ANTIBACTERIAL MECHANISMS IN THE INTESTINE AGAINST VIBRIO CHOLERAE

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ABSTRACT

The unspecific defence mechanisms operating in the gastro-intestinal tract of mice against V. cholerae and the effect of antibody on these have been analyzed using in vivo and in vitro models. In adult mice orally inoculated viable P^{32}-labelled V. cholerae 569B organisms were rapidly reduced in viability. The parameters involved in the elimination of the bacteria were killing by gastric acid, mechanical removal by peristalsis and killing in the small and large intestine. Inhibition of one or more of these parameters by different treatments delayed or prevented the elimination of the live organisms. Results in starved animals suggested that secretory processes in the small intestine contributed to the killing of the organisms. These three mechanisms also operated to different degrees against other gram-negative organisms.

In infant mice the oral inoculum of live organisms was removed to some degree by the same mechanisms as have been found in adult mice. Antibody increased the efficiency of these elimination mechanisms considerably. In the immune animals a more rapid mechanical removal of

the organisms from the small intestine occurred and, in addition, the bacteria were killed more efficiently.

The bactericidal mechanisms found in the small intestine of adult and infant mice in vivo has further been analyzed using the intestinal loop model in adult mice. The results suggested that V. cholerae 569B and other gram-negative organisms to some extent, which are attached to the mucosal surface, were rapidly killed by one or more substances secreted by epithelial cells. This mechanism operated also in germ-free animals and seemed therefore to be independent from a normal flora. Antibody had no promoting effect on this killing mechanism. The function of antibacterial antibody in immunity to cholera was further studied by relating the ability of several antibody preparations to reduce the adsorption of V. cholerae to isolated intestinal epithelial cells with their protective activity in infant mice. The results demonstrated that 1) the antibody-mediated reduction of V. cholerae adsorption to epithelial cells correlates with the degree of agglutination, 2) antibodies protect infant mice from cholera only at concentrations that agglutinate the bacteria and 3) purified anti-flagella antibodies protect infant mice from cholera. These results indicate that cross-linking (agglutination and/or inhibition of motility) plays a role in immunity to cholera in infant mice.

A similar function of antibody is suggested from results obtained in intestinal loops of adult mice actively immunized with living V. cholerae 569B. Growth of V. cholerae 569B was suppressed approximately sevenfold in loops of parenterally and orally immunized mice when compared to bacterial growth in non-immune animals. Similarly, growth of V. cholerae 569B was reduced in mice immunized with a hybrid vibrio strain, NCV 569B-165, which shares flagella but not somatic antigens with V. cholerae 569B. Immune-fluorescence studies of intestinal loop contents and intestinal sections indicated that in non-immune mice vibrios coated the intestinal mucosa. In contrast, the intestinal mucosa of immune animals was almost free of bacteria. The vibrios were found agglutinated in the intestinal lumen of immune animals. It was concluded that immunization suppressed the growth of V. cholerae in the intestinal lumen and that bacterial agglutination mediates this growth suppression.

Finally, the effect of antibody on the unspecific defence mechanisms in the intestine is discussed and it is suggested that the joint action of antibody which prevents adsorption of the organisms to the intestinal wall, and the killing mechanism acting on those organisms which nevertheless attach to the mucosal surface will efficiently remove V. cholerae from the small intestine.

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.

Jürgen G. Knop

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CHAPTER 1

INTRODUCTION

1.1 General introduction

Although many infectious diseases can be effectively controlled by drugs, vaccination and public health measures nowadays, there are some infections - predominantly those of the gastro-intestinal tract - which are still a great problem in all countries. In a recent report of a WHO scientific group (1972) on oral enteric bacterial vaccines it was stated that the chance of "limiting the incidence of intestinal infections in developing countries through education and implementation of simple measures of sanitation as well as of public and personal hygiene is minimal within the next four or five decades. The only alternative is to raise the immune status of the population at risk by widespread and effective immunization. Oral immunization methods offer many advantages over the conventional parenteral techniques that are currently available."

Although oral immunization against poliomyelitis has been a great success oral immunization with bacterial vaccines has provided only limited protection (WHO, 1972). It was felt that there is still a fun-

damental gap in our knowledge on the behaviour of pathogenic organisms and their hosts in enteric bacterial diseases. Indeed, the basic problems of host-parasite relationship in the intestine have only recently been studied in any detail. Information concerning the early stages of bacterial colonization of the mucosa, although still scanty, has accumulated. The non-specific and specific antibacterial mechanisms which may interfere with these early steps of mucosal colonization remain obscure. This lack of information may be explained by the difficulties experienced in an experimental approach to such problems. Most laboratory animals are naturally resistant to mucosal infections like cholera which affect man. Therefore, animal models often are inadequate. Furthermore, the intestine appears to be a most complicated organ to study bacterial infections and antibacterial defence mechanisms. The problems envisaged are a) those of mixed bacterial cultures (Hobson, 1969) and b) those of an open system. The intestine can be regarded as an open system similar to a continuous culture vessel (Novik, 1955) although, of course, much more complex. In such a system growth and killing rates are difficult to determine although lately a method for estimating growth and killing rates _in vivo_ in the intestinal tract using genetic markers has been described (Meynell, 1959; Meynell

and Subbaiah, 1963; Maw and Meynell, 1968). In addition, the conditions for survival and growth of an introduced pathogen may vary considerably in different parts of the intestinal tract depending for example on the quality and quantity of normal flora in that part.

The subject of this thesis is to analyse some unspecific and specific immunological host factors which affect colonization of the intestinal mucosa by enteric pathogens.

Basically two groups of enteropathogenic organisms may be differentiated according to their modes of infection:

i) The organisms that attach to the mucosal epithelium but do not penetrate the epithelium or the submucosa. Examples for such infections confined to the lumen are cholera (Gangarosa et al., 1960) and *E. coli* enteritis in calves (Arbuckle, 1970) and neonatal pigs (Smith, 1971). The common feature of these infections are attachment to the mucosal surface of the small intestine, multiplication on the surface without gross damage of the intestinal epithelium (Gangarosa et al., 1960; Lankford, 1960; Taylor et al., 1958) and production of an enterotoxin (Burrows, 1968; Finkelstein, 1970; Smith and Halls, 1968) which causes a watery diarrhea

(Smith, 1971; Pierce et al., 1971; Greenough and Carpenter, 1970; Norris and Manjo, 1968).

ii) The organisms that attach to the intestinal mucosa and penetrate into the epithelial cells or submucosa. The organisms may multiply within the epithelial cells - for example Shigella (La Brec et al., 1964; Ogawa, et al. 1966; Takeuchi et al., 1968) or penetrate through the epithelial cells - for example Salmonella (Takeuchi and Sprinz, 1967) and some strains of E. coli (Staley et al., 1970 b) - and multiply in the subepithelial tissue and may or may not cause systemic infection. In this group of infections epithelial cell injury and ulceration of the mucosa usually occur.

With regard to an experimental study of antibacterial mechanisms on the mucosal surface two points should be emphasized:

i. Adherance of bacteria to the mucosal surface is the first stage in the pathogenesis of both groups of infections. Thus antibacterial mechanisms acting on the mucosal surface to prevent this adherence stage would be of considerable importance in prevention of all enteric bacterial infections.

ii. Organisms of group i because they remain within the intestinal lumen, are more suitable for a

study of antibacterial factors operating on the mucosal surface.

Since a better understanding of the host defence mechanisms of the mucosal surface may be reached by some information about the factors which enable enteric pathogens to survive and multiply on the mucosal surface one section (1.2) in the introduction will be concerned with such factors. In the next two sections which follow, a short review will be provided describing our current knowledge on unspecific (1.3) and specific immunological antibacterial mechanisms on the intestinal mucosa (1.4).

1.2 Factors which enable bacteria to colonize the mucosal surface of the intestine

In order to produce a disease enteric pathogens must localize and grow on the mucosal surface of the intestine. Basic requirements for localization and growth are (H. Smith, 1968):

1) Attachment to the mucosal surface
2) Resistance to host defence mechanisms
3) The biochemical ability to grow under the nutritional conditions provided by the mucosa.

Our knowledge about the mechanisms of the early steps of infection of the intestinal mucosa is quite scanty, however, an examination of the mechanisms involved in

bacterial infections of other mucosal surfaces may be helpful in understanding the early stages of enteric bacterial diseases.

1.2.1 Attachment

Many bacteria colonizing the gastro-intestinal tract and other mucous membranes exert a certain host and tissue specificity of attachment. This selective attachment to only certain epithelia is particularly common among the bacterial members of the indigenous flora (Savage, 1972) but can also be demonstrated with many pathogenic organisms colonizing the intestinal mucosa. *Streptococcus salivarius* and *Streptococcus sanguis* adhere readily to the surfaces of the tongues and cheeks of humans and to epithelial cells in cheek scrapings obtained from humans, hamsters and germ-free rats (Gibbons and van Houte, 1971). Likewise, thick layers of lactobacilli colonize the keratinizing stratified epithelium of the non-secreting portion of the stomachs of normal mice (Savage, Dubos and Schaedler, 1968; Savage, 1970), rats (Brownlee and Moss, 1961), and swine (Dubos et al., 1965; Tannock and Smith, 1970).

Similarly, the epithelium of the ileum (Savage, 1969 a) and large intestine (Savage, 1970) is colonized by bacteria which only associate with that particular mucosal epithelium. If removed from the epithelium by treatment with antibacterial drugs the bacteria will recolonize the same epithelium after the therapy is discontinued (Savage and Dubos, 1968; Savage 1969 b).

Similarly, selective attachment to certain epithelia can also be found with some pathogenic organisms. *V. cholerae* attaches primarily to the mucosa in the small intestine (Taylor et al., 1958; Lankford, 1960; Freter et al., 1961; Freter, 1969; Freter, 1970) but rarely to the large intestine (Lankford, 1960).

Certain strains of *E. coli* that cause diarrheal disease in calves and neonatal pigs also adhere to the mucosa of the small intestine (Arbuckle, 1970; Smith and Halls, 1968; Smith 1971).

Other *E. coli* strains seem to attach preferentially to colonic epithelium (Staley et al., 1970 b). Similarly, *Shigella* organisms attach to and multiply mainly in colonic epithelial cells (La Brec et al., 1964; Ogawa et al., 1966; Takeuchi et al., 1968).

Members of the genus Salmonella associate with the mucosal epithelium of the small intestine prior to penetration (Takeuchi and Sprinz, 1967; Sprinz et al., 1966).
Attachment may be confined to not only a specific epithelium but in some cases to certain areas of a particular epithelium. Thus V. cholerae can be found associated mainly with the base of the villi (Freter, 1969). Shigella in guinea pigs seems to colonize preferentially the villus epithelium leaving the crypt epithelium free (Takeuchi, 1967). Similar observations have also been made in experimental Salmonella infections in guinea pigs (Takeuchi and Sprinz, 1967).
It should be mentioned that in germ-free animals such a defined specificity is not always observed. Salmonella panama in germ-free mice colonizes the small as well as the large intestine (Ruitenberg et al., 1971). This emphasizes that the specificity of attachment may be governed by several factors such as bacterium-epithelial cells interaction, availability of nutrients (see below) and antibacterial defence mechanisms of the mucosa (see below).
The relationship of bacteria to the mucosal surface varies: some organisms have a very close relationship by exhibiting physical attachment

to the luminal plasmalemma of the epithelium, others may be attached more superficially. Thus many types of autochthonus microorganisms (Davis et al., 1974; Dubos et al., 1965; Savage, 1969a;Savage, 1972; Savage et al., 1968; Savage et al., 1971) are closely associated with the mucosal epithelium. Electron microscopic pictures clearly showed interactions between the plasma membrane of the epithelial cells and the cell wall of Strep. moniliformis (Hampton and Rosario, 1965). The area of the attachment on the epithelial cells was characterized by a plaque like thickening of the plasma membrane; the epithelial cells with attached organisms were morphologically and functionally intact (Hampton and Rosario, 1965).

Recently scanning electron microscopy has been used to visualize the residents of the microbial flora on epithelial surfaces in the gastrointestinal tracts of mice (Savage and Blumershine, 1974). Most of the organisms were found attached by one end to the epithelium. In the keratinized epithelium of the stomach the organisms were inserted into a hole or depression by one end, in the small intestine most organisms were adhered to the epithelial cells by filaments which were inserted into holes in the epithelium.

Close contact between epithelium cells and organisms has been shown with Streptococcus pyogenes and oral epithelium (Gibbons and van Houte, 1971). Some enteropathogenic strains of E. coli seem also to adhere closely to the epithelial cells (Arbuckle, 1970; Smith and Halls, 1968). A transient association with the plasmalemma of the epithelial cells is also exhibited by all those organisms which subsequently penetrate the epithelial cells such as some strains of E. coli (Staley et al., 1971 a, b) or Salmonella typhimurium (Takeuchi, 1967) or Shigella (Ogawa, 1970).

In some instances there appears to be a more superficial association with the mucosal surface. The attachment of V. cholerae to the striated border of the villi (Patnaik andGhosh, 1966) and of gonococci to various epithelial cells (Swanson et al., 1971) appears to be rather loose.

Very little is known about the biochemical mechanisms mediating the attachment of the bacterial to mucosal epithelial although in a few instances the bacterial structures involved have been identified. Electron micrographs of adult murine gastro-intestinal tracts showed that specific structures of a certain type of indigenous microorganisms

interacted with the epithelial cells in a way which suggested that receptor-like structures on the epithelial cells might be involved in this attachment (Reimann, 1965; Davis and Savage,1974). The first segment of these filamentous organisms was inserted into an excavation of the epithelial cell and was even further projected into the cell by nipple-like appendages (Davis and Savage, 1974).

Attachment initiated by an enzymatic process has been described with Streptococcus mutans, an organism which adheres to the smooth surfaces of teeth (Gibbons and Fitzgerald, 1969; Mukasa and Slade, 1973). The adherence of Streptococcus mutans cells to a smooth glass surface requires the presence of dextran-sucrose and levansucrose enzymes which adsorb to the surface of the streptococcal cells. These enzymes synthesize insoluble dextran-levan polysaccharide. This polymer appears to be responsible for the attachment of the organisms to the smooth surface (Mukasa and Slade, 1974). Certain strains of E. coli which cause enteritis in piglets adhere to the mucous membrane of the small intestine (Arbuckle, 1970) and to isolated epithelial cells (Wilson and Hohmann, 1973). Arbuckle (1970) observed that only serotypes of E. coli that produce K88 antigen (Orskov et al.,

1964), a proteinaceous surface component of the bacterial cell (Stirm et al., 1967 a; Stirm et al., 1967 b) attach to the intestinal mucosa of piglets. The removal of the K88 plasmoid from such an enteropathogenic strain was accompanied by the loss of its diarrhoea-producing capacity (Smith and Linggood, 1971). Jones and Rutter (1972) demonstrated that K88 antigen was synthesized by a K88-positive enteropathogenic strain of E. coli in the small intestine of both gnotobiotic and conventional neonatal pigs where it functioned as an adhesive factor enabling the bacteria to adhere to and colonize the mucosa; the greatly reduced virulence of a K88-negative mutant strain was attributed to its inability to colonize the mucosa of the small intestine. These authors also suggested that mannose-resistant haemagglutination (Stirm et al., 1967 a) which could only be found in K88+ strains, may resemble the attachment of K88-positive bacterial to the gut wall (Jones and Rutter, 1974).

Another bacterial cell wall component found to be involved in the adherence of Streptococcus pyogenes to epithelial cells has been described by Ellen and Gibbons (1972). These authors demonstrated that virulent strains of Streptococcus pyo-

genes containing M-protein were found to adhere well to human cheek epithelial cells *in vitro* and *in vivo*, whereas an avirulent M-mutant strain adhered feebly. Pretreatment of M+ strains with trypsin to remove their M-protein surface coating or reacting them with type-specific antibody markedly impaired their abilities to attach to epithelial cells. Electron microscopy revealed that the attachment of an M+ strain to epithelial cells of germ-free rats was mediated by a fuzzy surface structure (Swanson et al., 1969) shown to contain M-protein.

Pili have been found to correlate with virulence in gonnococcal infections (Jephcott et al.,1971; Swanson et al., 1971) and it has been suggested that the pili might be responsible for the adherence of gonococci to epithelial cells (Swanson et al., 1971). Some studies demonstrated enhanced attachment of pilated gonococci to tissue culture cells (Swanson, 1973), buccal mucosal cells (Punsalang and Swayer, 1973) uterine epithelial cells (Ward and Watt, 1973) and human spermatozoa (James-Holmquest et al., 1974).

The bacterial surface properties that mediate the attachment of *V. cholerae* to the intestinal mucosa

have not been identified. *V. cholerae* produces erythrocyte agglutinins (Lankford and Legsomburana, 1965) but no direct evidence links these agglutinins with epithelial attachment. Freter (1972) found that vibrios killed by heat or in the presence of neomycin adsorbed significantly less than live vibrios to the mucosa of *in vivo* isolated loops of adult rabbits. This suggests that *V. cholerae* adsorbs to the mucosal surface via some biochemial processes or structures which require structural intact and metabolizing organisms. It should be mentioned that *V. cholerae* secretes a number of mucinolytic enzymes and neuraminidase (Burnet and Stone, 1947) which depolymerize glandular mucosubstances and may affect epithelial membranes. Such enzymes might be involved in the attachment of the vibrios to the mucosal surface.

1.2.2 Resistance to host defence mechanisms

In order to successfully infect a host tissue infective organisms must counteract the various host defence mechanisms. A number of such inhibitory factors called aggressins (Wilson and Miles,1964) have been described and seem to be of predominant importance during the decisive primary lodgement period (Miles, Miles and Burke, 1957).

These bacterial factors which are primarily non-toxic and which in most cases act by some kind of immune suppression (Glynn, 1972) are quite heterogenous in their chemical nature and include compounds such as proteins, polysaccharides, lipids and others.

Most aggressins described interfere with phagocytosis, intracellular ingestion and digestion of bacteria (Wilson and Miles, 1964; Dubos and Hirsch, 1965; Smith, 1968; and Glynn, 1972). A few examples are: Anti-inflammatory factors of Staphylococcus aureus (Argawal, 1967; Fisher, 1963; Hill, 1968) which may partly act by inhibition of chemotaxis of polymorphnuclear leukocytes (Weksler and Hill, 1969); cell wall compounds such as M-protein and capsular acid of S. pyogenes which inhibit ingestion of the organisms (Braun and Siva Sankar, 1960); the Vi-antigen of S. typhi (Dubos and Hirsch, 1965) and the K-antigens of E. coli which increase resistance to complement killing and phagocytosis (Glynn and Howard, 1970; Howard and Glynn, 1971 a, b).

Virtually nothing is known about bacterial aggressins inhibiting local defence mechanisms on the mucosal surface. If the classical antibacterial mechanisms such as complement mediated lysis or phagocytosis are not of primary importance on the mucosal surface of the intestine (see 1.4.3), such

aggressins as described before would be of minor importance for enteric pathogens which are confined to the lumen, and for the initial mucosal attachment of invading organisms.

A few hypothetical aggressins which would increase the chance of survival and growth of enteric pathogens on the mucosal surface may be suggested:

1) Factors which would increase resistance of organisms to gastric acid.
2) Factors which would interfere with killing and lysis by mucosal bacteriocidins and enzymes.
3) Factors which would allow attachment and thus counteract mechanical removal by peristalsis could possibly be defined as aggressins.-
4) Factors which counteract the antagonism of the normal flora. Such factors are probably of predominant importance since a relative small number of pathogenic organisms has to compete with a dense population of commensals on the mucosa.

1.2.3 Nutritional factors

The importance of nutritional factors on localization and growth of bacteria in certain hosts and tissues has been demonstrated in several bacterial infections (Pearce and Lowrie, 1972). Nutritionally deficient mutants of pathogenic species are avirulent unless injected with their required nutrients

(Braun and Siva Sinkar, 1960). Examples are certain strains of P. pestis which require free asparaginase for a full growth rate (Burrows and Gillet, 1971).

The ability to utilize urea appears to contribute to the localization of Corynebacterium renale and Proteus mirabilis in the kidney (Braude and Sieminski, 1960). Another growth stimulating factor which determines tissue specificity is erythritol which enhances B. abortus infections (Keppie et al., 1963). Availability of iron and other metal ions which are closely concerned with the activity of so many enzymes can affect bacterial growth in animal tissues (reviewed by Glynn, 1972). Nutritional factors may very well determine the host and tissue specificity of many intestinal commensals, such as cellulose degrading bacteria in the lumen (Hobson, 1969) and lactobacilli in young animals (Savage, 1972). This is also demonstrated by the observation that alteration in diet can affect alterations in the population levels and types of micro-organisms in the alimentary indigenous flora (Dubos and Schaedler, 1962; Lee et al., 1971).

It is quite possible that nutritional factors will partly determine the host and tissue specificity of certain enteric pathogens although to the

author's knowledge no such observations have been reported. For most pathogenic organisms sufficient nutrients will probably be available to support some growth although nutritional considerations may very well affect growth rates in the intestine particularly in competition with the normal flora.

1.3 Non-immunological antibacterial factors in the intestine

The intestinal mucosa has an enormous surface area which is frequently exposed to a variety of pathogenic or facultative pathogenic organisms. The importance of non-specific factors for the protection of the mucosa against colonization or invasion by enteropathogens has long been realized and a number of investigations on this subject were performed early this century. The significance of unspecific resistance is demonstrated by the fact that it is difficult to establish oral enteric infections in most animal models without pretreatment with opium, antibiotics and starvation.

1.3.1 Mechanical factors

Peristalsis will move the bacterial inoculum from the small intestine into the large intestine. Thus, depending on the vigour of peristalsis i.e. the transit time of the organisms (between 2 hours in mice - own observations) and approx. 8 hours in pigs (reviewed by Nielsen et al., 1968) - a proportion of the pathogenic organisms will be removed

before they can multiply to a significant number (Dixon, 1960). The importance of intestinal motility in controlling bacterial populations in the lumen of the small intestine has been confirmed experimentally. Results obtained in monkeys and dogs (Dack and Petran, 1934) suggest that mechanical cleansing of the lumen by peristaltic action aided by mucous secretions is an important antibacterial mechanism of the small intestine. Inhibition of motility with opiate is a prerequisite for the experimental production of enteric forms of shigellosis (Formal et al., 1963) and salmonellosis (Kent et al., 1966).

Glandular secretions from the salivary glands, stomach, duodenum, pancreas and liver which supply a relatively large amount of fluid may assist the mechanical removal of the organisms from the proximal part of the intestinal tract (Nielsen et al., 1968).

