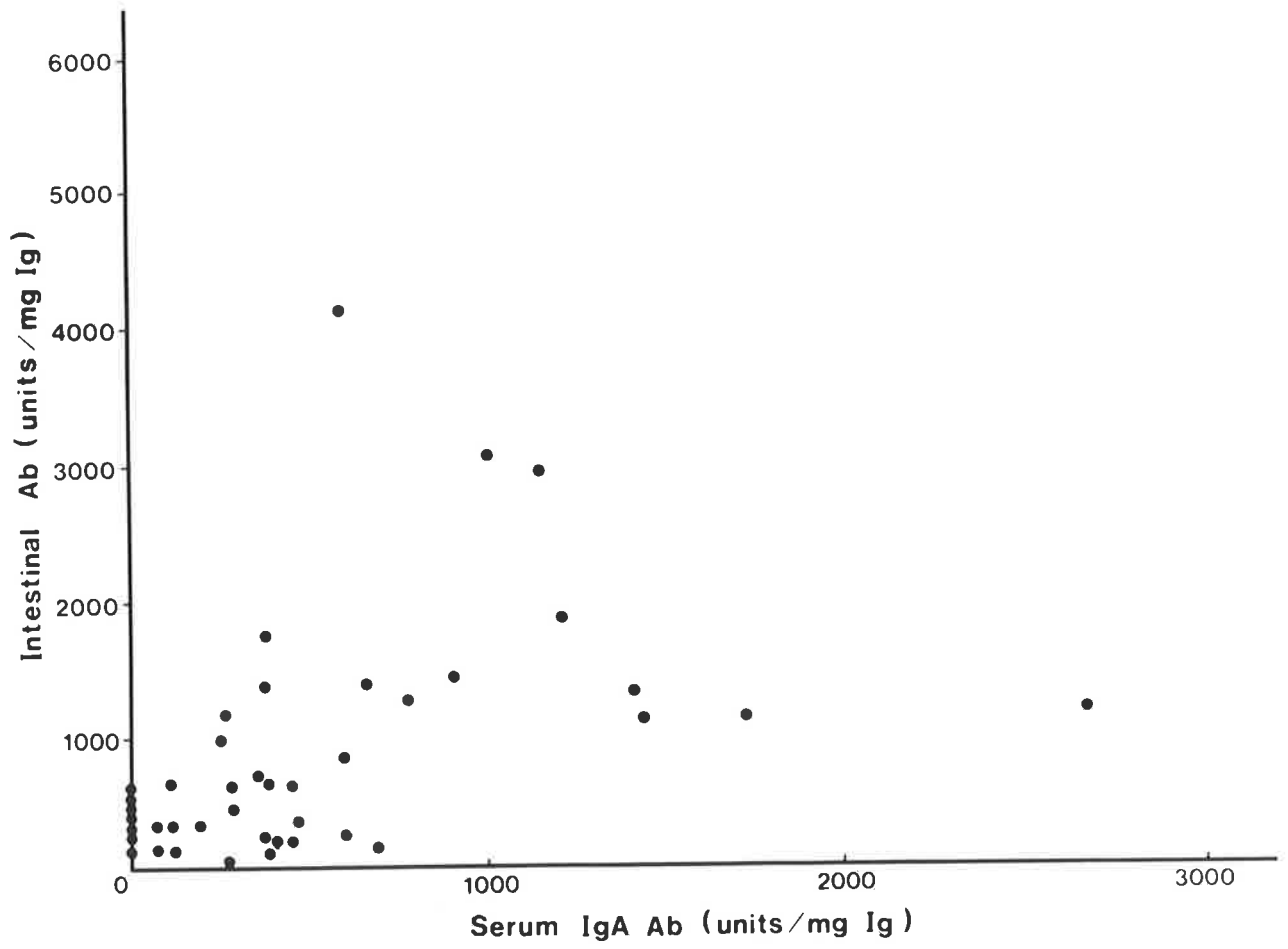
FIGURE VI – 18

Relationship between Salivary and Intestinal Antibody

.   Salmonella infection

O   Shigella infection

Figure VI - 18

Relationship Between Salivary Antibody and Intestinal Antibody
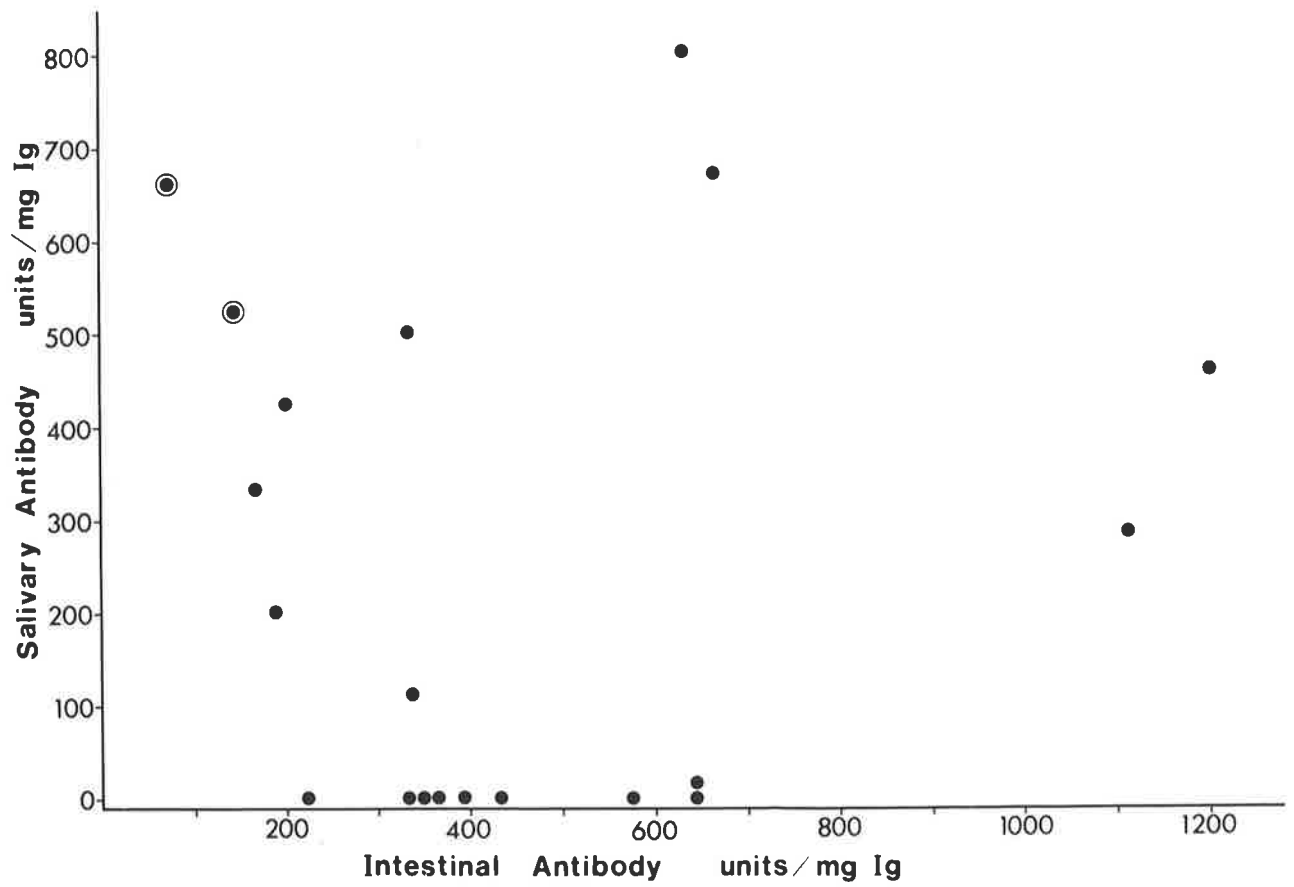
FIGURE VI - 19

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.

Figure $\overline{\text{VI}}$-19

ANTIBODY RESPONSE IN THE DIFFERENT IMMUNOGLOBULIN CLASSES (ADULTS)
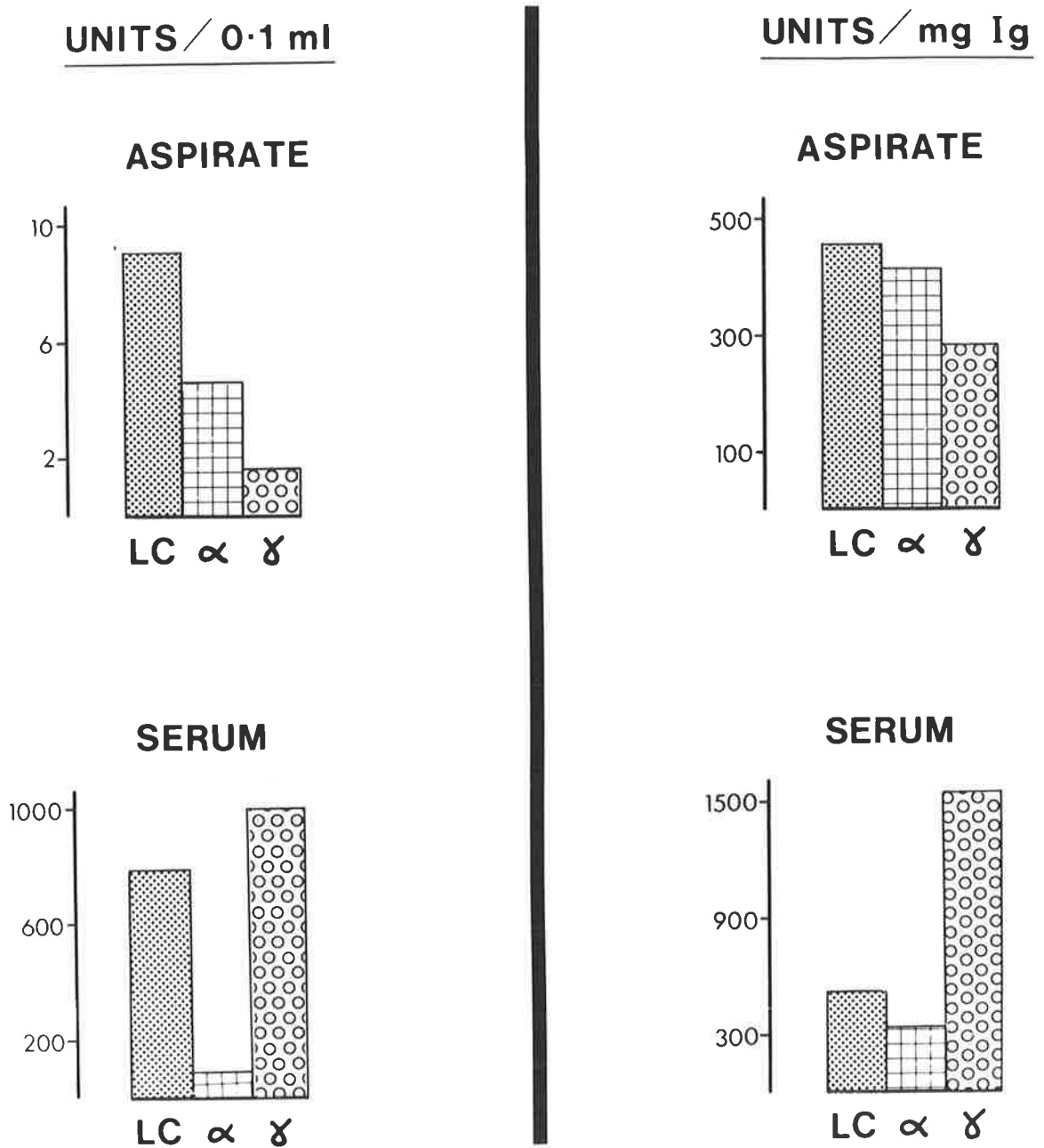
FIGURE VI - 20