A further mechanical barrier against bacterial colonization and invasion of the mucosa is the mucous layer which covers the epithelial cells. Florey (1933) carried out experiments in which he observed the cleansing action of the mucus using hydrocollagen. Graphite particles were encased in the mucous secretion and rolled up in a small ball of mucus. These small masses were then propelled on-

wards by peristaltic action of the bowel. But it was also observed that considerable areas of the surface of the villi appeared to be quite free from a mucous covering and that bacteria despite the mucus could come into close contact with the epithelial cells (Florey, 1933).
Undoubtedly, mechanical removal by peristaltic movement aided by mucus will diminish the number of organisms capable of attaching to the epithelial cells; but, because pathogenic organisms possess the ability to attach to the mucosa, mechanical removal - although quantitatively important - would not of itself be sufficient to complete the elimination of the organisms from the intestinal mucosa.

1.3.2 Bactericidal factors

The only clearly identified bactericidal factor described is the gastric acid which undoubtedly provides a strong barrier against invading organisms (Arnold and Finder, 1928; Arnold, 1929). Human saliva has been shown to be bactericidal to some extent (in Wilson and Miles, 1964). Little is known about bactericidal substances in the small intestine and the significance of such factors described in controlling enteric infections is not established. Antibacterial factors described in intestinal secretions are lysozyme, lactoferrin, poly-

amines and enzymes (review by Wilson and Miles, 1964; WHO Report, 1972; Glynn, 1972). Some controversy can be noticed in the literature concerning the killing of bacteria in the intestine. Intestinal juice seems to lack bactericidal activity for a number of organisms (Teale, 1934; own observation). Dixon (1960) using ^{51}Cr labelled red blood cells as a marker for mechanical removal showed that E. coli and some other organisms are eliminated solely by peristaltic action from the small intestine of rats without any measurable killing. Meynell and Subbaiah (1963) using a system which involved abortive transduction found a weak bacteriostatic and a weak bacteriocidal mechanism in the intestine of normal mice which was abolished by streptomycin treatment. Freter (1956) using V. cholerae and Shigella flexneri found that both strains were completely killed during their passage through the gastro-intestinal tract of untreated guinea pigs although it is not clear at which level of the gastro-intestinal tract this killing occured.

The question concerning a bactericidal mechanism in the small intestine remains unsolved. However, the intestinal epithelium does provide an effective barrier against many organisms. This is demonstrated by X-irradiation studies (Quastler and Hampton,1962) in which many organisms could be found in the sub-

epithelial tissue. Whether this barrier is a mechanical one or a biological one having the capability of destroying micro-organisms is yet to be determined.

1.3.3 Normal flora

The role of the normal flora in protection against enteric infectious disease is clearly established, at least in animal models. The indigenous microbiota prevents infection by V. cholerae and Shigella flexneri in guinea pigs (Freter, 1956; Hentges, 1969; Hentges and Maier, 1970); by Salmonella in mice (Miller and Bohnboff, 1963; Bohnhoff et al., 1964 a, b) and by E. coli in mice (Ashburner and Mushin, 1962). Further evidence is provided by experiments with germ-free animals which appear to be more susceptible to enteric infections than conventional ones (Kohler and Bohl, 1966; Abrams and Bishop, 1966).
Recently a detailed review has been published on the microbial interference regulating the bacterial population levels in the intestine (Savage, 1972).

In infant mice small numbers of micro-organisms - mainly lactobacilli - colonize the intestinal tract in the first 5 - 7 days of life, thereafter the numbers and variety of the flora increase and stabilize after about 2 - 3 weeks (Schaedler et al., 1965; Savage et al., 1968; Lee et al., 1971; Savage

and Mc Allister, 1971). In adult animals the population levels and localization of the indigenous microbiota which is composed predominantly of anaerobic bacteria (Howard, 1967; Savage, 1972) varies considerably. However, as a rule, the density of the bacterial population increases in the lower levels of the gastro-intestinal tract, i.e. in the ileum cecum and colon. Thus in mice only relatively few organisms can be recovered from and visualized in the upper small intestine (Savage, 1972; Savage and Blumershine, 1974). Similarly, the upper small intestine of normal fasting persons is free of a normal flora, the numbers increase in the jejunum and ileum and are highest in the colon (Drasar et al., 1969; Nelson and Mata, 1970).

The mechanisms of microbial interference are complex. The factors responsible for regulating the indigenous microbiota will probably also prevent colonization and invasion of the mucosa by pathogenic organisms. A number of bacteriocidins (colicines) produced by indigenous organisms have been described (Nomura, 1967). In a few experiments growth of colicine-sensitive bacterial pathogens for example in urinary infections (Braude and Sieminsky, 1960) and in experimental Shigella kerato-conjunctivitis has been suppressed by colicine producing strains. In the intestine of germ-free mice, however, coli-

cines were ineffective in suppressing growth of a colicine-sensitive E. coli (Ikari et al., 1969). Meynell (1963) showed that the indigenous flora interfered directly with intestinal Salmonella of mice by forming volatile organic acids in a reducing environment. A similar mechanism was observed with Shigella flexneri (Hentges and Maier, 1970).

Beside this direct effect, the indigenous microbiota may interfere indirectly with enteric pathogens. Abrams and Bishop (1967) demonstrated a more rapid propulsion of a non-absorbable radioactive marker through the gastro-intestinal tract of conventional mice in comparison with germ-free animals, suggesting that the normal flora might affect intestinal mobility. It should also be mentioned that the normal flora has a considerable influence on the anatomical and physiological development of the intestinal mucosa. Comparison of germ-free and conventional animals shows that the structure of the lamina propria (Lymphoid tissue, Gordon and Bruckner-Kardoss, 1961 a), the life cycle of the epithelial cells (Abrams et al.,1963) and the surface area of the mucosa (Gordon and Bruckner-Kardoss, 1961 b) is greatly influenced by a normal flora.

1.4 Antibody-mediated antibacterial mechanisms in the intestine

The concept that local immune mechanisms of the intestinal mucosa may be involved in protection against enteric infections was developed early this century. Besredka based his hypothesis that local rather than systemic immunity protects rabbits orally immunized with paratyphoid organisms on results indicating that "the amount of antibodies in the serum does not always correspond to the degree of immunity" (Besredka, 1925 cited by Thomson and Thomson, 1948). This together with the fact that bile was necessary for establishing effective immunity, induced him to propose the receptor theory in which antibody was not involved (Besredka, 1927).
Further support for the existence of a local immune mechanism in mucous membranes was given by several subsequent publications although the nature of these mechanisms remained obscure.
Immunity mediated by local antibody was demonstrated in pneumococcal respiratory infections (Bull and Mc Kee, 1929; Walsh and Cannon, 1934; Walsh 1938) in influenza virus infections (Fazekas de St. Groth et al., 1951) and in studies of Kerr and Robertson (1954) who showed that the route of immunization influenced the level of antibody in serum and vaginal mucus. Increased interest in local immunity arose with the discovery of IgA by Heremans and Schultze (1958) which consequently was shown to

be the predominant immunoglobulin class in mucous secretions (Tomasi and Bienenstock, 1968). Although the role of IgA in antibacterial immunity is still debatable, there is now strong evidence that antibody-mediated antibacterial mechanisms are effective on mucosal surfaces. Results from animal experiments and field trials with enteric pathogens which are confined to the intestinal lumen, such as V. cholerae and E. coli, clearly demonstrate that active immunization with the bacterial cell or passive immunization with antibacterial antibody can protect against these diseases. Thus in cholera both natural infection and parenteral immunization are associated with the development of antibodies to cholera antigens and of relative resistance to the disease (Mosley et al., 1968; WHO, Report of a scientific group, 1969).

Protection against the disease could also be demonstrated in guinea pigs (Burrows et al., 1947), parenterally or orally immunized with V. cholerae or given specific antiserum passively (Burrows and Ware, 1949). Freter (1954, 1956) obtained protection against V. cholerae infection in streptomycin-treated guinea pigs with antibody located in the intestinal tract. Protection against cholera by antibacterial antibody was also achieved in infant rabbits (Freter, 1964; Jenkin and Rowley, 1960; Feeley, 1965; Panse et al., 1964) and in infant mice (Ujiye et al., 1968, 1970; Neoh and Rowley, 1972;

Chaicumpa and Rowley, 1972; Guentzel and Berry, 1974) and in chinchillas (Blachman et al., 1974).
Studies on cholera in human volunteers have been particularly valuable because the natural disease only occurs in humans and the studies are not influenced by "natural" antibodies (acquired by subclinical infection) detectable in populations of endemic areas.
Cash et al. (1974) clearly demonstrated in human volunteers that immunity acquired after previous diarrhoea caused by infection with V. cholerae lasted about 12 months. An individuals immunity either to infection or to diarrhoea was not correlated with his serum titer of vibriocidal antibody or his serum titer of antitoxin. Immunity, either naturally acquired or vaccine induced, appeared to be directed against the vibrio rather than against the toxin.
Infection of neonatal pigs with enteropathogenic strains of E. coli which are confined to the lumen can be prevented by passive administration of antibody directed against the K88 antigens (Smith and Linggood, 1971; Smith, 1972; Rutter and Jones, 1973).
In summary, there is good evidence that antibody-mediated antibacterial mechanisms are capable of operating on the mucosal surface of the intestine. In the following paragraphs the availability of antibody, phagocytic cells and complement in the intestinal lumen will be discussed.

1.4.1 Antibody

In intestinal secretions immunoglobulins of all classes are found although IgA appears to be with regard to absolute concentrations the predominant immunoglobulin in many animal species investigated (Vaerman, 1970) and man (Girard and de Kalbermatten, 1970; Plaut and Keonil, 1969; Northrup, Bienenstock and Tomasi, 1970; Waldman et al., 1971). Most immunoglobulins are derived from local production in the lamina propria (Tomasi and Bienenstock, 1968) but there is clear evidence from experience using radioactive or biological markers that immunoglobulins are transported from the serum into the lumen of the gut (Wernet et al., 1971; Heddle, unpublished observation; Husband and Lascelles, 1974; Batty and Bullen, 1961). Thus antibody against various enteric pathogens may be derived from local or systemic sources.

a) Locally produced antibody

The predominant locally produced antibody class found in secretions is secretory immunoglobulin A (S-IgA) (Tomasi and Bienenstock, 1968). In addition, some of the IgG and IgM antibodies found in intestinal secretions may also be produced locally (Asofsky and Thorbecke, 1961; Smith et al., 1971; Allen and Porter, 1973) although little is known about their quantita-

tive contribution. A variety of antiviral and antibacterial antibodies are associated with secretory IgA in human colostrum, parotid saliva and nasal secretions, but little is known about IgA in intestinal secretions (Tomasi and Bienenstock, 1968).

The biological significance of IgA in protection against enteric bacterial infection depends on i) the amount and duration of the antibody response and ii) its antibacterial efficiency.

i) Although there is evidence from studies in both animals and man that antibacterial immunity to cholera correlates better with coproantibody levels than with titers of systemic antibody (reviewed by Pollitzer, 1959; Blachman et al., 1974; Cash et al., 1974) the amount of specific IgA antibody in these coproantibodies has never been determined. Recently, anti _V. cholerae_ antibodies of the IgA class have been demonstrated in convalescent patients (Northrup and Hossain, 1970). Active oral immunization with bacterial antigens in various animals has elicited an IgA antibody response, although with variable results. In some studies antibody activity in the IgA class after oral immunization with killed or living organisms has been assessed. Menzel and Rowley (1975) and Steele et al., (1974) found

that only prolonged oral immunization with high doses of living V. cholerae induced an IgA antibody response in rabbits. Similar observations have been made in dogs (Heddle,1975). Husband and Lascelles (1974) reported that repeated local infusion of living and killed Br. abortus into isolated intestinal loops in sheep failed to elicit a significant IgA antibody response. However, oral immunization with ferritin in mice (Crabbé et al., 1969) and ferritin or egg albumine in sheep (Husband and Lascelles, 1974) or vaccination with poliovirus (Ogra et al., 1968) usually resulted in a good IgA antibody response. These data suggest that stimulation of an IgA response may not only depend on the route of immunization but also on the nature of the antigen.

The availability of protective amounts of specific antibacterial antibody will also depend on a good anamnestic response. However, there is some evidence that the immunological memory of the secretory immune system is relatively poor. In several studies prolonged or repeated local vaccination with inactivated polio-virus (Ogra and Karzon, 1969), with V. cholerae (Freter and Gangarosa, 1963; Agarwal and Ganguly, 1972) or with heat-killed E. coli (Porter et al., 1974)

did not elicit a secondary immune response.

ii) Antibacterial efficiency

While the role of secretory IgA in resistance to mucosal viral infections seems fairly well established (see The Secretory Immunologic System, 1969) its participation in the antibacterial defence remains obscure.

IgA antibody directed against S. typhimurium (Eddie et al., 1971) V. cholerae (Steele et al., 1974; Heddle and Rowley, 1974) and E. coli (Knop and Rowley, 1974) lacked significant opsonic and complement-mediated bactericidal activity, even in the presence of lysozyme (Heddle et al., 1975).

Other antibacterial functions of IgA have been suggested such as bacteriolysis in the presence of complement and lysozyme (Adinolfi et al., 1966; Hill and Porter, 1974), bacteriostasis (Porter et al., 1974) or prevention of adsorption (Williams and Gibbons, 1972; Fubara and Freter, 1973). However, the relative importance of these mechanisms in antibacterial defence in vivo has not yet been assessed.

Recently, Steele et al. (1974) reported that specific IgA antibody protected infant mice against cholera. It was equally effective in

this respect with the other antibody classes (IgG and IgM) when compared on a weight basis.

b) Serum derived antibody

As mentioned above, in most intestinal secretions IgG and IgM are present and antibody activity in these classes against *V. cholerae* has been demonstrated in rabbits (Fubara and Freter, 1972; Steele et al., 1974; Menzel and Rowley, 1974), in dogs (Heddle and Rowley, 1975) and humans (Northrup and Hossain, 1970). Although some of the antibodies in these classes may be produced locally, there are many data in the literature which suggest that serum antibody is transported into the intestine in a functionally active state. Many authors could show that protection against cholera in various animal models could be achieved by passive intravenous and intraperitoneal immunization (reviewed by Freter, 1973; Feeley,1965; Jenkin and Rowley, 1960; Gilmore et al., 1966; Ghosh, 1970). Others, however, found only little protective effect of passively administered antisera with high bactericidal and agglutination titers (Pause et al., 1964; Finkelstein et al., 1964; Freter, 1964). These variable results may partly be explained by the quantitatively predominant antibody class in the

antisera used. Chaicumpa (1974) showed that passive immunization of infant mice via the i.p. or i.v. route with IgG antibody provided good protection while IgM antibody was quite poor in this respect. Antisera with high bactericidal and agglutination titer are likely to contain mainly IgM antibody which may only be transported into the intestinal lumen to a small degree (Wernet et al., 1972). Only very limited quantitative data are so far available on the amounts of antibody of the different immunoglobulin classes transported from the serum into the intestinal lumen (Wernet et al., 1972; Heddle unpublished observations) although this problem seems to be an important one to evaluate the route of optimal prophylactic immunization against enteric infections.

1.4.2 Phagocytic cells

The lamina propria of the normal intestinal mucosa is rich in a variety of immune cells, such as lymphocytes, plasma cells, eosinophiles and macrophages which can be seen in the core of the villi and infiltrating the epithelial cells (Curran and Creamer, 1965; Brandberg, 1969). Under inflammatory conditions the number of phagocytic cells found in the subepithelial tissue is greatly increased. Some studies

suggest that there may be a cell traffic of phagocytes through the epithelial cell layer. Thus the intestinal tract seems to be the principal site of granulocyte elimination (Teir and Rytömaa, 1966). These cells are occasionally found between two adjacent epithelial cells and migrating into the lumen (Takeuchi, 1967).
This normal traffic of granulocytes through the epithelial layer can experimentally be increased by specific immune reactions. Bellamy and Nielsen (1974) observed in pigs, sensitized to bovine serum albumin, a migration of large numbers of neutrophiles into the intestinal lumen after exposure of the intestinal mucosa to the immunizing antigen.

Phagocytic cells in the intestinal lumen containing bacteria have been observed in intestinal infections with S. typhimurium (Takeuchi, 1967) and Shigella (Takeuchi et al., 1965) although the numbers were quite small. In mucosal infections by organisms which do not penetrate and damage the epithelial cells such as cholera, light and electron microscopy never revealed the presence of phagocytic cells on the mucosal surface (Patnaik and Ghosh, 1966; Fresh et al., 1964; Gangarosa et al., 1960). Whereas macrophages appear to be involved in the early bacterial clearance from the mucosa of the

attachment sites. Smith and Linggood (1971) showed that antibody directed against the K88 antigen of *E. coli* protects against the disease and it is suggested that antibody might neutralize the attachment sites (Rutter and Jones, 1973; Smith, 1972). Prevention of adsorption of streptococci to buccal epithelial cells by secretory IgA antibody has been demonstrated recently by Williams and Gibbons (1972). Freter (see review 1973) analyzed in a series of papers the antibacterial function of antibody in experimental cholera. He observed using isolated ileal loops (Freter, 1972) or slices of ileum (Freter, 1970) in rabbits that antibody decreased the adsorption of the vibrios to the intestinal wall. This was due to blocking of adsorption and to an antibody-dependent antibacterial mechanism on the mucosal surface. This antibody-mediated antibacterial mechanism which also was demonstrated with IgA apparently required metabolizing epithelial cells since its effect could be inhibited by iodoacetate (Fubara and Freter, 1973).

The antibacterial mechanisms on the immune mucosal surface prevented only the growth of those vibrios which were in contact with the mucosa. Growth of the vibrios in the intestinal lumen was not affected. Recently Shedlofsky and Freter (1974) showed that

local antibacterial immunity in germ-free mice may act synergistically with bacterial antagonism in controlling the bacterial population in the lumen.- The mechanism of intestinal immunity against *V. cholerae* has also been investigated using an infant mouse *in vivo* model (Ujiye and Kobari, 1970). *V. cholerae* organisms given orally to 5 - 6 day old mice will elicit a diarrhoeal disease which is superficially similar to that in humans. The infant mice can be protected by antibacterial antibody (Neoh and Rowley, 1972). Protection is parallelled by a reduction of the *V. cholerae* population within the intestine which has prompted the suggestion that specific antibody may mediate the killing of the organisms within the lumen (Chaicumpa and Rowley, 1972). Recently Steele et al. (1974) demonstrated that all immunoglobulin classes (IgA, IgG, IgM) and $F(ab)_2$ antibody fragments protect infant mice from cholera. All three classes and $F(ab)_2$ were found to be equally protective on a weight basis. When comparing the efficiencies of the three antibody classes and $F(ab)_2$ in *in-vitro* antibacterial tests such as phagocytic tests involving either mouse peritoneal macrophages (Whitby and Rowley, 1959) or intravenous clearance (Jenkin and Rowley, 1961) or a bactericidal test with complement IgA antibodies and $F(ab)_2$ antibody

fragments showed no or only very little activity compared with IgG and IgM (Steele et al., 1974; Heddle and Rowley, 1975; Knop and Rowley, 1974). This strongly suggests that the classical anti-bacterial mechanisms - phagocytosis or complement-mediated killing - are not involved in the anti-bacterial immunity in this model. However, the results obtained in infant mice indicate that there is an antibody-mediated bactericidal mechanism operating in the intestinal lumen of the intestine.

In this thesis, based on the above observations, we used the infant mouse model to dissect the possible mechanism of antibody-mediated antibacterial immunity in the intestine. The outline of the experiments is based on the consideration that antibody may enhance unspecific antibacterial mechanisms already present in the intestinal lumen. Hence, the unspecific antibacterial mechanisms have been analyzed *in vivo* in the adult and infant mouse and the effect of antibody on these has been investigated in various *in-vivo* and *in-vitro* models.

CHAPTER 2

MATERIALS AND METHODS

2.1 Animals

2.1.1 Mice

An outbred strain of Swiss white mice was used for most experiments. Infant mice (6-day old) used throughout this work were litters of mothers from this strain. Germ-free BALB/C mice at an age of 8 weeks were obtained from the Walter and Eliza Hall Institute, Melbourne, Victoria, and used immediately after arrival. Conventional BALB/C mice of the same age were taken from the department's animal house.

2.1.2 Rabbits

Rabbits of various breedings were used to produce antisera.

2.2 Bacterial strains

2.2.1 V. cholerae 569B (classical, Inaba)

The original 569B was obtained from Mr. I. Huq of the Cholera Research Laboratories, Dacca. It was maintained as described elsewhere (Neoh and Rowley, 1972). The 569B from one ampoule was grown in nutrient broth, stabbed into agar stabs and stored at room temperature. Organisms were taken from these stabs when required by streaking on

agar plates and a single colony was used for experimental work.

2.2.2 V. cholerae 017 (El Tor, Ogawa)

This strain was kindly supplied by Dr. W.F. Verwey, Department of Microbiology, University of Texas, Medical Branch, Calveston, Texas. It was maintained as described for 569B.

2.2.3 Hybrid vibrio 569B-165

This strain was obtained from Dr. K. Bhaskaran, Central Drug Research Institute, Lucknow, India. In this hybrid vibrio the O-group antigens of 569B have been replaced by the O-antigens of a non-cholera vibrio strain NCV-165 (Gardner and Venkatraman, 1935) but the hybrid retained common flagellar antigens with 569B (Bhaskaran and Sinha, 1971). The hybrid was resistant to the bactericidal effect of 569B and O-165 antisera in the complement-mediated bactericidal assay (Rowley, 1968).

2.2.4 Vibrio strain V58 SR (III)

This non-motile Ogawa strain was kindly supplied by Dr. Bhaskaran.

2.2.5 E. coli 0111 BV

This strain (Rowley, 1954) was obtained from the

departmental collection and maintained as described for V. cholerae 569B.

2.2.6 Salmonella typhimurium C5

The bacteria were obtained from the stock of Jenkin and Rowley (1965). These organisms are resistent to the complement antibody-mediated bactericidal reaction (Auzins, 1968) and virulent for mice.

2.2.7 Salmonella typhimurium M206

This rough strain is sensitive to the complement-mediated bactericidal reaction of anti-C5 serum and is non-virulent for mice.

2.2.8 Streptomycin resistant mutants

Streptomycin resistant strains of the above bacteria were selected by making a thick lawn of the streptomycin sensitive organisms on nutrient agar plates containing 100 µg/ml of agar. Single colonies which grew over night were replated on fresh streptomycin plates. After further incubation the colonies were tested for slide agglutination against mono-specific antisera. The following streptomycin resistant mutants were used: V. cholerae 569B SR, 017 SR, E. coli BV SR, Salmonella typhimurium C5 SR and M206 SR.