Antibody Response in the Serum of Children

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 $\overline{VI}$-20

ANTIBODY RESPONSE IN THE SERUM OF CHILDREN
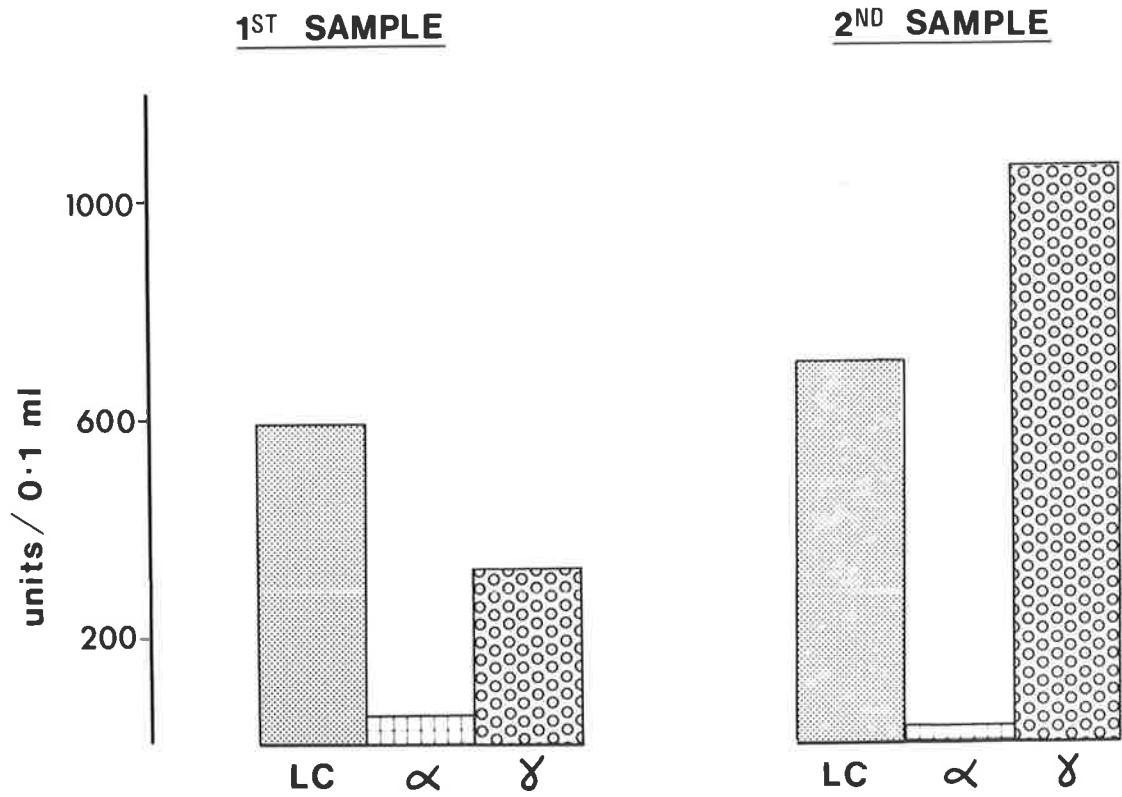
CHAPTER 7

DISCUSSION

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 $-80^{\circ}C$, 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 gastro-
enteritis 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., 1968 a & 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-μ 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

SAMPLING

No.	Date.	Wks after Infection.	Sample	Immunoglobulin(mg%)			Antibody(u/0.1ml)			Antibody(u/mg.Ig)		
				A	G	M	LC	α	γ	LC	α	γ
1	15.10.76	1	Serum	204	960	80	170			125		
			Int.Asp.	6	2	0	45			5625		
2	10.11.76	5	Serum	220	1270	66	170			109.3		
			Int.Asp.	4	2	8	32			2285.7		

Miss Devalynn

D.O.B.	Age 14 years
Past Medical History	Nil of note
Presenting Illness	Diarrhoea and abdominal colic that lasted twelve days
Bacteriology	Salmonella newport
Onset of Illness	3.11.76

SAMPLING

No.	Date.	Wks after Infection	Sample	Immunoglobulin(mg%)			Antibody(u/0.1ml)			Antibody(u/mg.Ig)		
				A	G	M	LC	α	γ	LC	α	γ
1	17.11.76	2	Serum	130	1620	95	400	50	360	216.8	384.6	222.2
			Int.Asp.	5	1		4			666.7		
2	1.12.76	4	Serum	107	1800	110	307.7		384.6	150		210
			Int.Asp.	–	–	–	–		–	–		–

SUBJECT 3

Mr Derek

D.O.B.	Age 36 years
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

SAMPLING

No.	Date	Wks after Infection	Sample	Immunoglobulin(mg%)			Antibody(u/0.1ml)			Antibody(u/mg.Ig)		
				A	G	M	LC	α	γ	LC	α	γ
1	2.5.77	1	Serum	197	900	40	660	280		580.5	1421.3	
			Int.Asp.	20	0	0	23	17	0	1150	850	
2	27.5.77	5	Serum	167	900	45	920	150	3770	827.3	898.2	4188.9
			Int.Asp.	25	0	0	36	0	0	1440		

SUBJECT 4

Mr. John

D.O.B.	Age 65 years
Past Medical History	Nil of note
Presenting Illness	Profuse watery diarrohoea that precipitated renal failure. Symptoms eased after 1 week.
Bacteriology	Salmonella havana Salmonella havana (stool) 6.3.1978
Onset of Illness	9.2.77

SAMPLING

No.	Date.	Wks after Infection	Sample	Immunoglobulin(mg%)			Antibody(u/0.1ml)			Antibody(u/mg.Ig)		
				A	G	M	LC	α	γ	LC	α	γ
1	17.2.77	1	Serum	255	1320	60	1050	320	1450	642.2	1254	1098.5
			Int.Asp.	68	9	16	1900	>160	32.7	20430.1		3633
2	7.3.77	4	Serum	255	1620	55	1140	510	3460	590.7	2000	2135.8
			Int.Asp.	47	9	8	560	>160	87.3	8750		11640
3	6.6.77	16	Serum	267	1520	55	450	100	2450	244.3	374.5	1611.8
			Int.Asp.	71	16	10	500	> 160	100	4800		6250
4	1.3.78	54	Serum	267	1400	55	364	109	1091	210	410	780
			Int.Asp.	44.5	8.7	6	109	82	21	1840	1840	2400
			Gastr.Asp.	29	21	3	11			208		

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
	Salmonella bredeni (stool) 14.9.77
Onset of illness	25.6.77

SAMPLING

No.	Date	Wks after Infection	Sample	Immunoglobulin(mg%)			Antibody(u/0.1ml)			Antibody(u/mg.Ig)		
				A	G	M	LC	α	γ	LC	α	γ
1	19.8.77	7	Serum	198	1800	60	390	50	3120	189.5	252.5	1733.3
			Int.Asp.	18	4	0	21	15	10	954.5	833.3	2500
2	21.9.77	11	Serum	220	2170	75	510	80	3930	206.9	363.6	1811.1
			Int.Asp.	24	11	3	53	43	23	1394.7	1791.7	2090.9

SUBJECT 6

Mrs. Anna-Liese

D.O.B.	Age 52 years
Past Medical History	Gastric Surgery (Polya Gastrectomy in 1971) Hysterectomy 1971. Nil else of note.
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

No.	Date.	Wks after Infection	Sample	Immunoglobulin(mg%)			Antibody(u/0.1ml)			Antibody(u/mg.Ig)		
				A	G	M	LC	α	γ	LC	α	γ
1	25.5.77	3	Serum	202	1120	95	7900	73.3	5000	5575.2	360	4464.3
			Int.Asp.	71	11	32	850	>160	>32	7456.1		
2	29.6.77	8	Serum	155	1000	85	800	<25	2170	645.2		2170
			Int.Asp.	18	1	3	26.7	3.8	3.0	1227.3	2111.1	3000
3	9.5.78	50	Serum	180	1170	85	160	<25	380	111.5		324.8
			Int.Asp.	44	3	6	27.8	7	4.0	528.3	159.1	1333.3