2.2.9 Growth conditions and viable counts

In most experiments log phase bacteria grown in nutrient broth for 3 hours on a shaker at 37° were used. For viable counts the organisms were plated out on blood-base agar. Streptomycin was added when necessary to a concentration of 100 µg/ml of agar. Intestinal tissues containing bacteria were homogenized in saline using an Ultra Turrax mixer (Janke and Kunkel, K.G.). Homogenization for 1 - 2 minutes did not affect the bacteria significantly. Dilutions of the homogenates for viable counts were made in saline.

2.3 Media

2.3.1 Hank's balanced salt solution

This medium was prepared as described by Weller et al. (1952) and was buffered to the desired pH with 2.8 % sodium bicarbonate and 5 % CO_2 in air.

2.3.2 Media for the preparation of intestinal epithelial cells

Two media were used for the preparation of intestinal epithelial cells as described by Reiser and Christiansen (1971). Medium I (incubation medium) contained: 96 mM NaCl, 8 mM KH_2PO_4, 50 mM sodium citrate, 5.6 mM Na_2HPO_4, 1.5 mM KCl, 2.5 mg/ml bovine serum albumin and 1.5 mg/ml hyaloronidase

("Hyalase", Fisons Ltd., Loughborough, England) per ml. The medium was adjusted to pH 7.2 and aerated with oxygen. Medium II (collection medium) contained: 137 mM NaCl, 11.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.2 mM KCl and 5 mg/ml of bovine serum albumin. The solution was adjusted to pH 7.2 and gassed with oxygen/CO_2.

2.4 Oral administration of bacteria

Adult mice were pretreated with 0.1 ml of a 20 % Na-bicarbonate solution a few minutes before administration of the bacteria to neutralize gastric acid. The bacteria were given through a round-tipped 19 gauge needle usually in a volume of 0.2 ml.

2.5 Immunization

2.5.1 Vaccination of mice against V. cholerae 569B

For parenteral immunization mice were injected once weekly with 2 x 10^8 living organisms i.p. for 6 weeks. For oral immunization 2 x 10^9 live 569B were given three times a week for 5 weeks. One week after the last vaccination blood samples were taken from some mice of each group and tested for antibody activity. The other mice were then used for experiments.

2.5.2 Vaccination of mice against NCV 569B - 165

Mice were immunized parenterally (i.p.) with 2 x 10^8 live bacteria once a week for 10 weeks.

2.5.3 Vaccination of mice against *Salmonella typhimurium* C5

Parenteral (i.p.) and oral vaccination with this organism followed the same schedule as described before, using heat-killed bacteria (100°, 30 min.), each single dose containing 2 x 10^7 organisms for i.p. injection or 2 x 10^9 organisms for oral vaccination.

2.6 Antisera

2.6.1 Preparation and storage

Mice were bled from the retro-orbital plexus, rabbits by heart puncture. The blood was collected in sterile bottles or tubes, left for two hours at room temperature and over night at 4°. The blood clot was separated from the serum by centrifugation. The serum was heat-inactivated at 56° for 30 min. and stored in small portions at -20°.

2.6.2 Mouse anti-569B I

This serum was produced in mice immunized parenterally with live organisms as described above (2.5.1). It had a haemagglutinating titre of 1:80 and a vibriocidal titre of 1:10^5. The mercaptoethanol sensitivity of this serum suggested that IgM was the predominant antibody class.

2.6.3 Mouse anti-569B II

This serum was obtained from mice immunized for 12 weeks parenterally with live organisms. It had a haemagglutinating titre of 1:1000 and a bactericidal titre of 1:2x10^{6}. Its haemagglutinating activity was only partly reduced by mercaptoethanol suggesting that antibody of the IgG class was predominant in this serum.

2.6.4 Rabbit anti-569B and anti-017

Both sera were kindly provided by Dr. W. Chaicumpa of this department. These sera were produced in rabbits with live 569B or 017 and absorbed several times with the heterologous vibrios (Neoh, 1971). Mono-specificity was demonstrated by the absence of haemagglutinating activity against SRBC sensitized with the heterologous LPS and vibriocidal titre against the heterologous vibrio (Chaicumpa, 1974). The haemagglutinating titre of the anti-569B serum was 1:1000 and of the anti-017 serum 1:512.

2.6.5 Rabbit anti-Salmonella typhimurium C5

This antiserum was produced by intravenous injection of boiled organisms. The agglutination titre tested against boiled C5 was 1:1280.

2.6.6 V. cholerae 569B specific immunoglobulins

These were kindly supplied by E. Steele of this department. Rabbit secretory IgA was purified from colostrum (Steele et al., 1974) and had a haem-agglutinating activity of 1:6 and a bactericidal activity of 1:2 per mg protein. IgG containing anti-V. cholerae 569B activity was purified from serum of rabbits immunized with live organisms (Steele et al., 1974). This preparation had a haemagglutinating titre of 1:500 and a vibriocidal titre of 1:6.2 x 10^4 per mg.

2.6.7 Preparation of anti-O and anti-H antibody

Anti-somatic antibody (anti-O) was prepared from the IgG described above (2.6.6) by adsorption onto boiled glutaraldehyde (0.25 %)-fixed 569B over night. After 5 saline washes the adsorbed antibody was eluted from the bacteria with 0.2 m glycin/HCl (pH 2.2) and neutralized. Anti-flagella antibody (anti-H) was obtained by absorbing the IgG five times with boiled glutaraldehyde-fixed 569B.

2.7 Protein estimations

Protein estimations of purified immunoglobulins were determined using published extinction coefficients (Eddie et al., 1971) at 280 mμ on a spectrophotometer.

2.8 Determination of anti-somatic antibody

Specific anti-somatic antibodies were determined by quantitative precipitation (Neoh and Rowley, 1970) with 569B LPS.

2.9 Preparation of LPS from V. cholerae 569B, E. coli 0111 BV and Salmonella typhimurium C5

LPS from these strains was prepared by the method of Westphal, Lüderitz and Bister (1952).

2.10 Haemagglutination

2.10.1 Passive haemagglutination (HA)

SRBC were coated with alkali-treated LPS of the test organisms (Crumpton et al., 1958). The sensitized red cells were added to twofold dilutions of the antibody preparation and incubated at 37° for 45 minutes. The haemagglutinating endpoint was read after a further 3 hours at room temperature. The details of this test have been described elsewhere (Auzins, 1968; Neoh, 1971).

2.10.2 Mercaptoethanol (2-ME) reduction of HA

To one volume of serum or antibody preparation an equal volume of 0.2 m 2-ME in PBS pH 7.4 was added and incubated at 37° for 1 hour. This mixture was then tested in a passive haemagglutination test.

2.11 Bacterial agglutination

Serial dilutions of antibody were made in PBS pH 7.8. An equal volume (0.5 ml) containing 5×10^9 washed bacteria (boiled for 2 hours or living) was added to each tube. Tubes were incubated at 37° for 90 minutes and macrocospic agglutination endpoints were read after 16 hours at 4°.

2.12 Bactericidal assay

This technique has been described (Rowley, 1968). Serial dilutions of antibody were mixed with equal volumes of guinea pig serum, diluted 1:10 and containing 1×10^5 washed bacteria per ml. After incubation for 90 minutes at 37° viable counts were determined on each dilution. The bactericidal endpoint was defined as the highest dilution of the antiserum giving 50 % killing of the organisms. Controls with guinea pig serum were included in each assay.

2.13 Radioactive labelling of bacteria with P^{32}

V. cholerae 569B or other bacteria were labelled with P^{32} as described by Jenkin and Rowley (1961). To 10 ml of a log phase nutrient broth culture of the organisms one mCi of P^{32} as phosphate was added and the bacteria grown for a further 3 hours on a shaker at 37°. The bacteria were washed four times and resuspended in 2.0 ml of nutrient broth. The number of viable organisms/ml was

estimated by plating out various dilutions of the radioactive labelled culture on agar plates. The radioactivity was measured on 0.1 ml samples on filter paper and counted in an end-window-ß Chicago-counter. The specific activity of the bacteria =

$$\frac{\text{Counts/min} \times 1000}{\text{viable organisms}}$$

was in the range of 0.8 - 1.8.

2.14 Infant mouse protection test

This test is based on the findings of Ujiye et al. (1970) and has been further developed by Neoh and Rowley (1972) and Chaicumpa and Rowley (1972). A log phase culture of 569B containing 2×10^7 living organisms in a volume of 0.1 ml were given orally to 5 - 6 day old mice through a round-tipped 22 gauge needle. The inoculum was incubated at 37° for 20 minutes with either heat-inactivated normal serum or antiserum. About 18 hours after oral challenge diarrhoea developed and between 24 and 48 hours after infection 70 - 100 % of the mice were dead. Antibody given together with the inoculum or injected i.v. or i.p. prior to oral challenge protected the mice. The dilution of antiserum that protected 50 % of the infant mice (PD_{50}) from death (at 48 hours) was calculated by the method of Reed and Muench (1938).

2.15 Preparation of epithelial cells

Intestinal epithelial cells were prepared by a modification of the method of Reiser and Christiansen (1971). Adult mice were killed and the entire small intestine was removed, bisected, placed in ice-cold saline, and the lumens flushed with ice-cold saline. Each intestinal segment was ligated at one end and inoculated with one ml of prewarmed medium I (2.3.2). The other end was ligated and the segment placed into an Erlenmeyer flask containing prewarmed Hank's medium (2.3.1) plus one percent foetal calf serum. An oxygen-carbon dioxide mixture was bubbled through the medium to obtain a pH of 7.0 - 7.5. The ligated segments were incubated at 35° in a shaker water bath for 20 minutes, then placed on ice and the lumens flushed with ice-cold saline to remove debris and mucus. Medium II (2.3.2) was inoculated into each segment which was gently patted on ice and the contents discarded. The wash with medium II was repeated twice more and the recovered epithelial cells placed on ice. The cells were gently pressed through a stainless steel wire mesh, washed in 15 ml medium II without albumin, resuspended in 5.0 ml Hank's medium containing one percent foetal calf serum, heparin (5 units/ml) and insulin (5 units/ml) and an oxygen-carbon dioxide mixture bubbled through. Cell aggregates were removed by layering the suspension over 4.0 ml foetal calf serum on ice for 10 minutes and removing the supernatant containing the single cell

suspension. Total cell counts were determined on a haemocytometer and cell viability was assessed with 0.2 % Trypan Blue to ensure that at least 70 % of the cells were viable.

2.16 Histology and immune-fluorescence

Frozen sections or smears were fixed in 10 % methanol for one minute, dried and flooded with a 1:20 dilution of rabbit antiserum to *V. cholerae* 569B (haemagglutination titre 1:512) for 30 minutes in a moist atmosphere. They were washed twice for five minutes with PBS (pH 7.1) and redrained. The preparations were then covered with fluorescein-labelled goat anti-rabbit globulin for 30 minutes. The fluorescein-conjugated antiserum kindly provided by Prof. N.C. Nairn, Monash University, Melbourne, Australia, had a protein concentration of 120 µg/ml and a fluorescein to protein ratio of 3.8 : 1.0. Samples were washed twice again as above and mounted in buffered glycerol (pH 8.6). The fluorescent bacteria were examined using an Olympus Model FLM fluorescence microscope with a 2 mm UVI exciter filter and a Wratten 2B barrier filter. The specificity of the fluorescein label was demonstrated in intestinal sections without *V. cholerae* which did not show fluorescence.

2.17 Statistical methods

The dilution of antiserum that will protect 50 % of the infant mice (PD 50) from death (at a time when more than 90 % of the control mice had died) was calculated by the method of Reed and Muench (1938). The U-test of Mann and Whitney (Weber, 1973) was used to determine the significance of differences between two groups.

CHAPTER 3

ELIMINATION OF V. CHOLERAE 569B FROM THE GASTRO-INTESTINAL TRACT OF ADULT MICE

3.1 Introduction

In general, adult animals are not or little susceptible to mucosal infections of the intestine such as cholera. Considerable alterations of the normal physiology of the intestine produced by starvation, pretreatment with natrium-bicarbonate and morphin or alterations of the normal flora had to be accepted in models which made use of adult animals (Burrows et al., 1947; Freter, 1954 and 1956). Similarly, isolated intestinal loops (De and Chatterjee, 1953) which have been extensively used to assess virulence of *V. cholerae* or *E. coli* strains (Drucker et al., 1967) or potency of vaccination and antisera (Jenkins and Rowley, 1960; Freter, 1964; Kasai and Burrows, 1966; Kaur and Burrows, 1969; Finkelstein, 1970) most likely affect the natural unspecific resistance of the animals by altering normal physiological processes in the intestine.

The resistance of untreated adult mice to cholera is parallelled by a rapid removal of the organisms from the gastro-intestinal tract (Chaicumpa and Rowley, 1972). On the contrary, infant mice are susceptible to cholera (Ujiiye et al., 1968) and the vibrios multiply in their

intestinal tracts (Chaicumpa and Rowley, 1972). Similarly, infant rabbits (Dutta and Habbu, 1955) challenged orally with living organisms develop fatal cholera and increased numbers of vibrios can be found in their intestine (Feeley, 1965). It is most likely that insufficient developed unspecific factors participating in the rapid removal of *V. cholerae* from the intestinal tract of adult mice may partly be responsible for the increased susceptibility of infant mice to a cholera infection. It would, therefore, be interesting to analyze these factors in adult as well as in infant mice. In this chapter the influence of unspecific factors on the rate of removal and killing of *V. cholerae* from the gastro-intestinal tract of adult mice has been studied.

3.2 Materials and methods

3.2.1 Bacterial strains

V. cholerae 569B (2.2.1)

Salmonella typhimurium C5 (2.2.6)

Salmonella typhimurium M206 (2.2.7)

E. coli O111 BV (2.2.5)

All strains were used as streptomycin resistant (SR) mutants (2.2.8).

3.2.2 Treatments and substances used to influence intestinal clearance

A) Starvation. Mice were kept without food and sawdust for 24 or 48 hours. The cages were frequently cleaned to remove the droppings. The mice received water *ad libitum*.

B) Laparotomy. Mice were anaesthetised with ether and a laparotomy was performed. The incision was quickly closed with a suture and the mice challenged orally with *V. cholerae* 569B 30 minutes after the laparotomy. After 3 hours the mice were killed and their intestines processed as described above (2.2.9) and in (3.2.2). Control experiments showed that anaesthesia alone had no effect on the ability of mice to remove bacteria from their intestines.

C) Bleeding. About 0.5 ml of blood was taken from the retro-orbital venous plexus of each mouse. After 30 minutes these mice were orally challenged with *V. cholerae* and the viable organisms were determined in their small intestines.

D) Lipopolysaccharide. Samples were prepared from *V. cholerae* 569B and *E. coli* 0111 BV (2.9).

E) Zymosan (Sigma Chemical Co) was washed several times in boiling 0.9 % NaCl and suspended at 10 mg/ml in 0.9 % NaCl.

F) Colloidal carbon for injection consisted of Pelikan drawing ink diluted with 0.9 % NaCl and gelatine to a concentration of 100 mg/ml. 10 mg was injected into mice either i.v. or i.p.

G) Atropine sulphate monohydrate was injected s.c. immediately and 90 minutes after oral challenge. The dose given was 4 ng/mouse/injection.

H) Prostigmin (La Roche) was given orally 30 and 90 minutes after oral challenge to starved mice. Each mouse received 0.025 mg in a volume of 0.3 ml per dose.

I) Histamin phosphate (Koch-Light Labs) was given orally 30 and 90 minutes after oral challenge to starved mice. Each mouse received 5 mg p.dose.

J) Streptomycin sulphate. One dose of 50 mg was given orally to adult mice 24 or 48 hours before challenge with the test organisms (Miller and Bohnhoff, 1963).

3.2.3 Experimental procedure

Adult mice were given 1 - 2 x 10^8 living organisms of a log-phase streptomycin-resistant culture by mouth through a round-tipped needle (2.4). In most experiments the mice were pretreated with sodium-bicarbonate. At various times after challenge the

mice were killed and stomachs, small intestines and large intestines were taken out separately. Faeces were collected into a bottle of saline during the experiment. All the samples were homogenized in an Ultra-Turrax homogenizer and 0.1 ml of suitable dilutions of the homogenates were plated out for viable counting(2.2.9).In experiments where P^{32}-labelled bacteria (2.13) were used 0.1 ml of the undiluted homogenate was counted for radioactivity as described above (2.13). The total number of living organisms and the total counts/min. recovered were calculated and expressed as a percentage of the challenge dose. Some results were given as the specific activity of the recovered organisms (see 2.13).

3.3 Results

3.3.1 Distribution of *V. cholerae* 569B in the gastro-intestinal tract of normal mice

In mice which had not been pretreated with sodium-bicarbonate 99 % of the oral inoculum was killed in the stomach within 15 minutes. Pretreatment with 20 % sodium-bicarbonate increased the number of living organisms reaching the small intestine to about 10 - 30 % of the challenge dose. Table 3.1 shows the fate of P^{32}-labelled *V. cholerae* organisms in the gastro-intestinal tracts of normal mice over

a period of 5 hours. The labelled inoculum passed very quickly through the stomach and after 10 min. 70 % could be found in the small intestine. The total number of living organisms in the gastro-intestinal tract remained constant during the first 60 minutes and fell thereafter to a very low level. The mechanical movement of the radioactivity and living organisms into the large intestine which was observed between 60 and 90 minutes could not account for the dramatic fall in numbers of living organisms in the small intestine. Over this period of time the P^{32} recovery in the small intestine was almost halved (from 74 to 40 %) but the numbers of living organisms fell more than a thousandfold (20 % to 0.01 %).

Another way of expressing this is to note that the specific activity of the viable bacteria in the small intestine increased from 3 to 4000 between 60 and 90 minutes. The most likely explanation for the abrupt decrease of living organisms and the increase of specific activity in the small intestine between 60 and 90 minutes is that although continuous killing occurs during the first 60 minutes this is masked by those organisms which are less fixed to the intestinal wall and rapidly moved to the ileum in a living state. This is demonstrated in Table 3.2 where the distribution of living

Table 3.1: Distribution of ^{32}P-labelled V. cholerae in the gastro-intestinal tract of normal mice at various times after oral challenge. Numbers represent percent recovery from inoculum. Each point represents an average of 20 - 30 mice. Average dose given orally: 1-2 10^8 organisms.

Time in minutes	Stomach		Small intestine		Large intestine		Faeces		Whole G.I.Tract*	
	liv.org.*	^{32}P	liv.org.	^{32}P	liv.org.	^{32}P	liv.org.	^{32}P	liv.org.	^{32}P
10	0.25	30	18	70	0.0002	1	0.0001	1	18	100
30	0.01	22	18	70	0.5	2	0.0001	1	18	95
60	0.001	13	20	74	10	13	0.0001	1	30	100
90	0.001	12	0.01	40	6	38	0.0001	1	6	90
180	0.002	11	0.0009	30	2.0	50	0.004	4	2	95
300	0.02	0.25	0.0001	17	1.5	30	0.01	43	1.5	90

* Liv.org. = Living organisms

** G.I. = Gastro-intestinal

Table 3.2: Distribution of living V. cholerae and ^{32}P in various parts of the small intestine. Recovery in percent of inoculum. Average dose 1 x 10^8 organisms/mouse.

	Percent recovery of			
	liv.org.	^{32}P	liv.org.	^{32}P
	after 30 mins		after 90 mins	
Stomach	0.01	22	0.002	12
Small intestine part 1	0.02	16	0.001	5
part 2	0.1	15	0.001	18
part 3	12	20	0.001	7
part 4	6	19	0.003	10
Large intestine	0.5	2	6	38

organisms and P^{32} is shown in four separate parts of the small intestine. Although the distribution of P^{32} is about equal in each quarter of the small intestine the living organisms are all found in the parts representing the ileum. Between 30 and 90 minutes these living organisms were moved into the large intestine and killing of the bacteria remaining in the small intestine became obvious. There appeared to be a similar antibacterial effect in the large intestine between 60 and 180 minutes (Table 3.1). It seems clear that V. cholerae organisms pass rapidly through the mouse small intestine but of the number of living organisms reaching the small intestine after 10 minutes a large proportion was killed during their passage through the small intestine.

3.3.2 Effect of different treatments on the clearance of V. cholerae from the gastro-intestinal tract

A) Atropine. This parasympathomimetic drug inhibits peristalsis and secretion in the gastro-intestinal tract. When injected into mice before challenge with V. cholerae atropine reduced the mechanical removal of the P^{32}-labelled organisms from the stomach and small intestine (Fig.3.1). The finding that a greater percentage of organisms survived in the stomach was presumably due to the inhibition of gastric acid secretion by the atro-

pine. During the period of the experiment there was no reduction in the number of living organisms in the small intestine (Fig. 3.2).

B) Laparotomy. Laparotomy had an inhibitory effect on peristalsis (Fig. 3.1) which was not so marked as in the atropine treated mice. This was indicated by the delay in stomach emptying but it may be noted that the organisms remaining in the stomach were mostly killed. No antibacterial effect was observed in the small intestine of these mice (Fig. 3.2).

C) Starvation. The antibacterial effect in starved mice was significantly reduced (Fig. 3.2 and 3.3). But starvation had no obvious effect on the peristaltic movement of the organisms in the intestine. This is indicated by a similar distribution of P^{32}-labelled organisms in various parts of the gastro-intestinal tract at different times as in normal mice (Fig. 3.1). A significant number of organisms survived in the stomach indicating that the gastric acid secretion in starved animals was reduced. The ability to eliminate *V. cholerae* rapidly from the small intestine could be restored in starved animals by giving food 30 minutes after oral challenge or by oral application of prostigmin or histamine (Table 3.3). Glucose

Fig. 3.1: Distribution of ^{32}P-labelled V. cholerae 569B in the gastro-intestinal tract of untreated ▒, starved ☐, atropine treated ▨ mice and in mice where a laparotomy had been performed ⊡.

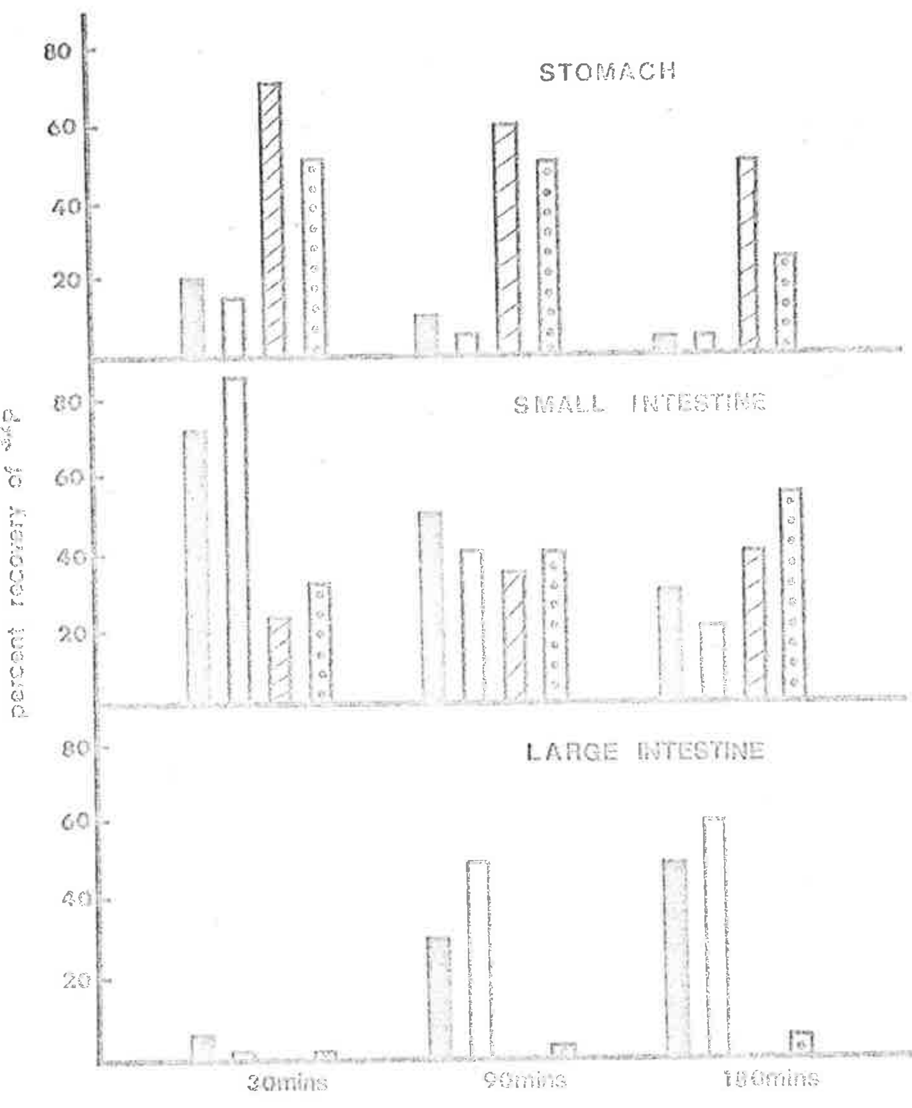

STOMACH
SMALL INTESTINE
LARGE INTESTINE
percent recovery of 32P
80
60
40
20
30mins
90mins
180mins

Fig. 3.2: Recovery of living *V. cholerae* 569B from the gastro-intestinal tract of untreated [ ], starved [ ], atropine treated [ ] mice and in mice where a laparotomy had been performed [ ].

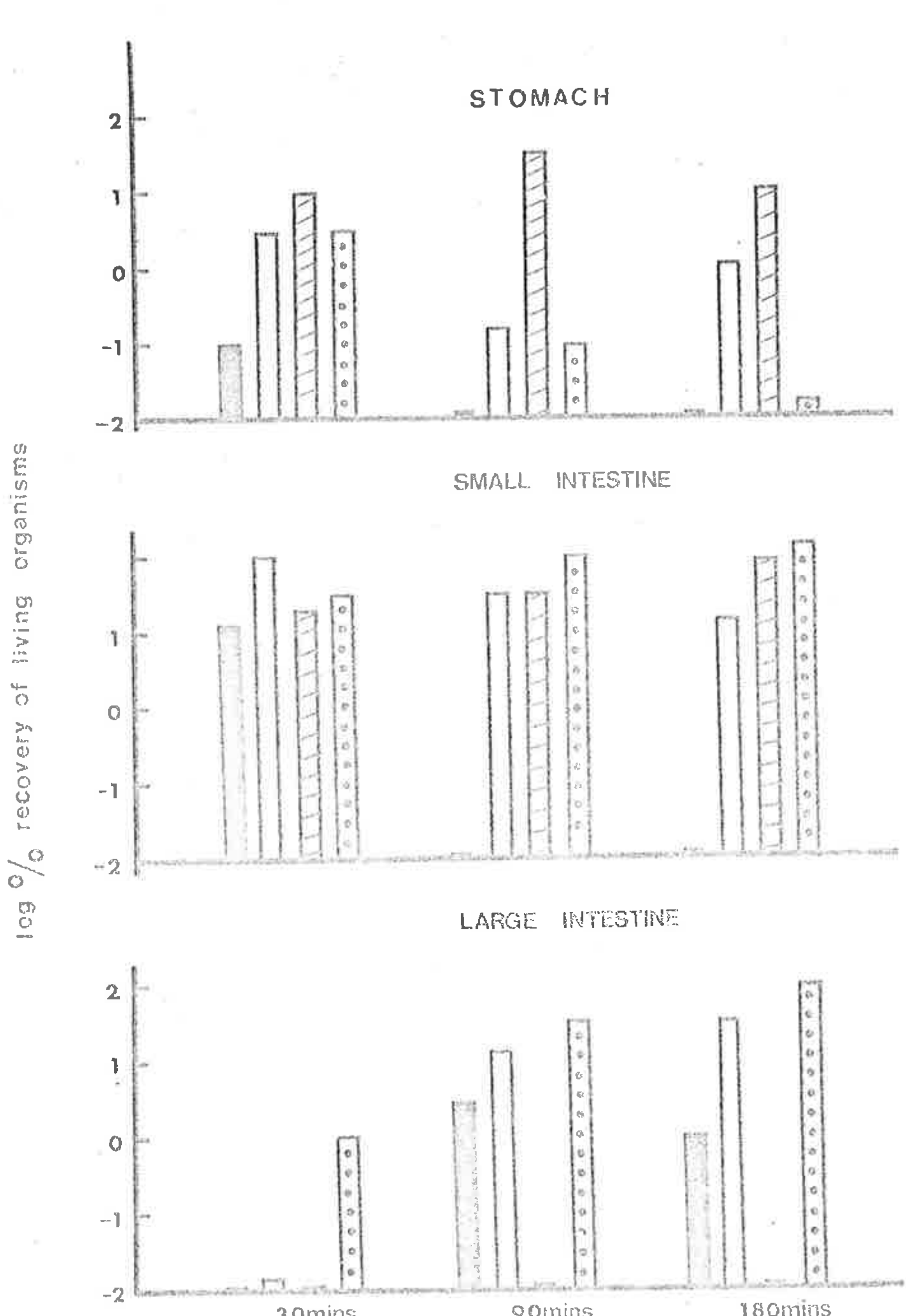
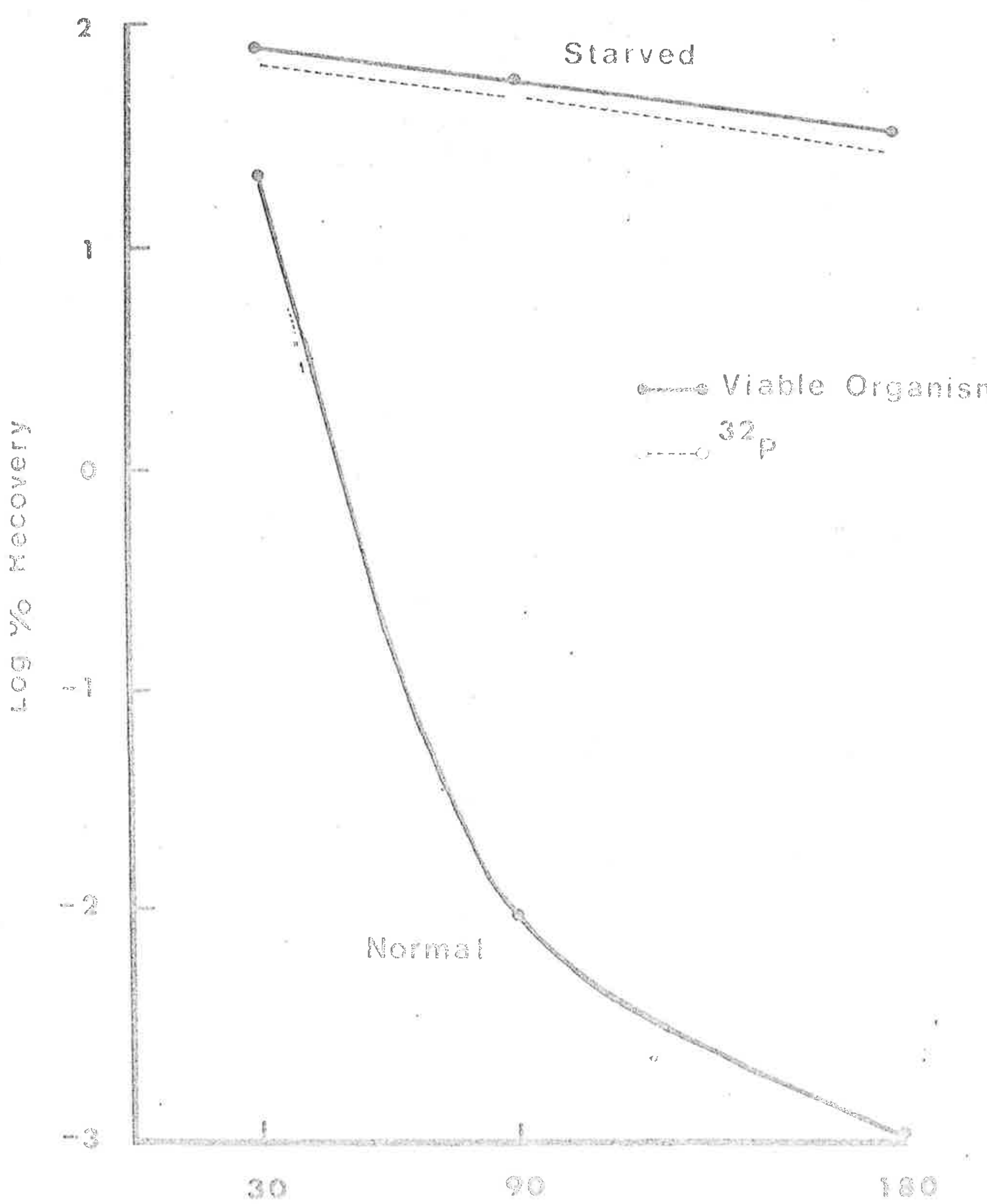

STOMACH
SMALL INTESTINE
LARGE INTESTINE
log % recovery of living organisms
2
1
0
-1
-2
30mins
90mins
180mins

Fig. 3.3: The recovery of viable *V. cholerae* and of the associated ^{32}P label from the small intestine of mice at intervals after feeding the ^{32}P-labelled organisms.



Starved
Viable Organisms
^{32}P
Normal
Log % Recovery
2
1
0
-1
-2
-3
30
90
180
Time After Feeding (Min.)

Table 3.3: Restoration of the killing ability in the small intestine of starved mice. Oral inoculum: 5×10^8 organisms.

Treatment	Recovery of living organisms in the small intestine in percent of the inoculum after 3 hours
Non-starved	0.001
Starved, no further treatment	20
Starved, 0.3 ml of saline orally prior to oral challenge	15
Starved, 100 mg glucose i.v. 10 min. before oral challenge	6
Starved, pellets given 30 min. after oral challenge	0.02
Starved, histamine orally 30 and 90 min. after oral challenge	0.2
Starved, prostigmin orally 30 and 90 min. after oral challenge	0.01

injected i.v. had no effect.

D) Effect of streptomycin. Streptomycin given orally inhibited the killing of the organisms in the small and large intestine (Table 3.4). Since most of the organisms reached the large intestine in 3 hours peristalsis seemed not to be effected.

E) Other inhibitors of intestinal clearance. All substances and treatments listed in Table 3.5 inhibited to various degrees the elimination of *V. cholerae* from the small intestine during the 3 hours of the experiment. Generally these substances were more effective when given i.p. than i.v. Oral administration was not effective.

Table 3.4: Recovery of V. cholerae 569B SR from the gastro-intestinal tract of streptomycin-treated mice in percent of challenge dose.

Time after streptomycin dose	Living organisms recovered after 3 hours in small intestine	large intestine
24 hours[x]	2 ± 1	60 ± 40
48 hours[x]	3 ± 2	30 ± 20

Challenge dose: 1×10^8 organisms

x = each group 12 mice

Table 3.5: Effect of different treatments on clearance of V. cholerae organisms from the gastro-intestinal tract of mice. Challenge dose per mouse: 1×10^8 organisms.

Treatment		% recovery of liv.org. from the small intestine at 3 hours
Non-treated		0.001
Bleeding		20
Zymosan 50 µg or 250 µg i.v.	30 min. before challenge	40
Carbon 10 mg i.v.	30 min. before challenge	0.001
Carbon 10 mg i.p.	30 min. before challenge	20
V. cholerae LPS 0.1 mg i.p. or 1 mg i.v.	60 min. before challenge	30
E. coli BV LPS 0.01 mg i.p. or 0.1 mg i.v.	60 min. before challenge	35
2×10^7 living V. cholerae organisms i.p.	60 min. before challenge	25
4 x injection of 0.1 ml pyrogen free saline during the experiment		0.5

3.3.3 Elimination of other gram-negative organisms from the gastro-intestinal tract of normal mice

A comparison was made of the removal of P^{32}-labelled *S. typhimurium* C5 and *V. cholerae* from the gastro-intestinal tract. There was a fourfold increase in specific activity of both organisms recovered from the small intestine 15 minutes after oral challenge, presumably due to killing of 75 % of the organisms in the stomach. After 3 hours the very high specific activity of the *V. cholerae* organisms found in the small intestine and the increased specific activity of the organisms recovered from the whole gastro-intestinal tract demonstrated a further substantial killing. The number of living *S. typhimurium* C5 organisms found after 3 hours in the small intestine was reduced (Table 3.6) although not so markedly as *V. cholerae*. However, mechanical removal seemed to be the dominant factor in the elimination of *S. typhimurium* C5 from the small intestine. Although there was an increase in specific activity (Table 3.6) of the organisms found in the small intestine the specific activity of the total organisms recovered indicated slight growth during the period of the experiment. Other non-virulent gram-negative organisms (Table 3.7) behaved similar to *S. typhimurium* C5.

Table 3.6: Elimination of *S. typhimurium* C5 from the mouse gastro-intestinal tract.

Time minutes	Organism	Specific activity of bacteria in Stomach	Small Intestine	Large Intestine	Total
15	V. cholerae 569B	120 ± 50	6 ± 4	n.d.	8 ± 3
	S. typhimurium C5	90 ± 70	5 ± 2	n.d.	6 ± 2
180	V. cholerae 569B	n.d.	33,000 ± 10,000	60 ± 5	100 ± 2
	S. typhimurium	n.d.	1,400 ± 400	2.4 ± 2.0	2.9 ± 2.0

$$\text{Specific activity} = \frac{\text{counts/min/ml} \times 1000}{\text{no. of living organisms}}$$

Specific activity of inoculum = 1.6

Table 3.7: Clearance of different organisms from the G. I. Tract of Mice

Organism	Oral dose	No. of mice	Percentage recovery of challenge dose after 20 minutes: Stomach	20 minutes: Small Intestine	180 minutes: Small Intestine	180 minutes: Large Intestine
V. cholerae 569B	1 x 10^8	24	0.001 ± 0.004	10 ± 6	0.0005 ± 0.0003	0.8 ± 4.0
E. coli 0111 BV	1 x 10^8	15	4.5 ± 4.0	35 ± 20	0.01 ± 0.02	22 ± 10
Salmonella typhimurium C5	1 x 10^8	24	0.5 ± 0.3	10 ± 5	0.02 ± 0.04	20 ± 10
Salmonella typhimurium M 206	1 x 10^8	18	1.5 ± 2.0	15 ± 10	0.02 ± 0.04	12 ± 5

3.4 Discussion

Adult mice removed living *V. cholerae* organisms rapidly from their gastro-intestinal tract over a period of 5 hours. As the results show very few living organisms could be found in the small intestines 3 hours after oral challenge. The stomach with its gastric acid provided an efficient barrier against bacteria. This was demonstrated by the great number of vibrios entering the small intestine alive once the gastric acid had been neutralized or its secretion inhibited by atropin (Fig. 3.2) or starvation (Fig. 3.2). The factors involved in the elimination of living organisms from the small intestine are:

1. Mechanical removal by peristalsis
2. Killing of the organisms in the small intestine.

A further substantial reduction of living vibrios occurred in the large intestine.

The rapid passage of the P^{32}-labelled organisms through the small intestine was an indicator of the vigour of peristalsis. The importance of peristalsis was shown by the fact that inhibition of peristalsis by atropine completely prevented the elimination of the organisms. The small intestine was not able to reduce the number of living organisms significantly once peristaltic movement stopped. Two explanations appear possible. Prevention of peristalsis could inhibit directly the mechanism by which the organisms were killed. Alternatively, the killing system operated only on those organisms which were close

to the intestinal wall and the preponderant remainder of the organisms were removed by peristalsis. Once mechanical removal was prevented by, for example, atropine although localized killing could continue, it would no longer be measurable since the small percentage of organisms close to the intestinal wall and susceptible to the killing action would be overwhelmed by the continued presence of the great majority of the organisms. This synergistic action of peristaltic removal and killing in the small intestine is further demonstrated in Fig. 3.4. An increase of specific activity of the vibrios in the small intestine could only be detected after the bulk of the organisms had been propelled into the large intestine.

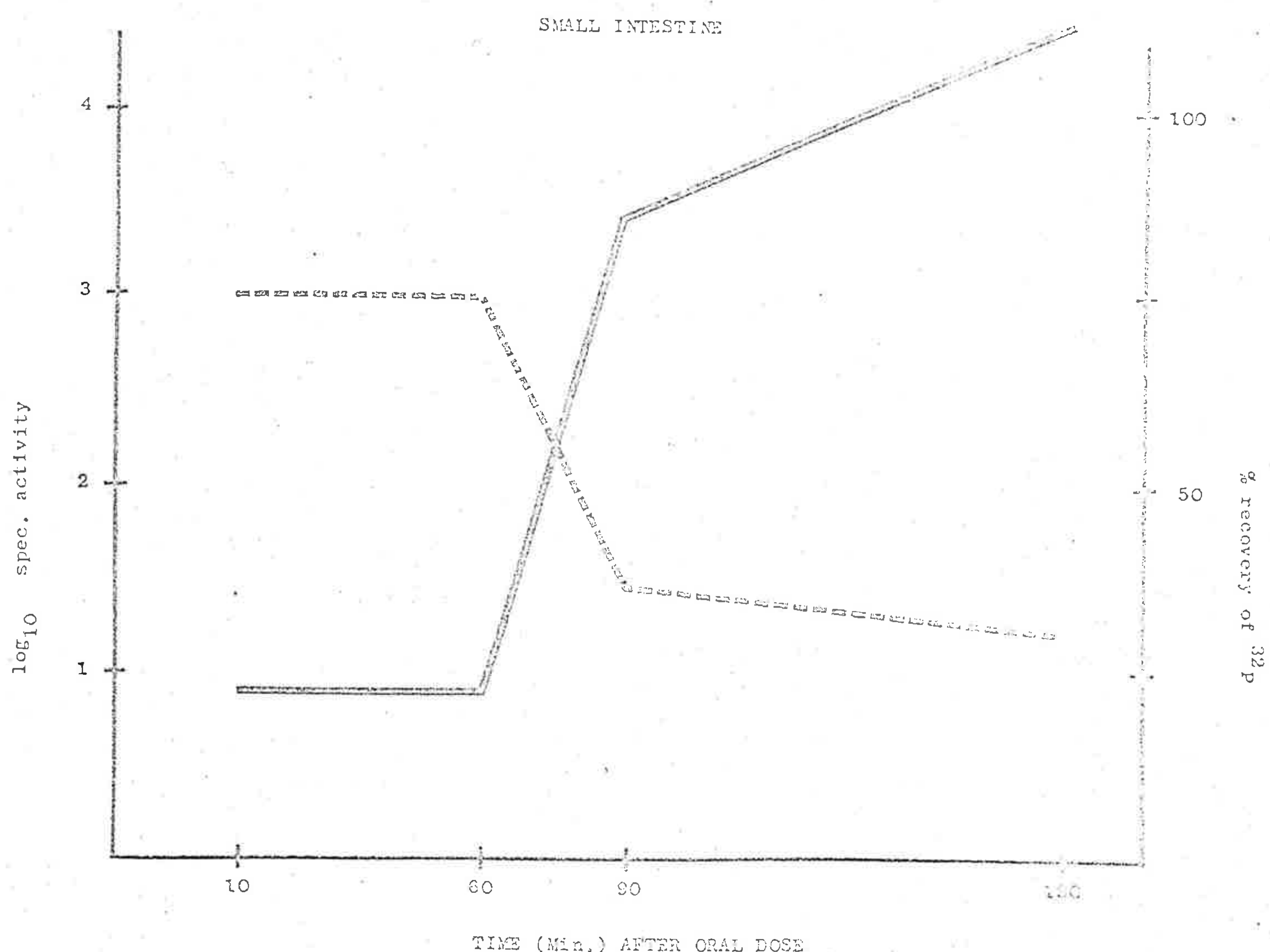
Other treatments like laparotomy, extensivo bleeding or injection of toxic substances like LPS, all of which delayed the rapid elimination of V. cholerae organisms from the small intestine, acted partly through inhibition of peristalsis. This could be deduced from the slower passage of the P^{32}-labelled organisms through the small intestine (Fig. 3.1) and was also demonstrated by the fact that most of the substances were more efficient when given i.p. than i.v.

It is interesting to note that starvation, like atropine, increased the numbers of organisms surviving the conditions in the stomach. This was obviously due to inhibition of gastric acid secretion. The results with starved animals indicate that inhibition of peristalsis is not an adequate

Fig. 3.4: Elimination of V. cholerae 569B from the small intestine of adult mice: Synergism between killing and mechanical removal.

——————— specific activity

- - - - - - - P^{32}


SMALL INTESTINE
4
3
2
1
log10 spec. activity
100
50
% recovery of 32P
10
60
90
TIME (Min.) AFTER ORAL DOSE

explanation for the delay of elimination of V. cholerae from the small intestine. The distribution of radioactive organisms through the gastro-intestinal tract of starved animals was similar to that found in normal animals (Fig. 3.1). Nevertheless, those organisms located in the small intestine were not killed appreciably.

The indigenous flora of conventional animals is known to have a considerable influence on their susceptibility to enteric infections (Miller and Bohnhoff, 1963). The nutritional state of animals similarly affects their susceptibility and it has been claimed that effects of inadequate diet are due to alterations in the numbers and types of the indigenous intestinal flora (Dubos and Schaedler, 1962). It seems unlikely that the inhibitory effect of acute starvation found in our experiments could be explained in terms of altered microflora. The main argument against this is the rapid restoration of normal behaviour which could be achieved by giving food at the time or even 30 minutes after challenge (Table 3.2). Similarly, histamin and prostigmin given 30 and 90 minutes after oral challenge rapidly reversed the effect of starvation suggesting that they operated by restoration of normal secretory function in the intestine. This is supported by Stening and Grossman (1969) who demonstrated that secretory processes in the small intestine were diminished during starvation and that food or histamine would instantly stimulate secretion. As stated earlier, there is

a measurable antibacterial process occurring in the small intestine and it now seems likely that intestinal secretions provide a necessary factor in this process.