SUBJECT 7

Mr. Robert

D.O.B.	Age 34 years
Past Medical History	Nil of note
Presenting illness	Urgency of bowel actions three days followed by explosive diarrhoea and rigors for 24 hours. Diarrhoea settled after 10 days.
Bacteriology	Salmonella heffarek
Onset of Illness	10.6.77

SAMPLING

No.	Date	Wks after Infection	Sample	Immunoglobulin(mg%)			Antibody(u/0.1ml)			Antibody(u/mg.Ig)		
				A	G	M	LC	α	γ	LC	α	γ
1	22.6.77	2	Serum	68	112	85	820	30	3860	644.1	441.2	3446.4
			Int.Asp.	5	2	2	2	2	2	222.2	400	1000
			Saliva	2.5	4		0					
2	12.10.77	18	Serum	63	142	85	430	<25	1860	274.2		1309.9
			Int.Asp.	5	9	12	15	10	5	576.9	2000	555.6
			Saliva	6	1.9	0	0					

SUBJECT   8

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

SAMPLING

No.	Date	Wks after Infection	Sample	Immunoglobulin(mg%)			Antibody(u/0.1ml)			Antibody(u/mg.Ig)		
				A	G	M	LC	α	γ	LC	α	γ
1	31.8.77	2	Serum	195	1325	205	1700	70	2960	985.5	359	2234
			Int.Asp.	7	0	2	6	2	0	666.7	285.7	
			Saliva	2.5	0	0	2			666.7		
2	2.11.77	11	Serum	175	260	195	630	< 25	1560	386.5		1238.1
			Int.Asp.	17	17	8	14	2	6	333.3	117.6	352.9
			Saliva	21.5	2.2	0	0			0		

SUBJECT 9

Mrs Joan

D.O.B.	Age 33 years
Past Medical History	Nil of note
Presenting Illness	Diarrhoea with hourly bowel actions and rigors with vomiting after 48 hours. Diarrhoea stopped after a week.
Bacteriology	Salmonella typhi-murium
Onset of Illness	6.12.76

SAMPLING.

No.	Date	Wks after Infection	Sample	Immunoglobulin(mg%)			Antibody(u/0.1ml)			Antibody(u/mg.Ig)		
				A	G	M	LC	α	γ	LC	α	γ
1	21.12.77	2	Serum	175	1300	125	780	80	780	487.5	457.1	600
			Int.Asp.	23	0	5	11	5	0	392.9	217.4	
			Saliva	8.5	0	0	0					
2	24.2.78	9	Serum	175	1540	150	270	20	380	144.8	114.3	246.8
			Int.Asp.	6	0	0	2	0	0	333.3		
			Saliva	8.5	0	0	1	1		111.1	111.1	

Miss Lisa

D.O.B.   Age 16 years
Past Medical History   Appendicitis - 14 years.
Presenting Illness   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.
Bacteriology   Salmonella typhi-murium
Onset of Illness   28th December, 1977.

SAMPLING

No.	Date.	Wks after	Sample	Immunoglobulin(mg%)			Antibody(u/0.1ml)			Antibody(u/mg.Ig)		
				A	G	M	LC	α	γ	LC	α	γ
1.	24.1.78	4	Serum	213	1540	175	860	60	1520	446.1	281.7	987
			Int.Asp.	6	2	2	12	9	0	1200	1500	
			Saliva	11	0	0	5	2		454.5	181.8	
2	20.3.78	12	Serum	213	1640	200	760	60	760	370.2	281.7	463.4
			Int.Asp.	10	2	2	9	6	0	642.9	600	
			Saliva	4	1.9	0						

141

SUBJECT 11

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

No.	Date	Wks after Infection	Sample	Immunoglobulin(mg%)			Antibody(u/0.1ml)			Antibody(u/mg.Ig)		
				A	G	M	LC	α	γ	LC	α	γ
1	8.2.78	2	Serum	187	860	60	1370	320	1910	1237.6	1711.2	2220.9
			Int.Asp.	21	2	3	29	17	1	1115.4	809.5	500
			Saliva	6.5	0	0	2	1		285.7	142.9	
2	15.3.78	7	Serum	175	890	80	860	80	1000	751.1	457.1	1123.6
			Int.Asp.	18	2	2	14	9	1	636.4	500	500
			Saliva	5	0	0	4	3		800	600	

SUBJECT 12

Mrs Alma

D.O.B.	Age 54 years
Past Medical History	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

No.	Date.	Wks after Infection	Sample	Immunoglobulin(mg%)			Antibody(u/0.1ml)			Antibody(u/mg.Ig)		
				A	G	M	LC	α	γ	LC	α	γ
1	10.2.78	4	Serum	215	860	260	408	60	580	359.6	279.1	600
			Int.Asp.	15	3	5	10	4	1	434.8	266.7	333.3
			Saliva	6	0	0	0					
2	29.3.78	11	Serum	225	860	260	150	0	580	111.5		700
			Int.Asp.	32	10	10	19	10	5	365.4	312.5	500
			Saliva	4.5	0	0	0					

SUBJECT 13

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

SAMPLING

No.	Date.	Wks after Infection	Sample	Immunoglobulin(mg%)			Antibody(u/0.1ml)			Antibody(u/mg.Ig)		
				A	G	M	LC	α	γ	LC	α	γ
1	1.3.78	4	Serum	250	1600	140	340	30	690	170.9	120	431.3
			Int.Asp.	13	2	2	3	2	2	176.5	153.8	1000
			Saliva	8.5	0	0	3	2		333.3	222.2	
2	4.4.78	9	Serum	268	1700	130	310	20	580	147.8	74.6	341.2
			Int.Asp.	12	27	3	8	2	3	190.5	166.7	111.1
			Saliva	8.5	1.9	0	2	2		200	250.0	

Mrs Ursula

D.O.B.	Age 43 years
Past Medical History	Nil of note
Presenting illness	Colicky right sided abdominal pain and diarrhoea which lasted 9 days.
Bacteriology	Shigella sonnei
Onset of Illness	9.11.77

SAMPLING

No.	Date.	Wks after Infection	Sample	Immunoglobulin(mg%)			Antibody(u/0.1ml)			Antibody(u/mg.Ig)		
				A	G	M	LC	α	γ	LC	α	γ
1	24.11.77	2	Serum	155	1300	140	450	60	720	282.1	387.1	553.8
			Int.Asp.	11	9	7	4	1	1	148.1	90.9	111.1
			Saliva	10	2.5	0	7	3		538.5	300	
2	20.12.77	6	Serum	110	1300	150	560	30	1520	359	272.7	1169.2
			Int.Asp.	8	1	4	1	1	0	76.9	125	
			Saliva	6	0	0	4	3		666.7	500	

Mrs Denise

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

SAMPLE

No.	Date	Wks after Infection	Sample	Immunoglobulin(mg%)			Antibody(u/0.1ml)			Antibody(u/mg.Ig)		
				A	G	M	LC	α	γ	LC	α	γ
1	30.6.78	2	Serum	213	1340	210	760	40	540	431.1	187.8	403
			Int.Asp.	8	8	4	7	2	3	350	250	375
			Saliva	5.5	0	0	0	0				
2	7.8.78	7	Serum	188	1200	175	670	20	480	428.7	106.4	400
			Int.Asp.	13	9	6	18	2	1	642.9	153.8	111.1
			Saliva	3	0	0	0	0				

Mr. Ronald

D.O.B.	Age 32 years
Past medical history	Nil of note
Presenting Illness	Diarrhoea and vomiting lasting 72 hours.
Bacteriology	Salmonella typhi-murium
Onset of Illness	19.6.78

SAMPLING

No.	Date.	Wks after Infection	Sample	Immunoglobulin(mg%)			Antibody(u/0.1ml)			Antibody(u/mg.Ig)		
				A	G	M	LC	α	γ	LC	α	γ
1	29.6.78	1	Serum	390	960	160	1520	270	830	1006.6	692.3	900
			Int.Asp.	2	0	3	1	0	0	200		
			Saliva	6.5	0	0	3	0		428.6		
2	8.8.78	7	Serum	390	1280	175	2220	30	790	1203.3	76.9	617.2
			Int.Asp.	13	5	0	6	2	1	333.3	153.8	200
			Saliva	5.5	0	0	3	0		500		

Tony :

D.O.B.   Age 6 years
Past Medical History   -
Presenting Illness   Vomiting and diarrhoea accompanied by fever and rigors and right
                                iliac fossa.   Appendicectomy performed prior to isolating pathogen.
Bacteriology   Salmonella typhi-murium
                                Salmonella typhi-murium (stool) 16.3.78. (aspirate negative 22.3.78).
Onset of Illness   12.2.78

SAMPLING

No.	Date.	Wks after Infection.	Sample	Immunoglobulin(mg%)			Antibody(u/0.1ml)			Antibody(u/mg.Ig)		
				A	G	M	LC	α	γ	LC	α	γ
1	21.2.78	1	Serum	105	1370	140	760	70	320	470.6	666.7	233.6
			Int.Asp.	20	3	12	48	20	0	1371.4	1000	
2	22.3.78	4	Serum	80	1470	40	290	< 25	290	182.4		197.3
			Int.Asp.	6	8	3	8	5	2	470.6	833.3	250

Samantha   ;

D.O.B.   :   '.   Age 7 months
Past Medical History   —
Presenting Illness   Diarrhoea
Bacteriology   Salmonella typhi-murium
                                  Salmonella typhi-murium (stool) 22.3.78 (aspirate negative on 29.3.78).