On the other hand the indigenous flora is of importance to prevent the colonization of the intestinal tract by the challenge organisms. Streptomycin destroyed the anti-bacterial effect on V. cholerae in the intestine.

A few other gram-negative organisms were removed mechanically as rapidly as V. cholerae. In the case of S. typhimurium C5 which is virulent for mice there was evidence from the increase of specific activity which occurred at 3 hours (Table 3.6) of a bactericidal effect in the small intestine but this was clearly not as marked as with V. cholerae. This is in agreement with Meynell and Subbaiah (1963) who showed that S. typhimurium was removed mainly mechanically but that there was also a weak bactericidal reaction in the intestine.

CHAPTER 4

ELIMINATION OF V. CHOLERAE 569B FROM THE GASTRO-INTESTINAL TRACT OF INFANT MICE: ENHANCING EFFECT OF ANTIBODY

4.1 Introduction

In the adult mouse bacterial pathogens such as *V. cholerae* are removed from the small intestine by peristalsis and a killing mechanism. In this killing the normal flora and probably bactericidal secretions of the mucosa play a role (chapter 3). The adult mouse is not a suitable model with which to study the role of antibody in the elimination of enteric organisms from the small intestine, for two reasons:

1) The normal adult mouse has measurable amounts of natural antibodies against *V. cholerae* and these might participate in the efficient removal of the organisms from the intestine.

2) The elimination of *V. cholerae* by peristalsis and killing occurs so rapidly in adult mice that it would be difficult to measure any additional effect of antibody.

The infant mouse is susceptible to cholera but can be protected by antibody. This is accompanied by a decrease in the number of living organisms recoverable the gastro-intestinal tract (Chaicumpa and Rowley, 1972).

In this chapter using the same experimental approach as described in chapter 3 we have analyzed the elimination of V. cholerae 569B from the gastro-intestinal tract of infant mice and compared the results with those obtained after giving opsonized bacteria.

4.2 Materials and methods

4.2.1 Bacterial strain: V. cholerae 569B SR (2.2.1 and 2.2.8).

4.2.2 Antiserum: Rabbit anti-569B (2.6.4).

4.2.3 Opsonization:

5.0 ml of a P^{32}-labelled culture (2.13) of V. cholerae 569B containing approx. 2×10^8 orgs/ml were mixed with 0.1 ml of rabbit anti-569B serum (4.2.2) and incubated at 37° for 15 minutes. The non-opsonized bacteria were treated similarly with normal rabbit serum.

4.2.4 Experimental procedure

Two groups of 6-day old mice were given orally 0.1 ml of an opsonized or non-opsonized ^{32}P-labelled culture of V. cholerae 569B containing 1.8×10^8 living organisms and 2.1×10^6 c.p.m. At various times thereafter the mice were killed, stomachs, small intestines and large intestines removed separately and these as well as the remaining carcasses

homogenized separately in saline (2.2.9). Aliquots of each were plated out for viable and radioactive counts. Each point in the figures represents the mean ± SD of 7 - 9 mice. The significance of the results was determined as described (2.17).

4.3 Results

Fig. 4.1 shows the movement of the radioactive label from the stomach into the large intestine over a time of 6 h. The stomach was emptied quite rapidly although about 15 % of the opsonized bacteria moved relatively slowly into the small intestine compared with the non-opsonized organisms. The reason for this is not clear but it will have some implications on the interpretation of the elimination of vibrios from the small intestine.

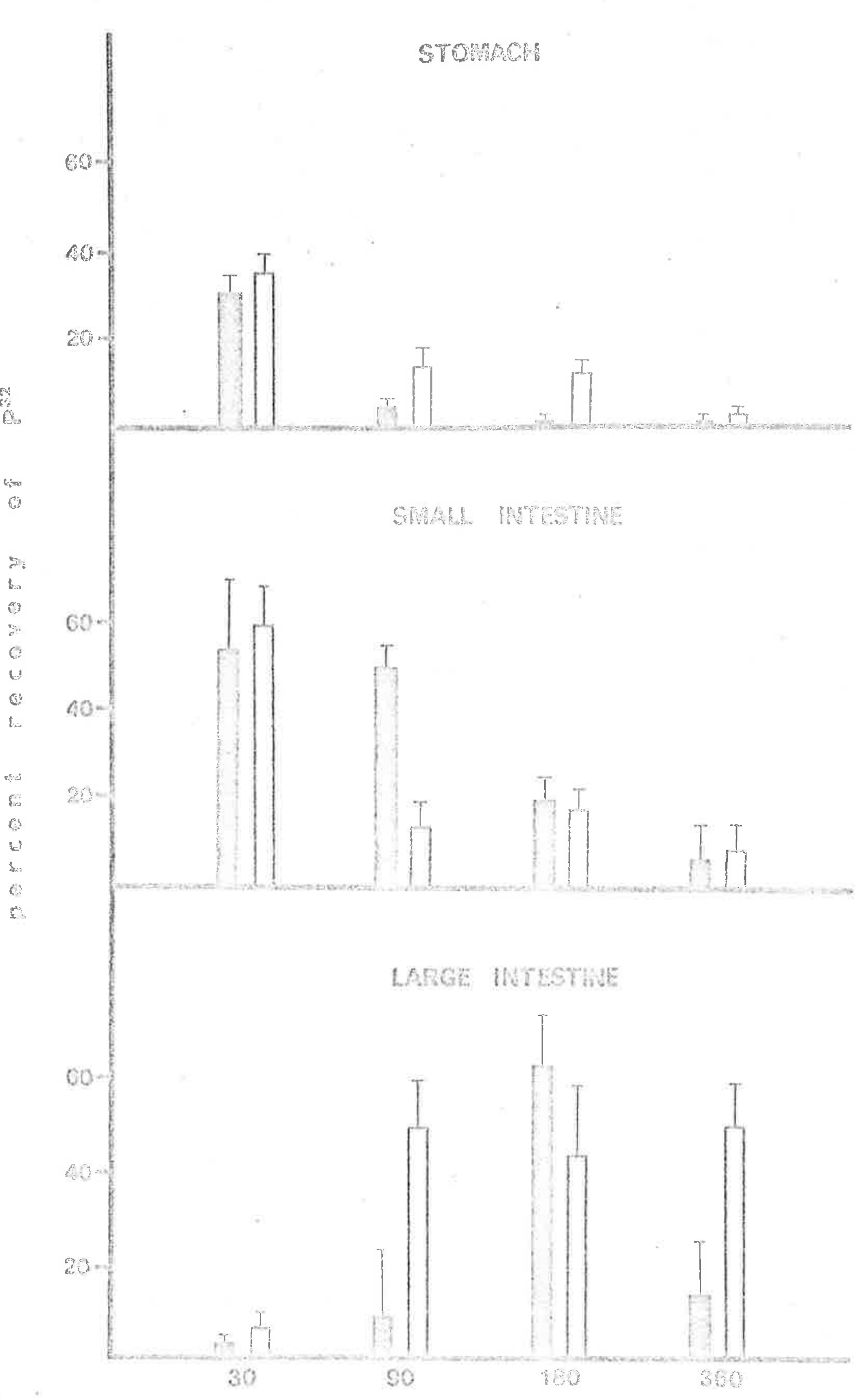
Bacteria treated with antibody passed more rapidly through the small intestine into the large intestine (Fig. 4.1). The difference between the two groups are significant after 90 min in both small ($P < 0.02$) and large intestine ($P < 0.02$ The amounts of radiotracer found in both groups after 180 and 360 minutes was not significantly different although one would expect less ^{32}P-labelled vibrios in the antibody groups. This, however, may be due to the 15 % opsonized bacteria in the stomach most of which were released into the small intestine between 90 and 360 minutes.

In the large intestine significantly less ($P < 0.02$) radiolabel was present in the control after 360 min by comparison with the antibody group. It is most probable that the mice challenged with non-opsonized V. cholerae already had diarrhoea and consequently excreted the radioactive label more rapidly.

The recovery of living V. cholerae 569B after various times in different parts of the gastro-intestinal tract are shown in Fig. 4.2. Most of the organisms found in the stomach after 90 min were dead in both groups. Only a small proportion (10 - 20 %) of the total bacterial population was killed in the stomach. This is indicated by the specific activity of the vibrios which reached the small intestine after 30 or 90 min (Fig. 4.3) which was not significantly different from the initial inoculum. In the small intestine the antibody-treated vibrios were removed more rapidly than the non-treated vibrios (Fig. 4.2). Significant differences between the two groups were found after 90 min ($P=0.02$) and 360 min ($P < 0.002$).

The number of organisms found in the large intestine reached a maximum after 90 min in the opsonized and 180 min in the control group. The numbers decreased much more rapidly in the opsonized group than in the non-opsonized. After 360 min approx. 0.08 % of the opsonized and 4.3 % of the non-opsonized bacteria were found to be still viable

Fig. 4.1: Distribution of ^{32}P-labelled V. cholerae 569B in the gastro-intestinal tract of infant mice. ▒ Bacteria treated with normal rabbit serum. ☐ Bacteria treated with rabbit anti-569B serum.


STOMACH
SMALL INTESTINE
LARGE INTESTINE
percent recovery of P32
60
40
20
30
90
180
360
minutes

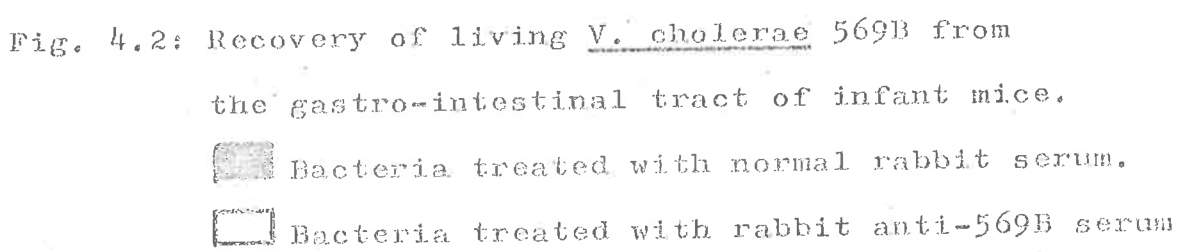


Fig. 4.2: Recovery of living V. cholerae 569B from the gastro-intestinal tract of infant mice.

Bacteria treated with normal rabbit serum.

Bacteria treated with rabbit anti-569B serum.

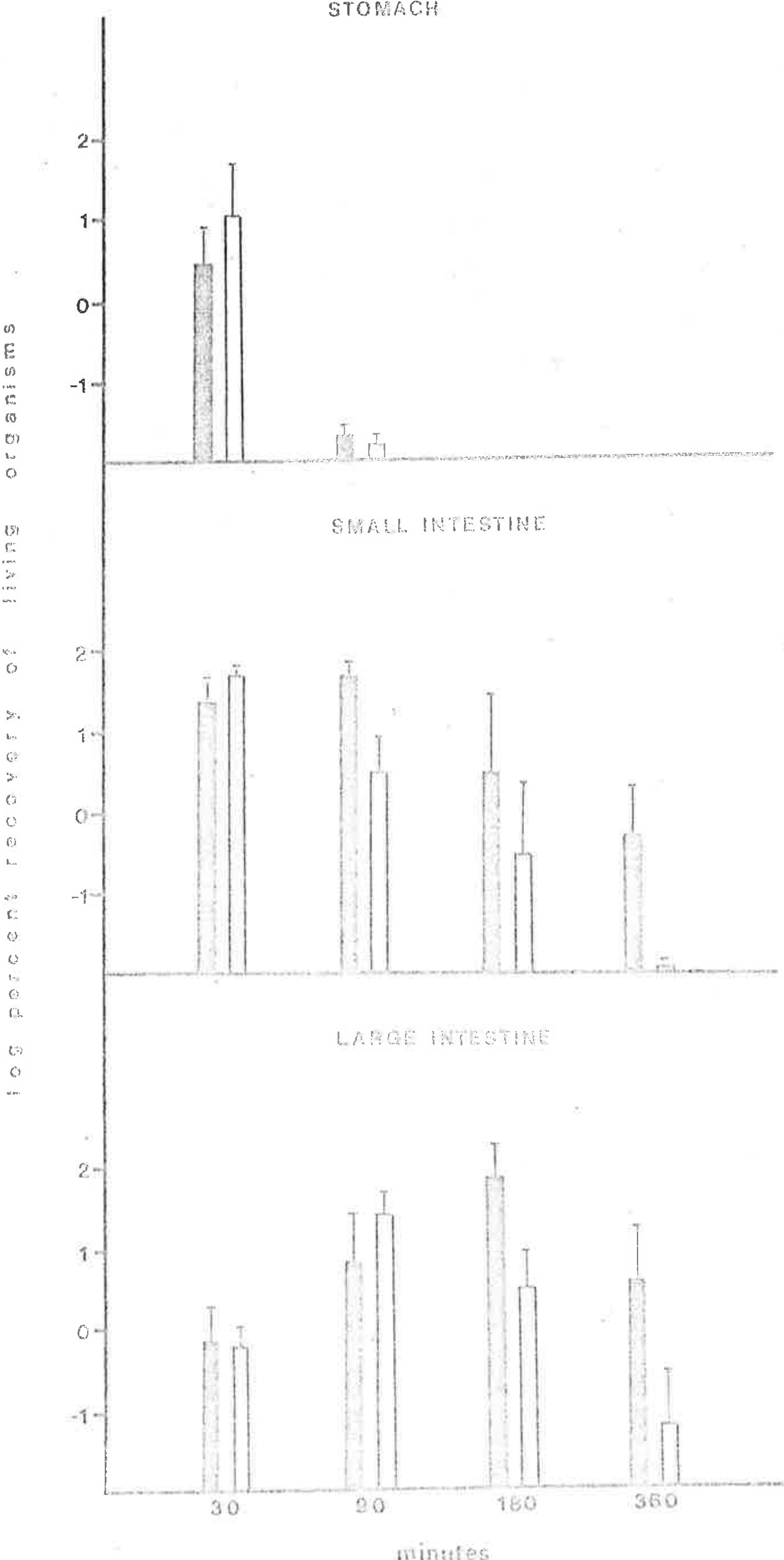
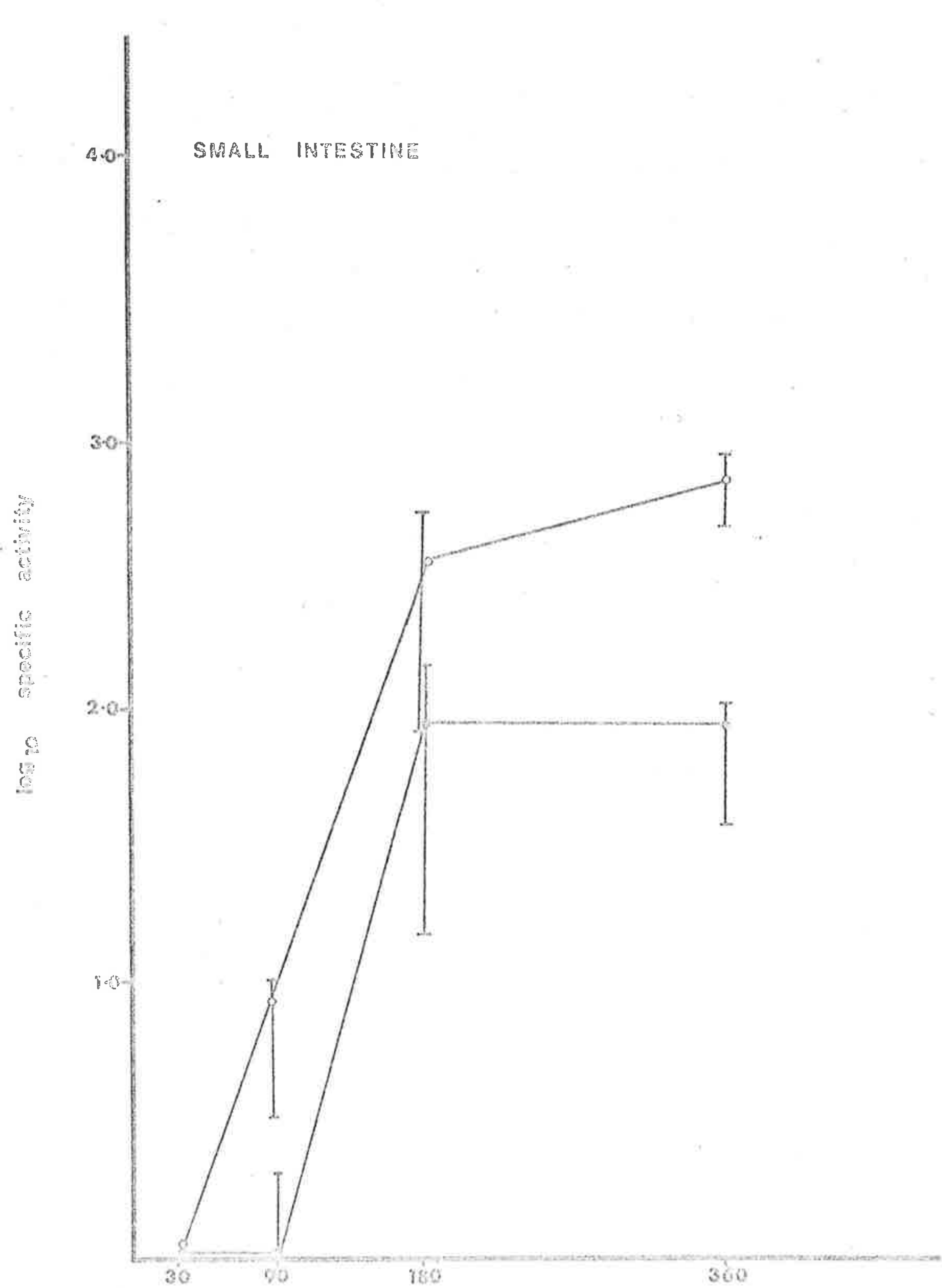
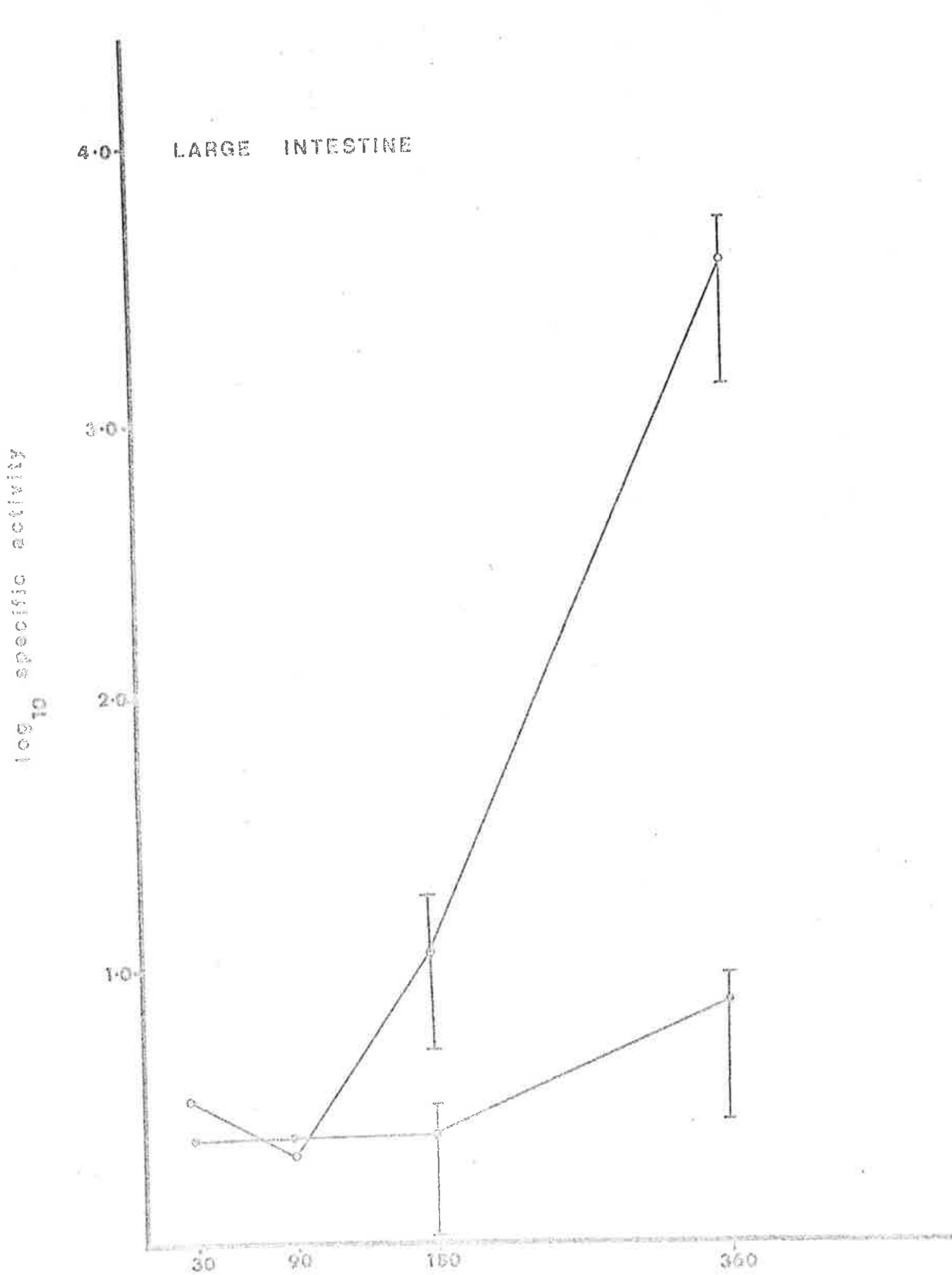

STOMACH
SMALL INTESTINE
LARGE INTESTINE
log percent recovery of living organisms
2
1
0
-1
30
90
180
360
minutes

Fig. 4.3: Change of specific activity of *V. cholerae* 569B non-opsonized ●——● and opsonized ○——○ in the small intestine of infant mice.