Onset of Illness   19.2.78

SAMPLING

No.	Date.	Wks after Infection.	Sample	Immunoglobulin(mg%)			Antibody(u/0.1ml)			Antibody(u/mg.Ig)		
				A	G	M	LC	α	γ	LC	α	γ
1	28.2.78	1	Serum	30	750	120	1000	80	1210	1111.1	2666.7	1613.3
			Int.Asp.	7	1	6	17	10	0	1214.3	1429	
2	29.3.78	4	Serum									
			Int.Asp.	5	1	0	8	6	0	1333.3	1200	

148

Mandy

D.O.B.   Age 2yrs 8 months
Past Medical History   -
Presenting Illness   Diarrhoea and tonsillitis
Bacteriology   Salmonella typhi-murium (stool negative on 8.2.78)
Onset of Illness   14.1.78

SAMPLING

No.	Date.	Wks after Infection	Sample	Immunoglobulin(mg%)			Antibody(u/0.1ml)			Antibody(u/mg.Ig)		
				A	G	M	LC	α	γ	LC	α	γ
1	23.1.78	1	Serum	185	860	310	350	<25	<100	258.3		
			Int.Asp.	13	3	5	6	3	0	285.7	230.8	
2	8.3.78	7	Serum	105	1070	230	930	40	560	661.9	381	523.4
			Int.Asp.	6	2	3	3	0	0	272.7		

Kimberley

D.O.B.	Age 2 yrs 2 months.
Past Medical History	-
Presenting Illness	Fever and diarrhoea
Bacteriology	Salmonella typhi-murium (stools and aspirate negative on 19.4.78)
Onset of Illness	14.3.78

SAMPLING

No.	Date.	Wks after Infection	Sample	Immunoglobulin(mg%)			Antibody(u/0.1ml)			Antibody(u/mg.Ig)		
				A	G	M	LC	α	γ	LC	α	γ
1	23.3.78	1	Serum	50	750	120	320	30	190	347.8	600	253.3
			Int.Asp.	13	6	13	26	15	3	812.5	1153.8	500
2	19.4.78	4	Serum	43	900	60	370	0	650	368.9	0	722.2
			Int.Asp.	8	23	5	7	2	7	194.4	250	304.3

150

SUBJECT 21

Jason

D.O.B.	Age 4 years
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

No.	Date.	Wks after Infection	Sample	Immunoglobulin(mg%)			Antibody(u/0.1ml)			Antibody(u/mg.Ig)		
				A	G	M	LC	α	γ	LC	α	γ
1	18.4.78	1	Serum	100	720	300	1480	140	960	1321.4	1400	1333.3
			Int.Asp.	6	4	4	19	4	5	1357.1	666.7	1250
2	30.5.78	6	Serum	45	680	155	700	<25	1330	795.5		1955.9
			Int.Asp.	6	5	0	6	0	0	545.5		

Leah ㄴ

D.O.B. Age 1 year, 6 months.
Past Medical History -
Presenting Illness Diarrhoea
Bacteriology Salmonella typhi-murium (aspirate negative on 1.5.78 and 6.6.78)
Onset of Illness 4.4.78

SAMPLING

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

152

Habib

D.O.B.   :   Age 6 months
Past Medical History   –
Presenting Illness   Fever and diarrhoea
Bacteriology   Salmonella typhi-murium
    Salmonella typhi-murium (stool) 26.6.78 (aspirate negative 26.6.78)
Onset of Illness   13.5.78

SAMPLING.

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

153

David

D.O.B.   Age 2 years 5 months
Past Medical History   —
Presenting Illness   Diarrhoea, fever and vomiting
Bacteriology   Salmonella typhi-murium (aspirate negative 26.6.78)
Onset of Illness   11.5.78

SAMPLING

No.	Date.	Wks after Infection	Sample	Immunoglobulin(mg%)			Antibody(u/0.1ml)			Antibody(u/mg.Ig)		
				A	G	M	LC	α	γ	LC	α	γ
1	22.5.78	1	Serum	83	1000	260	800	30	380	595.7	361.4	380
			Int.Asp.	8	16	9	57	12	2	1727.3	1500	125
2	26.6.78	5	Serum	50	860	170	480	30	380	444.4	600	441.9
			Int.Asp.	6	2	3	3	1	1	272.7	166.7	500

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

No.	Date.	Wks after Infection	Sample	Immunoglobulin(mg%)			Antibody(u/0.1ml)			Antibody(u/mg.Ig)		
				A	G	M	LC	α	γ	LC	α	γ
1	7.4.78	1	Serum	135	1000	200	690	80	440	516.9	592.6	400
			Int.Asp.	10	2	8	83	31	1	4150	3100	500
2	25.5.78	8	Serum	105	1000	230	2670	120	5000	2000	1142.9	5000
			Int.Asp.	10	13	4	80	13	50	2963	1300	3846.2

Terence

D.O.B.	Age 32 years
Past Medical History	Nil of note
Presenting Illness	Diarrhoea which lasted 3 days and one episode of vomiting
Bacteriology	Negative for bacterial pathogens

SAMPLING

No.	Date	Wks after Infection	Sample	Immunoglobulin(mg%)			Antibody(u/0.1ml)			Antibody(u/mg.Ig)		
				A	G	M	LC	α	γ	LC	α	γ
1	22.4.77		Serum	275	1170	200	60	0	440	36.5		376.1
			Int.Asp.	10	2	5	1	0	0	58.8		

SUBJECT 27

Rowan

D.O.B.   Age 25 years
Past Medical History   Nil of note.
Presenting Illness   Diarrhoea accompanied by fever and general malaise, which
                                          persisted for a week.
Bacteriology   Negative for bacterial pathogens

SAMPLING

No.	Date	Wks after Infection	Sample	Immunoglobulin(mg%)			Antibody(u/0.1ml)			Antibody(u/mg.Ig)		
				A	G	M	LC	α	γ	LC	α	γ
1	6.6.77		Serum	288	1060	105	100			68.8		
			Int.Asp.	3	0	0	0			0		

Justin

D.O.B.   Age 31 years
Past Medical History   Nil of note

Healthy control

SAMPLING

No.	Date.	Wks after Infection	Sample	Immunoglobulin(mg%)			Antibody(u/0.1ml)			Antibody(u/mg.Ig)		
				A	G	M	LC	α	γ	LC	α	γ
1	2.11.77		Serum	158	1970	170	170	10	>160	74	63.3	
			Int.Asp.	8	1	4	1	0	0	76.9	0	0

John

D.O.B.   :   Age 27 years

Healthy control.

SAMPLING

No.	Date	Wks after Infection	Sample	Immunoglobulin(mg%)			Antibody(u/0.1ml)			Antibody(u/mg.Ig)		
				A	G	M	LC	α	γ	LC	α	γ
1	14.6.78		Serum	543	1320	110	340	50	540	172.3	92.1	409.1
			Int.Asp.	10	1	0	4	0	0	393.6		

Peter

D.O.B.   Age 31 years

Healthy control

SAMPLING.

No.	Date.	Wks after Infection	Sample	Immunoglobulin(mg%)			Antibody(u/0.1ml)			Antibody(u/mg.Ig)		
				A	G	M	LC	α	γ	LC	α	γ
1	5.7.78		Serum	75	920	330	170			128.3		
			Int.Asp.	5	0	0	0			0		

Robert :   ¡

D.O.B.   :   ¦   Age 30 years

Healthy control

SAMPLING

No.	Date	Wks after Infection	Sample	Immunoglobulin(mg%)			Antibody(u/0.1ml)			Antibody(u/mg.Ig)		
				A	G	M	LC	α	γ	LC	α	γ
i	15.6.77		Serum	75	920	330	140			119.1		
			Int.Asp.	6	1	0	0			0		

Jill

D.O.B.   Age 23 years
Healthy Control

SAMPLING

No.	Date.	Wks after Infection	Sample	Immunoglobulin(mg%)			Antibody(u/0.1ml)			Antibody(u/mg.Ig)		
				A	G	M	LC	α	γ	LC	α	γ
1	21.11.77		Serum	100	1170	120	90			64.7		
			Int.Asp.	9	8	5	1			45.5		

SUBJECT 33

Paul :

D.O.B.   :   Age 30 years

Healthy control

<u>SAMPLING</u>

<u>No.</u>	<u>Date</u>	<u>Wks After Infection</u>	<u>Sample</u>	Immunoglobulin(mg%)			Antibody(u/0.1ml)			Antibody(u/mg.Ig)		
				A	G	M	LC	α	γ	LC	α	γ
1	15.6.77		Serum Int.Asp.	5	1	0	0			0		

163

Alan

D.O.B. :   Age 26 years

Healthy control

SAMPLING

No.	Date.	Wks after Infection	Sample	Immunoglobulin(mg%)			Antibody(u/0.1ml)			Antibody(u/mg.Ig)		
				A	G	M	LC	α	γ	LC	α	γ
1	15.6.77		Serum Int.Asp.	8.3	2.5	0	3			272.7		

David

D.O.B.   Age 31 years

Healthy control

SAMPLING

No.	Date.	Wks after Infection	Sample	Immunoglobulin(mg%)			Antibody(u/0.1ml)			Antibody(u/mg.Ig)		
				A	G	M	LC	α	γ	LC	α	γ
1	12.7.78		Serum	212.5	1120	75	140			96		
			Int.Asp.	2	1	0	0			0		

SUBJECT 36

Deborah

Age 25 years

Healthy control

SAMPLING

No.	Date.	Wks after Infection	Sample	Immunoglobulin(mg%)			Antibody(u/0.1ml)			Antibody(u/mg.Ig)		
				A	G	M	LC	α	γ	LC	α	γ
1	11.7.78		Serum	187.5	1320	115	70			42		
			Int.Asp.	3.5	1.0	1.5	0			0		

SUBJECT 37

Neville

Age 30 years

Healthy control

SAMPLING

No.	Date.	Wks after Infection	Sample	Immunoglobulin(mg%)			Antibody(u/0.1ml)			Antibody(u/mg.Ig)		
				A	G	M	LC	α	γ	LC	α	γ
1	11.7.78		Serum	230.5	1220	200	120			70		
			Int.Asp.	7	3.8	3.0	0			0		

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