SMALL INTESTINE
4·0
3·0
2·0
1·0
log10 specific activity
30
90
180
360
minutes

($P < 0.002$). In Fig. 4.3 the specific activities are shown of the ^{32}P-labelled vibrios recovered from the small and large intestine after various times. In the small intestine the specific activity of the non-opsonized bacteria after 30 and 90 min was similar to the inoculum. Between 90 and 180 min killing of the organisms occurred as indicated by an increase of specific activity. No further increase was found after 180 min. The situation with opsonized bacteria was different. The specific activity increased from 1.0 at 30 min to about 100 at 180 min. A further but less dramatic increase was found between 180 and 360 min. The specific activities of both groups are only significantly different at 90 min ($P \leqslant 0.02$) and 360 min ($P = 0.001$). There was no significant difference at 180 min. In the large intestine (Fig. 4.4) the initial specific activities in both groups were approx. the same and only slightly increased. Only a slight increase of the specific activity (ca. 5) of the non-opsonized bacteria was found in a period of 360 min. In the opsonized group, however, a dramatic increase of the specific activity of the bacteria was found between 90 and 360 min.

Fig. 4.4: Change of specific activity of V. cholerae 569B non-opsonized ●——● or opsonized ○——○ in the large intestine of infant mice.


LARGE INTESTINE
4·0
3·0
2·0
1·0
log10 specific activity
30
90
180
360
minutes

4.4 Discussion

Similar to the findings in adult mice mechanical removal by peristalsis and killing appear to be the basic mechanisms also in infant mice by which *V. cholerae* is eliminated from the intestine. Antibody seems to have an effect on both these mechanisms. It is evident from Fig. 4.1 that in the presence of antibody the vibrios passed down the small intestine and accumulated in the large intestine faster than in its absence. Thus, peristalsis becomes a more efficient clearing mechanism in the presence of antibody. This could be due to prevention of adherence of the *V. cholerae* to the intestinal wall (Freter, 1969, and see chapter 6) which would tend to facilitate the removal by the peristaltic stream.

Concurrently with the more rapid removal of bacterial substance there was a rapid killing of those organisms left behind in the small intestine as indicated by a dramatic change in their specific activity (Figs. 4.3 and 4.4). This already existing process is clearly enhanced in the presence of antibody. As shown in Fig. 4.3 the opsonized organisms increase in specific activity after 30 min. compared with the 90 min. time at which a similar increase was seen with the control organisms. These times are those at which the bulk of the organisms has left the small intestine in the two cases (Fig. 4.1), and, therefore, the killing observed was of the remaining minority. It was probable that it was this small proportion of organisms

which were held back in the small intestine by association with the epithelium and that only when the bulk of the organisms had been removed mechanically were any changes in specific activity realizable. This observation is similar to that made in adult mice (see Fig. 3.4). Since antibody had an effect on removal rates it becomes difficult to determine whether antibody had any direct effect on the epithelial cell-associated killing. Two possibilities exist. Either antibody has a direct sensitizing action on bacteria, thereby rendering them more susceptible to some epithelial cell metabolic product, or antibody, by lowering the bacterial load associated with the epithelial surface, allows the killing process to occur efficiently on the reduced number of attached bacteria, and that this process itself is not influenced by antibody. Perhaps the only piece of evidence which supports one or other of these possibilities is the slope of the specific activity lines in Fig. 4.3 which are the same with or without antibody. If anything, this indicates that the killing process is the same with or without antibody and there favours the second alternative.

The situation is even more complex in the large intestine since the results may be influenced by increased fluid secretion which occurs in baby mice after challenge with V. cholerae in absence of antibody. This is indicated by an increased excretion of ^{32}P in the normal mice (Fig. 4.1),

most likely to diarrhoea. This could change the normal environment in the large intestine (composition of the normal flora) and also its normal physiological function (increased amount of fluid, no normal pellet formation). These altered conditions may have a considerable influence on the killing of V. cholerae in the large intestine. Some support for this is given by the results of Chaicumpa and Rowley (1974) who showed that substances such as cholera-toxin, lysolecithin and prostaglandins inhibited to some degree the killing of V. cholerae in infant mice. Shedlofsky and Freter (1974) using germ-free mice found a more rapid elimination of V. cholerae in the large intestine of immunized animals when they were contaminated with an autochtonus flora. They postulate a synergism between local immunity and the normal intestinal flora. Our data support this suggestion for the large intestine. However, the upper small intestine in man (Nelson and Mater, 1970) and infant mice (Schaedler, Dubos and Costello, 1965) is relatively free of a normal flora. The killing which occurred in the infant mice even without antibody could therefore be independent of a normal flora. It would therefore be interesting to accumulate more data on this killing mechanism which apparently operates on the mucosal surface of the small intestine of adult and infant mice and to determine the effect of antibody on it.

CHAPTER 5

A BACTERICIDAL MECHANISM ON THE MUCOSAL SURFACE OF THE SMALL INTESTINE OF MICE

5.1 Introduction

In chapter 3 and 4 we observed that those *V. cholerae* and, to a lesser extent, other gram-negative bacteria which remained in the small intestine after the majority of bacteria had been removed into the cecum by peristalsis, were dead and that the normal flora and possible the mucosa itself participated in this killing.

In this chapter a killing mechanism operating on the mucosal surface of the small intestine of adult mice will be described and analyzed using ligated intestinal loops and isotope-labelled bacteria.

5.2 Materials and methods

5.2.1 Mice: See 2.1.1.

Conventional and SPF adult Swiss White Mice

Infant Swiss White Mice

Germ-free and conventional BALB/C mice

5.2.2 Bacteria

V. cholerae 569B (2.2.1)

V. cholerae 017 (2.2.2)

NCV 569B-165	(2.2.3)
S. typhimurium C5	(2.2.6)
E. coli O111 BV	(2.2.5)

5.2.3 Antisera

Mouse anti *V. cholerae* 569BI	(2.6.2)
Rabbit anti 569B and O17	(2.6.4)
Rabbit anti *S. typhimurium* C5	(2.6.5)
Rabbit anti 569B - sIgA	(2.6.6)

5.2.4 Experimental procedure

Mice were laparotomized under ether narcosis and the small intestine ligated at about 5 cm and 10 cm below the stomach, thus producing a loop approx. 5 cm long. Into this loop 0.1 ml of ^{32}P-labelled bacteria (2.13) containing approx. $4 - 5 \times 10^8$ and $1 - 2 \times 10^6$ c.p.m. were injected with a 26 gauge needle. The loop was put back into the abdominal cavity and the abdomen covered with a tissue soaked in warm saline. After 10 minutes the loop was taken out, cut open and washed gently in 10 ml of saline. It was then washed vigorously by 10 sec of mechanical agitation in 10 ml of saline. Finally, the washed piece of intestine was homogenized in 2.0 ml of saline (2.2.9). All wash fluids and the homogenate were plated out for radioactive and viable counts.

The results in Tables 5.1 - 5.6 were calculated on the assumption that the % of radioactivity found in any sample indicated the initial % of viable organisms in that sample and that any change found in the viable numbers was due to killing or to growth of the attached organisms. Since it is unlikely that any significant amount of ^{32}P will leave the bacteria without the organism being killed, this assumption is probably valid (Spitznagel and Wilson, 1965).

5.2.5 <u>Streptomycin treatment</u>

48 hours before the experiments each mouse was given 50 mg of streptomycin orally.

5.2.6 <u>Extraction of the radiotracer from the loop</u>

The method used to recover the ^{32}P label fixed to an intestinal loop after a standard experiment was basically the one used to isolate epithelial cells from the intestinal mucosa (Reiser and Christiansen, 1971) and described in 2.15. The loop which had been cut open and thoroughly washed was incubated for 30 min at 35° in 2.0 ml of medium I containing hyaloronidase (2.3.2). After incubation the piece of intestine was transferred into 2.0 ml of medium II (2.3.2) and was vibrated on a whirlmix for 2 min to release epithelial cells. It was then homogenized in

another 2.0 ml of medium II. The released epithelial cells were centrifuged at 500 rev/min for 5 min, tested for viability with trypan blue and stained with Wright-Giemsa. Aliquots of the incubation medium, homogenates, wash fluids and supernatants were assayed for the radio tracer.

5.3 Results

5.3.1 Fate of ^{32}P-labelled V. cholerae 569B attached to the intestinal wall

Table 5.1 shows the amount of radioactive label and the number of living organisms recovered from the various wash fluids and the homogenized tissue after 10 min incubation of ^{32}P-labelled *V. cholerae* 569B in an intact intestinal loop. Most of the radioactivity and the living vibrios were recovered in the first and second wash fluid with complete viability. The third wash fluid contained very little radioactivity and only 40 % of this was still in living organisms. About 3 % of the radio tracer was still associated with the tissue after the final wash compared to 1,7 % in the final washing fluid, indicating that this radioactivity was firmly bound to the tissue. More washings removed little further radio tracer. The number of living organisms found in the washed piece of intestine amounted to 0.07 % of the inoculum.

Accordingly, only 2.5 % of the finally attached bacteria were still viable (Table 5.1).

Different results were obtained in a similar experiment using ^{32}P-labelled S. typhimurium C5. Most of the bacteria were recovered in the first and second wash and the organisms recovered in the washed and homogenized piece of intestine showed only a 50 % drop in viability (Table 5.1).

In Table 5.2 the recovery of radio tracer, living organisms and the change of viability after various times of incubation of V. cholerae 569B in a loop is shown. The amount of label fixed to the washed loop reached its maximum after 5 min and remained constant for 60 min. The numbers of attached living organisms showed a steady decrease for the first 10 min but increased after 30 min. The viability showed a decrease after 1 min and was lowest between 5 and 30 min but rose again by 60 min. This increase by 60 min is probably the result of an overall growth of the total bacterial population in the loop. Since the viability of the firmly attached vibrios was lowest 10 min after injection this time point was chosen for all subsequent experiments.

Table 5.1: Killing of ^{32}P-labelled organisms absorbed to the intestinal wall after 10 minutes' incubation in an *in vivo* mouse intestinal loop.

Organism	Sample	% of initial dose recovered as ^{32}P	% of initial dose recovered as living organisms	% viability of recovered bacteria
V.cholerae 569B SR	1. Wash fluid	58 ± 5	65 ± 7	100 ± 30
	2. Wash fluid	37 ± 3	33 ± 9	100 ± 20
	3. Wash fluid	1.7 ± 0.9	0.5 ± 0.3	40 ± 8
	Washed intestine	2.7 ± 1.4	0.07 ± 0.03	2.6 ± 1.0
S.typhimurium C5	1. Wash fluid	83 ± 7	88 ± 7	100 ± 25
	2. Wash fluid	14 ± 5	10 ± 6	90 ± 26
	3. Wash fluid	1.6 ± 0.9	0.6 ± 0.3	90 ± 32
	Washed intestine	2.5 ± 1.4	1.4 ± 0.8	54 ± 25

Mean ± SD of 8 mice

Injected into loop: $5 - 7 \times 10^8$ living organisms

$2 - 4 \times 10^6$ c.p.m. of ^{32}P

Table 5.2: Killing of ^{32}P-labelled V. cholerae 569B attached to the intestinal wall at various times after injection into the loop

Time (min)	% of initial dose recovered as ^{32}P	% of initial dose recovered as living organisms	% viability of attached bacteria
1[a]	1.2 ± 0.5	0.17 ± 0.07	10 ± 6
5	2.7 ± 0.5	0.14 ± 0.10	4.2 ± 1.5
10	2.7 ± 1.2	0.07 ± 0.03	2.6 ± 0.8
30	2.5 ± 0.8	0.08 ± 0.03	4 ± 1.3
60	2.3 ± 0.9	0.27 ± 0.11	12 ± 4

[a] each time point 3 - 6 mice

Injected: approx. 7.8×10^8 living vibrios and 1.6×10^6 c.p.m. of ^{32}P.

5.3.2 Attachment and killing of *V. cholerae* 569B in the intestinal loop without blood supply

An intestinal loop was made as usual and the blood vessels supplying this loop were coagulated. After 10 min the ^{32}P-labelled vibrios were injected into the loop and incubated for 10 min. The loop was then cut out, washed and homogenized as described. The result of such an experiment is shown in Table 5.3. Although the amount of radioactive label was about half that recovered from the intact loop, the numbers of living vibrios found in the loop without blood supply was twentyfive times higher. Accordingly, the survival of the organisms attached to these loops was high. This indicates that a rapid killing occurs of those *V. cholerae* attached to the intact intestinal mucosa, and that this killing requires an intact blood supply for its full expression.

5.3.3 Effect of prior streptomycin treatment on the killing process

Inhibition of the normal flora with streptomycin has been shown to allow colonization of the intestine by pathogenic organisms such as *S. typhimurium* (Miller and Bohnhoff, 1963) or *V. cholerae* 569B (Freter, 1956). The normal flora may act on invading bacteria by secreting bactericidal sub-

Table 5.3: Killing of V. cholerae 569B associated with mouse intestinal mucosa after 10 minutes' exposure

Mice	No. viable orgs. initially attached ($x 10^6$) [b]	No. of living orgs. recovered ($x 10^6$)	% viability of attached bacteria	Significance
Normal Conventional	15 ± 5[a]	0.3 ± 0.015	2	S.
Conventional no blood supply	9 ± 2	7.5 ± 3.0	83	
Normal S.P.F.	7.9 ± 1.9	0.4 ± 0.25	5	N.S.
Streptomycin-treated S.P.F.	6.6 ± 1.2	0.53 ± 0.16	7	
BALB/C Conventional	5.6 ± 2.8	0.16 ± 0.07	2.6	N.S.
BALB/C Germ-free	5.6 ± 2.5	0.30 ± 0.08	5.3	

[a] Mean ± SD from 5 - 6 mice in each group

[b] Calculated from % radioactivity associated with the washed tissue

Inject.: 4 - 6 x 10^8 live organisms

3 - 6 x 10^6 c.p.m.

stances such as organic acids (Meynell, 1963). In order to find out if the normal flora played a part in the killing of V. cholerae 569B in our model, SPF mice were pretreated with 50 mg of streptomycin 48 h before the experiment. All mice had an extended cecum filled with feces mixed with gas and fluid (Savage and Dubos, 1968). The results in Table 5.3 demonstrate that the killing mechanism still operates after streptomycin treatment.

5.3.4 Killing of mucosa-associated vibrios in germ-free mice

Since streptomycin did not abolish the killing mechanism on the attached vibrios one might expect this killing mechanism to operate in germ-free mice. As shown in Table 5.3 the attached vibrios were clearly killed in germ-free animals.

5.3.5 Effect of passive antibody or immunization on the killing of ^{32}P-labelled bacteria attached to the intestinal wall

Opsonization of the bacteria or immunization of the mice did not increase the killing of the organisms tested (Table 5.4). Even organisms such as S. typhimurium or V. cholerae 017 which normally showed only a slight decrease in their viabilities (Table 5.1 and 5.6) were not affected by antibody

Table 5.4: Effect of antiserum or immunization on the killing of V. cholerae 569B, 017 and S. typhimurium C5 attached to the intestinal wall after 10 minutes' incubation in a loop.

	% viability of attached organisms			
	Test organism		Mice immunized	
	non-opsonized	opsonized	orally	parenterally
V. cholerae 569B (Inaba)	2.5 ± 0.6	3.3 ± 0.7 [a]	4.0 ± 1.0 [d]	3.3 ± 1.1 [e]
V. cholerae 017 (Ogawa)	40 ± 10	33 ± 12 [b]	n.d.	n.d.
S. typhimurium C5	30 ± 20	31 ± 10 [c]	25 ± 12 [f]	32 ± 15 [g]

a with 20 haemagglutinating units rabbit anti V. cholerae 569B

b " 20 " " " " " " 017

c " 10 bacterial agglutination units rabbit anti S. typhimurium

d serum haemagglutination titre 1/20

e " " " 1/256

f " bacterial agglutination titre 1/4

g " " " " 1/2000

n.d. = not done

or active immunization. It should be noted that antibody had also no significant quantitative effect on the fixation of the organisms to the mucosa in this model.

5.3.6 Killing of mucosa-attached *V. cholerae* 569B in infant mice

As shown in chapter 3 the killing rate of *V. cholerae* in the small intestine of infant mice appeared to be increased in the presence of antibody. Therefore, the effect of antibody on the killing of *V. cholerae* attached to the intestinal wall of infant mice was tested. Since the intestine in these mice is very soft no attempt was made to produce loops. The ^{32}P-labelled organisms (volume 30 ul) were injected directly into the small intestine. Most of the small intestine was taken out 10 min later, cut open and washed as described. The results are given in Table 5.5. Only a slight decrease of viability of non-opsonized organisms was found and this was not changed by opsonization with whole antiserum or purified yA antibodies.

5.3.7 Sensitivity of different gram-negative organisms towards the killing mechanism

On Table 5.6 some other *V. cholerae* strains and other gram-negative organisms are listed which had

Table 5.5: Killing of V. cholerae 569B associated with the intestinal wall of the small intestine of 6-day old infant mice and the effect of antibody on it

Vibrios opsonized with	% viability of vibrios recovered from the washed loop	
NRS	50 ± 7	(6)[x]
Rabbit anti 569B γA [a]	83 ± 21	(4)
Mouse anti 569B serum [b]	100 ± 32	(4)

x Number of mice

a 2 haemagglutinating units added to inoculum

b 20 " " " " "

Table 5.6: Killing of different bacteria associated with mouse intestinal mucosa after 10 minutes exposure

Organism	No. viable orgs. initially attached ($x\ 10^6$) [b]	No. live organisms recovered ($x\ 10^6$)	% Survival
V. cholerae 569B	8.8 ± 4 [a]	0.34 ± 0.14	4.0
V. cholerae 017	9.2 ± 2.8	4.4 ± 3.1	48
NVC 569B-165	4.0 ± 0.8	0.20 ± 0.16	5.0
E.coli OIII BV	3.6 ± 1.8	2.4 ± 1.7	66
S.thyphimurium C5	5.0 ± 2.0	2.8 ± 2.1	56

a Mean ± SD of 6 - 8 mice

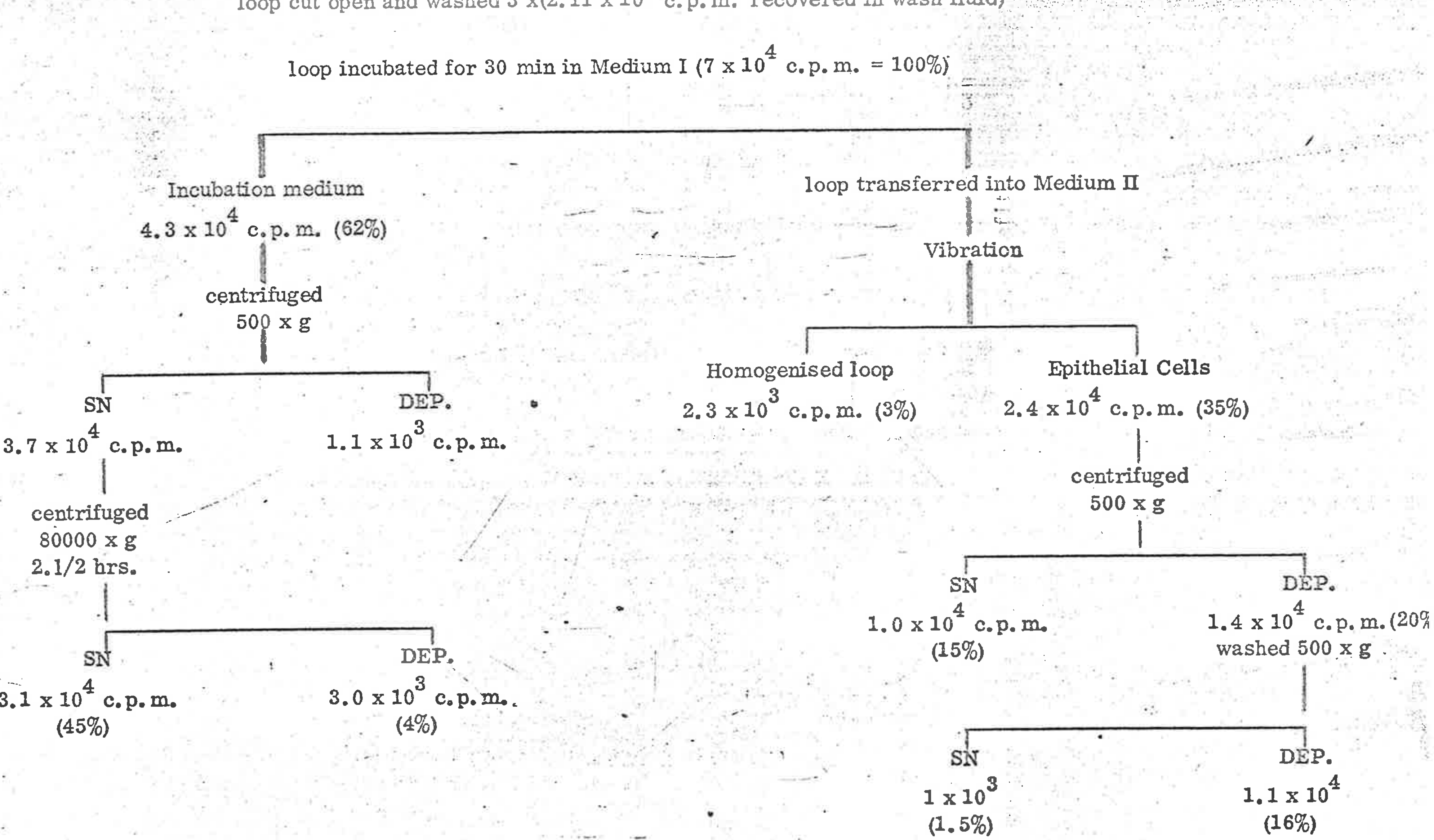
b Calculated from % radioactivity associated with the washed tissue

been tested in this loop model. In addition to *V. cholerae* 569B the hybrid vibrio 569B-165 which does not share somatic antigens with 569B was similarly sensitive towards the killing. On the other hand, *V. cholerae* 017 appeared to be quite resistant. Both *E. coli* 0111 BV and *S. typhimurium* C5 were quite resistant to the killing and the amount of radio label bound to the mucosa was similar for both organisms and comparable to *V. cholerae 569B*.

5.3.8 Localization of the killed vibrios attached to the washed intestinal loop

The radio tracer attached to the washed tissue was originally located in approx. 2 - 4 x 10^7 *V. cholerae* organisms of which only 5 x 10^5 were still viable at 10 min. In order to obtain some data on the nature of the killing mechanism an attempt was made to locate the dead organisms. One approach was to section the washed loop after the usual type of experiment and to detect organisms under the U. V. microscope using fluorescein-labelled antibody. Although one would expect approx. 1000 vibrios per section only an occasional vibrio could be seen. It seemed, therefore, possible that the attached ^{32}P label was no longer in antigenically intact bacteria but was in the form of fragments or even

Table 5.7: Location of the radio label in the intestinal loop



SN = supernatant
DEP = sediment

free ^{32}P. We, therefore, tried to determine the location of the label. A method was adopted which has been used to isolate epithelial cells from the intestinal mucosa. The result of such an experiment is shown in Table 5.7. After incubation of the washed loop for 30 min in a medium containing hyaloronidase about 60 % of the label was found in the incubation medium and 35 % was associated with the epithelial cells after they had been vibrated off the mucosa. About 80 % of the epithelial cells were alive as tested by the trypan blue exclusion test and 95 % of the cells could be identified as epithelial cells. The rest were either leukocytes, lymphocytes or non-identified cells. Further washing of the epithelial cells removed another 15 % of the label leaving 20 % of the total label firmly cell-bound.

5.4 Discussion

Our results suggest that a bactericidal mechanism exists on the mucosal surface of the small intestine which is able to control bacterial growth to some extent. This suggestion rests on the assumption that the greater proportion of radioactivity attached to the intestinal wall by comparison with the numbers of viable organisms is not due to preferential absorption of free ^{32}P or of dead organisms in the inoculum. Several data, however,

indicate that these alternative explanations are not likely:

1) Using other organisms such as V. cholerae 017 or S. typhimurium C5 the increase of specific activity is considerably less than with V. cholerae 569B although the amount of free ^{32}P or already dead organisms is approximately the same.
2) Results obtained in loops without blood supply demonstrate directly that the radioactivity firmly bound to the mucosa represents intact organisms which in this case are not killed.
3) In the infant mouse, using opsonized V. cholerae 569B, the percentage viability found in the firmly fixed organisms was not different from that in the inoculum. This could not happen if free ^{32}P or dead organisms in the inoculum were preferentially absorbed to the mucosa.

It appears also unlikely that the killing occurred in the lumen followed by absorption of dead organisms or fragments on to the mucosa. As shown in Table 5.1 no measurable killing occurred of those organisms free in the lumen. The dead organisms found on the mucosal surface and indicated by the radio tracer represent a very small part of the whole population (3 %). These organisms would not significantly increase the amount of dead bacteria already present in the inoculum, and for reasons given above it is not likely that these are preferentially absorbed to the mucosa.

Although the fixed vibrios are initially killed very rapidly the killing does not continue with the same rate as indicated by the increase of viability after 30 min. This is parallelled by an overall growth of the whole bacterial population present in the loop. This inability of the killing mechanism to control growth of the whole bacterial population can partly be explained by the unphysiological nature of the loop model. Under physiological conditions, the majority of the organisms would be flushed into the large intestine, the remaining ones would be spread over a much wider surface area and thus the infecting organisms could be removed quite effectively. On the other hand, this result points out that this mucosal killing mechanism has its limitations and can be overcome once too many organisms are attached on to the mucosa at one spot.

Some conclusions about the nature of this killing mechanism may be drawn from the results obtained. Most of the radioactivity was recovered from the medium containing hyaluronidase in which the tissue was incubated. Another part could be removed from the isolated epithelium cells by washing. Since most of the epithelial cells were intact after they had been released from the mucosa by vibration this would indicate that lysis of most of the organisms occurred on the epithelial cell surface or in the mucous layer. After enzymatic digestion of the mucous layer the labelled bacterial fragments were released into the in-

cubation medium. Only 20 % of the label was firmly fixed on to or inside the epithelial cells.

The killed organisms could not be located microscopically with fluorescent antibody, probably because the bacteria were lysed to such an extent that the fragments were no longer antigenically intact. This is supported by the fact that 90 % of the radio tracer is of small molecular size as shown by centrifugation. A similar loss of small molecular size compounds after lysis by antibody and complement has been shown by Spitznagel (1966 a and b) using smooth ^{32}P-labelled enterobacteraceae. Following further conclusion about the nature of the killing mechanism can be drawn from the results obtained:

1) An intact blood supply is required for maximum efficiency. This could mean that the epithelial cells must be metabolising for the mechanism to be efficient (see also GENERAL DISCUSSION).
2) The normal flora seems not to have an effect on this killing mechanism as shown in streptomycin-treated and germ-free mice.
3) This killing mechanism seems to be much less developed in infant mice.
4) Antibody given passively or active immunization has no effect on the killing of the attached organisms.
5) Different gram-negative organisms are variably sensitive towards this killing. This agrees with the results we

obtained in intact adult mice where ^{32}P-labelled *S. typhimurium* C5 and *E. coli* O111 BV were much less killed than *V. cholerae* 569B (see 3.3.3 and Table 3.7).

CHAPTER 6

EFFECT OF ANTIBODY ON THE ADSORPTION OF V. CHOLERAE 569B ON THE MUCOSAL SURFACE: A PROTECTIVE MECHANISM IN INFANT MICE

6.1 Introduction

In the infant mouse antibody clearly enhanced the mechanical removal of *V. cholerae* 569 B from the small intestine (4.2), most likely due to prevention of adsorption of the organisms to the intestinal wall. Further evidence that antibody may prevent the adsorption of pathogens to epithelial cells is given by the results of Freter (1969) and Williams and Gibbons (1972).

In this chapter we have investigated several antibody preparations for their abilities to reduce adsorption of *V. cholerae* 569B to isolated intestinal epithelial cells and correlated these to agglutination and protective activities in infant mice.

6.2 Materials and methods

6.2.1 Bacterial strains

V. cholerae 569B	(2.2.1)
V. cholerae 017	(2.2.2)
V. cholerae V58 SR (III)	(2.2.4)
NCV 569B 165	(2.2.3)

Some of these strains were used as streptomycin resistant mutants (2.2.8).

6.2.2 Antisera and purified antibodies

Mouse anti-569B I (2.6.2)

Rabbit secretory IgA and serum IgG with anti-569B activity (2.6.6)

Anti-O and anti-H antibody, preparation see 2.6.7

6.2.3 Antibody assays

Haemagglutination assay (2.10.1)

Bactericidal assay (2.12)

Tube agglutination (2.11)

6.2.4 Preparation of isolated intestinal epithelial cells

Described in 2.15

6.2.5 Adherence of vibrios to epithelial cells and microscopic agglutination

To one ml of either an antibody dilution or saline or normal serum control 1 - 2 x 10^8 *V. cholerae* 569B were added and the suspension incubated for 15 min at 37°. To 0.1 ml of this bacterial suspension 1 x 10^6 intestinal epithelial cells were added and the mixture incubated for 60 min at 37° on a shaker. Each sample was centrifuged at low speed and the pellet resuspended in 0.2 ml of Hank's medium.

Smears of each sample were prepared, fixed in 10 % methanol and treated with fluorescein-labelled anti-serum as described in 2.16. The fluorescent bacteria were examined with a fluorescence microscope (2.16). The effect of antibody on adherence of *V. cholerae* to epithelial cells was evaluated by determining the percent epithelial cells with adherent bacteria. All values were adjusted to a control value of 100 %.

6.2.6 Microscopic agglutination

Microscopic agglutination was measured on samples prepared the same way except that epithelial cells were absent from the suspension. Agglutination was scored on a scale from zero to four according to the size of agglutinates formed. Assessments of bacterial adsorption and microscopic agglutination were determined using a double blind method.

6.2.7 Infant mouse protection test

This test has been described in 2.14. For convenience and maximum sensitivity, the protection tests in this chapter involved oral administration of *V. cholerae* together with the antibody dilution. Significant protection, however, can be obtained if the antibody is given orally or systemically (i.v. or i.p.) several hours before challenge (Chaicumpa, 1974).

In most protection tests the oral inoculum was assayed simultaneously for microscopic agglutination and bacterial adherence to epithelial cells.

6.3 Results

6.3.1 Correlation between adsorption, agglutination and protection

The data in Table 6.1 show some correlation between the ability of whole antiserum or secretory IgA to reduce bacterial adsorption to epithelial cells and their ability to protect infant mice from cholera. Furthermore, the decrease in adsorption of *V. cholerae* to epithelial cells appears to be inversely related to the amount of agglutination present.

6.3.2 Effects of anti-O and anti-H antibody

Although the degree of agglutination, reduction in bacterial adsorption to cells, and protection seem to parallel one another there may not be any causal relationship between agglutination and protection; the agglutination may simply indicate the presence of antibody which acts in some other manner *in vivo*. If agglutination of organisms exerts a protective function anti-flagella antibodies should protect infant mice from cholera. In order to test this possibility anti-O and anti-H antibodies were purified from the IgG (anti-O, H) and each antibody pre-

Table 6.1: Effects of mouse anti-serum and rabbit secretory IgA to V. cholerae 569B on agglutination, protection and adsorption of bacteria to epithelial cells.

Sample tested	Dilution	Percent survival*	Microscopic agglutination**	Percent cells with adsorbed bacteria
a) Mouse anti-569B	1 : 100	100	3.3	54
	1 : 400	18	2.4	69
	1 : 1000	0	2.0	86
NMS	1 : 100	0	0.0	100
b) Rabbit secretory IgA	1 : 40	89	1.8	59
	1 : 400	45	0.6	76
	1 : 4000	45	0.3	100
NRS	1 : 40	10	0.0	100

* Determined at 65 (a) and 52 hours (b) after challenge with 2 x 10^7 V. cholerae 569B

** Scored on a scale from 0 to 4 according to the size of the agglutinates

NMS Normal Mouse Serum

NRS Normal Rabbit Serum

paration was tested in the same three assays. The characteristics of these antibodies are indicated in Table 6.2. The anti-O retained the HA, vibriocidal and O-agglutinating activity found in the original IgG (anti-O, H). The anti-H retained only H-agglutinating activity; it had no measurable activity in any of the assays involving bacterial somatic antigens. All three preparations protected infant mice from cholera.

The relationship between protective activity, agglutination and adsorption to epithelial cells is shown in Table 6.3. Each of the antibody preparations had good protective activity (90 to 100 % survival) only at concentrations that caused obvious agglutination and a reduction in bacterial adsorption to epithelial cells of at least 40 to 50 %. In order to accurately interpret the above results it was important to ensure that the anti-flagella antibody was pure with no antibody directed against somatic antigens.

6.3.3 Absorption of anti-H with a hybrid vibrio

The anti-H was absorbed with a hybrid vibrio (569B-165 SR) which shares flagellar antigens with *V. cholerae* 569B but does not possess any of the somatic antigens of 569B (see Table 6.5 and 2.2.3). The data in Table 6.4 show that progressive ab-

Table 6.2: Biological activities* of rabbit IgG preparations containing anti-O, H anti-O or anti-H to *V. cholerae* 569B.

Antibody preparation	Haem-agglutination	Bactericidal	Agglutination***		Protective activity (PD 50)
			Boiled	Live	
Anti-O,H	500	6.2×10^4	54	930	1323 ± 380**
Anti-O	480	3.3×10^4	33	33	260 ± 96
Anti-H	<0.4	<8	<1	930	305 ± 142

* Activities given as the reciprocal of the end point dilution per mg of original total IgG (anti-O, H)

** Mean plus standard deviation of four (anti-O and anti-H) or five (anti-O, H) experiments

*** Corrected tube agglutination titres against washed boiled organisms or washed live organisms

Table 6.3: Effects of rabbit IgG containing anti-O, H, anti-O or anti-H activity to V. cholerae 569B on agglutination, protection, and adsorption of bacteria to epithelial cells.

Sample tested	Dilution	Percent Survival*	Microscopic agglutination**	Percent cells with adsorbed bacteria
Anti-O,H	1: 100	100	3.1	22
	1: 1000	92	1.9	48
	1:10000	25	0.8	85
NRS	1: 100	8	0.0	100
Anti-O	1: 100	100	2.5	53
	1: 1000	30	0.8	83
	1:10000	30	0.3	94
NRS	1: 100	0	0.0	100
Anti-H	1: 10	100	2.3	44
	1: 100	90	1.9	49
	1: 1000	60	0.9	85
NRS	1: 10	0	0.0	100

* Determined at 48 - 52 hours after challenge with 2×10^7 V. cholerae 569B

** Scored on a scale from 0 to 4 according to the size of the agglutinates

NRS Normal Rabbit Serum

Table 6.4: Effect of absorption of anti-H with a hybrid vibrio sharing only flagellar antigen.

Absorption with:	Number of absorptions	IgG* (µg/ml)	Live** agglutination	*** PD50	PD50 / Agglutination
Nil	-	1012	640	390	0.6
Boiled 569B/165SR	1	1080	640	ND	-
	3	948	640	280	0.4
	4	1104	640	290	0.4
Live 569B/165SR	1	1080	320	320	1.0
	3	912	40	20	0.5
	4	924	10	3	0.3

* Determined by single radial immunodiffusion using a goat anti-γ

** Reciprocal of highest dilution showing macroscopic tube agglutination against live washed V. cholerae 569B

*** Average values of two experiments

ND Not determined

sorptions of anti-H with live hybrid vibrios removes agglutinating and protective activity in parallel. Absorption with the boiled hybrid (flagella destroyed) had no effect on the agglutinating or protective activity of anti-H.

6.3.4 Agglutination of various vibrio strains

The anti-H (prepared against *V. cholerae* 569B) was tested for its agglutination characteristics against various flagellated and non-flagellated vibrios (Table 6.5). Only the flagellated motile vibrios were agglutinated by the anti-H preparation. Strain V58SR (III) which is non-motile and which lacks flagella as determined by electronmicroscopy (see 2.2.4) showed no agglutination with the anti-H at the highest concentration tested (1 : 3).

6.4 Discussion

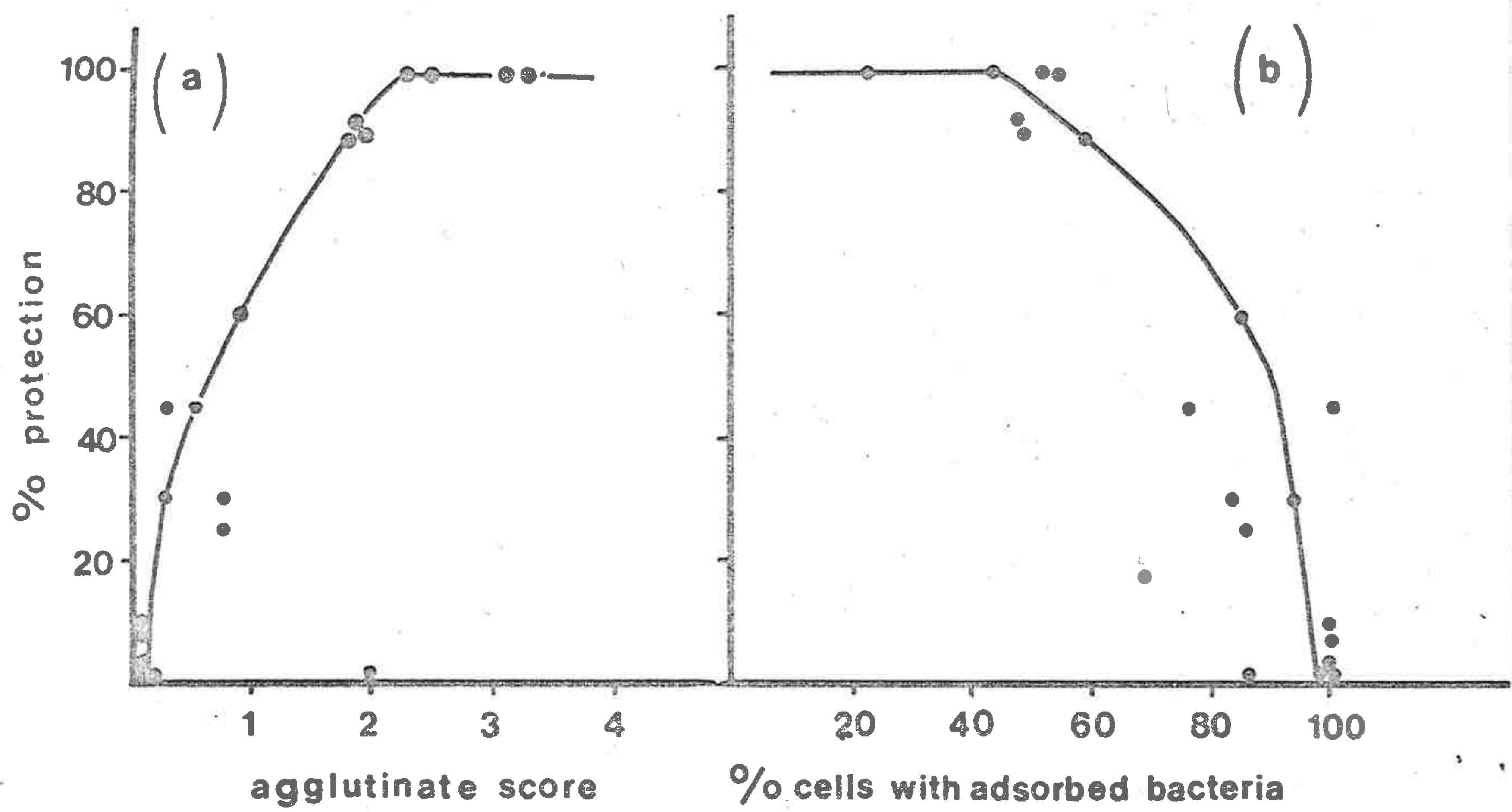
The results of this chapter are summarized graphically in Fig. 6.1. The data show (i) a significant inverse correlation between protection and the degree of attachment to epithelial cells *in vitro* ($P < 0.001$), (ii) a significant positive relationship between protection and the degree of agglutination *in vitro* ($P < 0.001$) and (iii) a significant inverse relationship between the degree of attachment to epithelial cells *in vitro* and the degree of bacterial agglutination *in vitro* ($P < 0.001$).

Table 6.5: Agglutination of various vibrio strains with anti-O, H, anti-O and anti-H.

Strain	O-serotype	Motility	Live agglutination titre* with: Anti-O,H	Anti-O	Anti-H
569B	Inaba	+	2400	140	2400
017SR	Ogawa	+	800	400	800
569B/165SR	NCV	+	2400	< 5	2400
V58SR (III)	Ogawa	-	80	40	< 3

* Reciprocal of highest dilution showing macroscopic tube agglutination against live washed organisms

Fig. 6.1: Correlation between protection, agglutination (a) and prevention of adsorption (b):
Summarized from the data in Tables 6.1 and 6.3.
The line of best fit is indicated by ....


(a)
(b)
% protection
100
80
60
40
20
1
2
3
4
agglutinate score
20
40
60
80
100
% cells with adsorbed bacteria

The possibility existed that bacterial agglutination was merely a measure of the presence of antibody which was providing protection through some other mechanism. To determine if there was any causal relationship between agglutination and protection purified anti-flagella antibody was prepared. Such antibody should only act by agglutination and/or inhibition of motility and not via a direct bactericidal property (see Table 6.2). The finding that anti-flagella antibody could protect infant mice from cholera and that this was parallelled by the agglutinating activity indicated that agglutination can be an important mechanism of intestinal immunity. The validity of this conclusion depends on the assumed purity of the anti-H preparation used in the assays. Strong support for this assumption is the absence of HA and bactericidal activity (Table 6.2) and the fact that protective and agglutinating activity were removed in parallel by absorption with a hybrid vibrio (Table 6.4) which shared only the flagella antigen (Table 6.5). At first sight anti-H antibody might appear to be not as efficient as anti-O antibody in protection (Table 6.2). However, based upon specific antibody content, anti-H appears at least as protective as anti-O. From Table 6.4 the maximum possible concentration of specific anti-H can be estimated as 50 - 100 ug/mg total IgG. The concentration of anti-O antibody determined by quantitative precipitation with V. cholerae 569B LPS was approx. 200 ug/mg total IgG.

From these values the PD_{50}/ug of antibody can be calculated to be 1.3 and 3 - 6 for anti-O and anti-H respectively.

It seems probable that agglutination and prevention of adherence to epithelial cells are causally related to protection. Microscopic examination of the epithelial cells and the bacterial population at protective antibody concentrations revealed that bacterial aggregates were present containing 100 to 1000 bacteria or more. Agglutination would, therefore, reduce the challenge dose of organisms at the epithelial surface 100 to 1000 fold.

CHAPTER 7

ANTIBACTERIAL EFFECT OF ANTIBODY IN THE ADULT MOUSE: SUPPRESSION OF GROWTH IN THE INTESTINAL LOOP

7.1 Introduction

Using the intestinal loop as a model to analyze the antibacterial effect of the mucosa on V. cholerae (Chapter 5) we observed that the total population of organisms in such a loop multiplied over a period of 5 hours. Active immunization of the mice with V. cholerae suppressed the growth. In this chapter we tried to elucidate the antibacterial role of antibody in this model using mainly histological methods.

7.2 Materials and methods

7.2.1 Mice: Adult Swiss White Mice (2.1.1)

7.2.2 Bacteria:

V. cholerae 569B SR (2.2.1)

NCV 569B-165 (2.2.3)

7.2.3 Immunizations: For parenteral and oral with V. cholerae 569B see 2.5.1.

For parenteral immunization with NCV 569B-165 see 2.5.2.

7.2.4 Antiserum: Mouse anti 569B II (2.6.3)

7.2.5 Antibody assays:

Indirect haemagglutination (2.10.1)

Bactericidal assay (2.12)

Tube agglutination (2.11)

7.2.6 Experimental procedure

Mice were anaesthecized with ether, the abdomen incised, and 5 cm intestinal loops were ligated beginning approx. 10 cm below the pylorus. Using an infusion pump 50 µl of a log phase culture of *V. cholerae* containing approx. 2×10^7 living organisms were injected into the loops. The abdomen was closed and the mice were killed after 5 hr. The loops were removed and homogenized with an Ultra Turrax homogenizer (Janke and Kunkel, Staufen, W. Germany) in 10 ml of saline. In one group of mice viable bacterial counts were determined from the homogenate. In another group of mice frozen sections of intestinal loops were examined with fluorescein-conjugated antisera to determine the distribution pattern of *V. cholerae* on the intestinal mucosa. Smears of loop fluid stained in the same manner were examined microscopically to assess bacterial agglutination. Because of our subsequent finding that bacterial growth was suppressed in immunized animals (Table 7.1), the immune mice received approximately 10 times (2×10^8) more bacteria per loop than the non-immune animals in this experiment. This was done so that each group would have the same number of organisms after 5 hours and, therefore, the relative changes in bacterial distri-

bution and agglutination could be assessed more accurately. All sections and smears were assessed using double-blind procedure.

In separate experiments, mice were passively immunized intravenously or the bacteria were pretreated with antibody prior to inoculation into intestinal loops and the numbers of viable bacteria determined from intestinal loop homogenates five hours later.

7.3 Results

7.3.1 Growth of V. cholerae 569B in the intestinal loops of non-immunized and immunized mice

In intestinal loops of non-immunized animals *V. cholerae* grew in 5 hours to 1600 percent of the numbers inoculated. In animals immunized parenterally or orally with *V. cholerae* 569B the bacteria multiplied to some extent but growth was retarded about sevenfold when compared to non-immune animals (Table 7.1). In mice immunized with the hybrid vibrio 569B-165 which shares only the flagella antigen with the test organism a comparable suppression of growth occurred (Table 7.2). Table 7.3 shows the antibacterial activities of the antisera from each of these groups of mice. Serum from mice immunized with *V. cholerae* 569B had good haemagglutination, bactericidal, and agglutination titres against 569B whereas the serum from

mice immunized with 569B-165 had only one activity against 569B - agglutination of live bacteria.

Passive immunization was much less effective at retarding bacterial growth in the intestine (Table 7.4). Antiserum given either directly into the loop or intravenously produced a mild retarding effect on growth of the organism only when high concentrations of antibody were used.

Table 7.1: Effects of immunization with <u>V. cholerae</u> 569B on growth of <u>V. cholerae</u> 569B in intestinal loops of mice.

Route of immunization	Number of mice	Number of bacteria ($x 10^7$) inoculated	Bacteria recovered (% of no. inoculate
Non-immune	11	2.8 $\pm$ 1.4 [a]	1600 $\pm$ 1000 [a]
Parenteral	10	2.8 $\pm$ 1.4	190 $\pm$ 175
Oral	6	2.4 $\pm$ 1.6	300 $\pm$ 210

a Values expressed as mean $\pm$ standard deviation

b Bacterial recovery determined 5 hours after inoculation

Table 7.2: Effects of immunization with hybrid vibrio 569B-165 on growth of *V. cholerae* 569B in intestinal loops of mice.

Route of immunization	Number of mice	Number of bacteria ($x 10^7$) inoculated	Bacteria recovered [b] (% of no.inoculated)
Non-immune	8	1.0 ± 0.4 [a]	1030 ± 300 [a]
Parenteral	8	1.0 ± 0.4	160 ± 80

a Values expressed as mean $\pm$ standard deviation

b Bacterial recovery determined 5 hours after inoculation

Table 7.3: Antibacterial activities of sera* from mice immunized with V. cholerae 569B or with the hybrid vibrio 569B-165 when tested against V. cholerae 569B.

Immunized with	Haemagglutination titre	Bactericidal titre	Agglutination titre with	
			Live 569B	Boiled 569B
569B	256	1.5×10^6	2000	512
569B-165	<2	50 - 250	64	<2
Non-immune	<2	50	<2	<2

* Pooled sera from 10 mice in each group

Table 7.4: Effects of passive immunization on recovery of V. cholerae 569B from intestinal loops of mice.

Route of passive immunization [a]	Number of mice	Number of bacteria ($\times 10^6$) inoculated	Bacteria recovered[b] (% of no. inoculated
Local			
Bacteria pre-treated with:			
4 H.U.	6	5 ± 1.6 [c]	2400 ± 1400 [c]
16 H.U.	5	1.4 ± 1.1	1700 ± 800
50 H.U.	5	3 ± 1.4	700 ± 215
Parenteral			
300 H.U. (0.3 ml) per mouse intravenously 24 hr before test	11	2.3 ± 1.4	500 ± 175
Non-immune			
0.3 ml normal mouse serum I.V. 24 hr before test	5	3 ± 1.1	1200 ± 480

a The antiserum was raised in mice against living V. cholerae 569B. The antiserum had a haemagglutination titre of 1/1000, therefore, 1 ml of this serum had 1000 haemagglutination units (H.U.).

b Bacterial recovery determined 5 hours after inoculation.

c Values expressed as mean ± standard deviation.

7.3.2 Location of *V. cholerae* 569B in the intestinal loops of immunized and non-immunized mice

The effects of active immunization on the location and distribution of *V. cholerae* within the lumen of the small intestine are summarized in Table 7.5. In the non-immune animals much of the epithelial surface of villi was covered by a layer of bacteria varying from two to ten organisms thick. Often the bacterial layer extended down the lateral edges of villi but organisms were never observed within the crypts. Smears of intestinal loop contents indicated that the bacteria which were free in the lumen were not aggregated.

In the immunized mice the distribution of vibrios differed markedly from the non-immune animals. The mucosa was conspicuously free of organisms. There were only a few individual bacteria observed on the epithelial surface. Smears of luminal contents of immunized animals showed large aggregates of *Vibrio cholerae* with only a few single organisms.

7.4 Discussion

In Chapter 4 and 6 it was demonstrated that antibody exerted its protective effect by preventing the adsorption of the vibrios to the mucosa thus allowing a more efficient mechanical removal of the organisms by peristaltic forces.

In the experiments described in this chapter active immunization resulted in a suppression of growth of V. cholerae in the intestinal loop. It was concluded that inhibition of growth was achieved by secreted antibody which prevented the attachment of the organisms to the intestinal mucosa. This conclusion is based on following observations:

1) The mucosa in the immunized animals was free from attached vibrios while covered with organisms in the non-immune mice. The differences in numbers of organisms attached to the mucosa in immune and non-immune mice could not be quantitated but may be estimated to be more than 1000 fold. It should be mentioned that the total number of organisms per loop was approximately the same in both groups because 10 x more vibrios had been injected into the loops of the immune mice.
2) The bacteria in the lumen of the small intestine or in the smears from immune animals were agglutinated.
3) Growth of the vibrios in loops of mice immunized against NCV-569B-165 was similarly suppressed. Antibody in these mice was not directed against the somatic antigens of V. cholerae 569B but only against the flagella (Table 7.3). A mechanical effect of antibody - agglutination of the bacteria and inhibition of motility - appears, therefore, to be the likely mechanism of growth suppression.

It should be mentioned that the viable counts were not affected by agglutination. In control experiments in which

viable counts were performed on deliberately agglutinated vibrios, after homogenization in the presence of a piece of intestine the viable counts were not reduced.

There are at least two possible ways that bacterial growth may have been suppressed:

a) Suitable conditions (nutrients, pH, etc.) for multiplication are lacking, and
b) multiplication rates and killing rates are equivalent resulting in a constant number of organisms.

The growth inhibition observed in this model could be caused by a reduced multiplication rate due to less suitable growth conditions in the lumen and heavy agglutination of the organisms as well as by an active killing mechanism on the mucosal surface. Freter (1972) suggested that antibody not only has an effect on the adsorption of the organisms to the mucosa but also mediates an antibacterial mechanism on the mucosal surface. In our model antibody does not seem to mediate the killing of vibrios for the reasons given above (anti-H antibody is effective) but it is possible that this killing mechanism described in Chapter 5 participates in the growth control in immunized mice (see General Discussion, section 4).

Antibody given passively was quite inefficient in suppressing growth of the vibrios in the loop. Only high doses given either together with the organisms or injected i.v.

had some effect. This may be explained by enzymatic degradation of the antibodies to insufficient levels during the period of the experiment. Rapid enzymatic degradation of serum antibody in the mouse intestine has been demonstrated by Fubara and Freter (1972). In actively immunized animals, however, antibody could be provided continuously by secretion and this may also occur in mice with antibody given i.v. 24 hours before the experiment.

Table 7.5: Effects of active immunization on the location and distribution of *Vibrio cholerae* 569B in ligated intestinal loops of mice.

Immune state	Distribution of *V. cholerae*[a]		Microscopic agglutination
	Mucosal surface	Intestinal lumen	
Immune	Occasional bacteria observed	Bacterial aggregates	+++
Non-immune	Thick layer of bacteria	Mostly single bacteria	±

a The distribution and degree of agglutination of *V. cholerae* 569B were determined on smears of intestinal loop contents and frozen sections of intestinal loops using a fluorescent antibody technique. Smears and sections were examined 5 hours after bacterial inoculation.

CHAPTER 8

GENERAL DISCUSSION

In Chapter 1 the determinants of host-parasite relationships in the intestine have been reviewed. Susceptibility to an infectious agent depends on a variety of host and bacterial factors The infant mouse changes from a state of susceptibility to almost complete resistance to a cholera infection during maturation. This allows the conclusion that host factors are of major importance for the resistance of the adult mouse to infection in this model. Susceptibility in infant mice, on the other hand, appears to be mainly due to lack of those protective factors which enable adult mice to resist an infection. To analyze the role of unspecific defence mechanisms in protection against cholera and the effect of antibody on these the infant and adult mouse models appeared to be quite suitable. As reviewed in Chapter 1 bacteria will be eliminated from the gastro-intestinal tract by mechanical removal and killing. To assess the relative importance of each of these factors a second marker - besides viability - had to be used.

Radiolabelling with 32P provided a relatively simple method with which to label bacteria and count radio-activity in tissues. But a few points with regard to the interpretation of the experiments where 32P-labelled bacteria had been used should be discussed.

i) Irradiation damage of the labelled organisms

The uptake of 32P and accordingly the specific activity of the bacteria was low. Although a slight decrease of virulence of the labelled vibrios was observed this seemed not to have any significant effect on the results. Radiolabelled and non-labelled vibrios were similarly sensitive towards killing in the adult mouse and no differences in bacterial agglutination was observed.

ii) Loss of radiolabel without killing of the organisms

Liberation of the P^{32} label appears to be associated with killing of the organisms only (Spitznagel, 1966 a, b). Loss without killing would be insignificant, particularly in the short-termed experiments described in Chapter 5.

iii) Loss of radiolabel into the circulation

In the long-termed experiments with adult and infant mice described in Chapter 3 and 4 approximately 90 % of the radio-activity were recovered after 3 - 6 hours in the gastro-intestinal tract and faeces. About 10 % were recovered in liver and no measurable activity was found in the spleen. This amount, however, does not greatly affect the interpretation of the results obtained in Chapter 3 and 4. The specific activities shown in Fig. 4.3 and 4.4 would only be insignificantly affected. In the loop experiments with 32P-labelled bacteria (Chapter 5) no measurable amount of P^{32} could be found in the liver and spleen.

iv) Growth and death rate

The total number of viable organisms recovered in a tissue will be the net result of growth and death. Methods for estimating growth and death rate on mucosal epithelium and other tissues have been described (Meynell and Subbaiah, 1963). Radiolabels such as ^{32}P are not suitable to estimate those rates accurately because the marker could be reutilized etc. Thus, although specific activity may reveal killing ($\frac{\text{viable organisms}}{\text{counts per min}}$), it cannot be excluded that simultaneously also growth had occurred. In most experiments this seemed to be of minor significance for the interpretation of the results. On the other hand, the estimation of such rates would have been of great value particularly in the infant mouse experiments with and without antibody (Chapter 4) where multiplication and killing may occur simultaneously.

There are several animal models available using V. cholerae which do not mimic the human disease exactly. Nevertheless, they permit the study of local enteric immune mechanisms. The infant mouse protection test which provides an assessment of the protective ability of antibacterial antibody (Neoh and Rowley, 1972; Chaicumpa and Rowley, 1973) has been used here to analyze the anti-bacterial function of antibody in the intestine. The results concerning the antibacterial immune mechanisms on the mucosal surface may, with some restrictions, be transferrable to the human situation, for the following reasons:

i) The basic features of the disease closely resemble those in man. The infection can be established by mouth with living organisms and the course of the disease appears to be similar to that in man (multiplication of the challenge organisms in the intestine, fluid distension of the small intestine, diarrhoea, death 24 - 48 hours after challenge). The infecting organisms are entirely confined to the intestinal lumen (Chaicumpa and Rowley, 1973; Chaicumpa, 1974).

ii) Although in most experiments antibody has been given passively with the bacterial inoculum for convenience, it has been shown that antibody injected i.v. or i.p. protected the infant mice (Chaicumpa, 1974). Thus, secreted antibody as observed in actively immunized humans receiving parenteral antigen (see Chapter 1.4.1), can be protective. There is no conceptual difficulty in assuming that antibody will operate basically through the same mechanisms on the mucosal surfaces of both infant mice and humans. The possibility that active immunization has an additional effect by eliciting a cellular response cannot be excluded.

8.1 The role of unspecific factors in the elimination of V. cholerae from the gastro-intestinal tract of mice

A number of unspecific antibacterial factors which prevent V. cholerae from colonizing the small intestine has been found in adult mice. Gastric acid reduces the number of living organisms to less than 1 % of the inoculum before

they reach the small intestine. In a susceptible host gastric acid could provide an efficient barrier against an enteric infection. Gastric acidity may account for the wide range in the infective dose of V. cholerae (10^6 - 10^{11} viable organisms) in human volunteers (WHO, 1972). On the other hand, gastric acidity appears less decisive as an antibacterial factor mediating the resistance of adult mice to a cholera infection. After neutralization of the gastric acid by Na-bicarbonate, approx. 100 x more living organisms (20 % of the inoculum see Table 3.1) could be found in the small intestine. These and even higher numbers of living vibrios were removed rapidly from the small intestine. The most important antibacterial factors appear, therefore, to be located in the small intestine itself.

The two basic mechanisms responsible for the elimination of the vibrios from the small intestine are mechanical removal by peristalsis and a bactericidal mechanism. The requirement for the combined action of both mechanisms to eliminate the living organisms from the small intestine is an important finding in Chapter 3 and 4. Inhibition of peristalsis prevents the elimination of V. cholerae from the intestine (Fig. 3.1 and 3.2). Similarly, inhibition of killing by starvation (without affecting peristalsis) reduces the efficiency of the elimination process. The synergistic action of these two factors is further demonstrated in Fig. 3.3 and can also be observed in infant mice. As de-

monstrated in Fig. 4.4 an increase of specific activity in the antibody-treated as well as in the non-treated groups occurred only after the bulk of living vibrios had been removed mechanically.

The complexity of this in vivo model does not allow a clear definition of the killing mechanism although the results of the starvation experiment (3.4) suggest that secretions are involved. It seems that, after the majority of organisms has been removed, those bacteria in close contact with the mucosa are killed preferentially (Table 3.2).

The role of the indigenous flora in this elimination process is not clear although, as the results in streptomycin-treated mice demonstrate, an intact flora seems to be important. As outlined in the introduction, the normal flora may exert its antagonistic effect in several ways: Additional killing (Meynell, 1973), enhancing the peristaltic movement, etc. (Abrams and Bishop, 1967).

The basic mechanisms responsible for the efficient elimination of pathogens from the gastro-intestinal tract of adult mice can also be found in infant mice (Chapter 4) although with reduced efficiency. The organisms are only slightly affected by gastric acid, the mechanical removal is delayed, and the killing mechanism in the small intestine, although present, appears less efficient. It is probably reasonable to assume that the susceptibility of infant mice towards a cholera

infection finds its explanation - at least partly - in these less developed antibacterial mechanisms.

The number of living organisms is considerably reduced further in the large intestine. All of the single factors participating in this killing have not been analyzed. The normal flora is probably of greater importance here than in the small intestine and it is not clear whether the killing mechanism found in the small intestine operates in the large intestine as well. The data in Chapter 4 (and discussed in 4.4) suggest that normal physiological functions are required for an efficient killing of the organisms in the large intestine.

It seems probable that the antibacterial parameters observed in mice will operate in other animals, including man. Maintenance of a stable indigenous microbial flora, gastric acidity, peristalsis, and an intestinal antibacterial mechanism may all be required for maximum efficiency. Each of these mechanisms operates to different degrees with different organisms and V. cholerae appears to be extremely sensitive to several of these. Stress situations produced by shock (laparotomy and bleeding) or toxin (LPS; Chapter 3) delay the rapid elimination of the organisms and thus increase the chance of a successful colonization of the mucosa by enteric pathogens.

8.2 Nature and biological significance of the mucosal killing system

The data obtained from the *in vivo* experiments in adult and infant mice (Chapters 3 and 4) suggested that a bactericidal mechanism was operating on the mucosal surface, killing mucosa-associated *V. cholerae*. The complexity of the *in vivo* model hindered a more detailed definition of this killing system, and the effect of antibody on it. Therefore, the loop model was adopted to allow a more accurate and more reproducible measurement of the bactericidal effect.

The data given in Chapter 5 allow the following conclusions about the nature of this killing system:

i) It operates on the mucosal surface (Table 5.5) and kills only mucosa-attached organisms (see detailed discussion in 5.4).

ii) It probably requires metabolically intact epithelial cells. This conclusion is based on the results obtained in loops with a compromised blood supply. Epithelial cells left *in situ* for 10 minutes without transferring them into an oxygenated nutrient medium are likely altered metabolically. The sensitivity of these cells to suboptimal conditions is demonstrated by the fact that they are very difficult in *in vitro* conditions (Reiser and Christiansen, 1971). It cannot be excluded that a blood-derived factor, secreted by epithelial cells, is involved in this killing. This, however, seems to be unlikely since mouse serum is not

bactericidal for V. cholerae and other gram-negative organisms. This problem can be solved only with tissue culture methods.

iii) It kills other gram-negative organisms with varying efficiencies (Table 5.10). It is possible that this depends on the cell wall structure of the organisms; however, this could only be clarified by examining more organisms systematically.

iv) It appears to be antibody-independent.

In short, it is suggested that one or several metabolic products secreted from the intestinal epithelial cells are involved in this killing process. Such a mucosa-associated killing has not been described in the literature although there are a few data which point to such a chemical barrier against bacterial colonization.

Hampton and Rosario (1965) observed destruction of Streptococcus moniliformis attached to mucosal epithelium in electron microscopic pictures. Fubara and Freter (1973) recently provided data which indicate that an antibacterial mechanism operates on the mucosal surface which requires metabolizing mucosal cells because it can be inhibited by metabolic inhibitors such as iodoacetate. This antibacterial mechanism, however, seems to be antibody-dependent.

What kind of bactericidins could be secreted from epithelial cells? Several bactericidal systems are known which cause killing and lysis of bacteria. Activation of the whole C'sequence results in damage to bacterial cell walls (Müller-Eberhardt, 1971). But, as discussed in 1.3, it seems unlikely that the whole complement sequence is functionally active in the environment of the intestine. The alternate pathway (Götze and Müller-Eberhardt, 1971) can be activated by endotoxin, zymosan etc. and does not require antibody for activation. Thus, antibody independence of the mucosa-associated killing system would not be an obstacle to the assumption that the complement system may be in some way involved. Other factors reported to enhance complement-mediated killing, such as lysozyme (Adinolfi et al., 1966) or beta-lysins (Donaldson et al., 1974), require antibody. The observation that complement-resistant bacteria (NCV 5698-165 see Table 5.10 and Chaicumpa, 1974) are equally sensitive to this bactericidal system could be taken as an argument against an involvement of the complement sequence in this killing.

Other bactericidal substances are found in polymorphnuclear leucocytes and macrophages although in the latter the antimicrobial agents are unknown (Hirsch, 1972). Such agents are lactic acid (Dubos, 1953), hydrogen peroxide (McRipley and Sbarra, 1967), myeloperoxidase (Klebanoff, 1967, 1968) and cationic proteins (Zeya and Spitznagel,

1968, 1971). The release of such antimicrobial substances from phagocytic cells on the mucosal surface is unlikely in view of the fact that hardly any cells can be found on this surface (see 1.4.2). However, such agents could be synthesized and released from epithelial cells. In addition to such bactericidal agents, digestive enzymes would be required since the organisms were degraded to relatively small fragments (Table 5.7). The mucosal epithelial cells of the small intestine contain and secrete a variety of digestive enzymes, such as ß-galactosidase, ß-glucoronidase, phosphatases, and other lysosomal enzymes. Most of these enzymes can be found in highest activities in the cells along the villi (Nordström and Dahlquist, 1973). The brush border of the epithelial surface is the site of terminal hydrolysis of disaccharides and peptides prior to absorption (Miller and Crane, 1961).

More data are required to reach a definite conclusion about the nature of this killing mechanism. Tissue culture methods, metabolic inhibitors, or drugs which affect membranes such as cytochalasin B would yield more information about the metabolic and secretory processes involved in this killing. Recently, Chaicumpa and Rowley (1974) were able to inhibit the killing of V. cholerae in the gastro-intestinal tract of infant mice by agents such as choleratoxin, lysolecithin, and prostaglandins.

A few words should be said about the biological significance of such a mucosa-associated killing system. As outlined in Chapters 1.3 and 8.2, the intestinal mucosa is protected by a variety of factors and this killing mechanism may be an additional one. However, the upper levels of the intestinal mucosa are relatively free from a normal flora and will, therefore, be at greater risk of being infected by enteric pathogens which specifically attach to it. Such a chemical barrier against colonization and invasion by enteropathogenic organisms could be an important defence mechanism, particularly in such areas.

8.3 Effect of antibody on the elimination of *V. cholerae* from the gastro-intestinal tract

In the infant mouse (Chapter 4) antibody clearly promotes the elimination of *V. cholerae* from the gastro-intestinal tract. The results described in this chapter suggest that antibody enhances non-immunological antibacterial processes which can be observed even without antibody in the control animals.

A number of workers have shown prevention of growth or decreasing numbers of organisms in the intestine of antibody-treated animals (Bhattacharya and Mukerjee, 1968; Ghosh, 1970; Smith and Linggood, 1971; Chaicumpa and Rowley, 1972) and it has been suggested that antibody mediates the killing of the organisms by an as yet un-

defined system. Freter (1969) showed that immunity to experimental cholera was due to an antibody-dependent antibacterial mechanism on the mucosal surface which could be inhibited by iodoacetate (Fubara and Freter,1973).

As discussed above (8.2) the two important unspecific parameters protecting the mucosa of the small intestine are mechanical removal and killing of the organisms. The data obtained in the infant mouse model (Chapter 4) clearly demonstrate that the mechanical removal is accelerated by antibody. Although the more rapid mechanical removal is paralleled by an increased killing (Fig. 4.3 and 4.4) it seems unlikely, as discussed in this chapter (4.4) that antibody directly promoted this killing. This is further substantiated by the observation in intestinal loops (Chapter 5) that antibody did not have any effect on the killing mechanism. The main support, however, for this assumption is provided by the results in Chapters 6 and 7. In these experiments protection of infant mice and growth control in intestinal loops could be obtained by anti-H antibody.

In summary, there is no evidence for an antibody-mediated bactericidal mechanism in infant mice.

The data given in the Chapters 4, 6 and 7 suggest that the only function of antibody in intestinal immunity against

V. cholerae, as based on the infant mouse model, is to prevent the adsorption of vibrios to the intestinal mucosa. The most convincing data - supporting the assumption that antibody acts by prevention of adsorption only - are provided by the experiments in Chapter 6. The statistically significant correlation between agglutination, prevention of adsorption and protection and the fact that anti-H antibody protects infant mice against the disease are strong indications for the validity of this assumption.

As reviewed in the introduction it is generally accepted that bacterial infections on mucosal surfaces require association between the pathogen and the mucosal surface. Adhesive properties have long been recognized in many species of enterobacteriaceae (Duguid and Gillies, 1957; Duguid, 1959,; Duguid et al., 1966; Duguid, 1968), however, the significance for the pathogenesis of intestinal infections had not been experimentally shown. Recently the importance of K88 antigen for the virulence of *E. coli* for piglets and calves (Arbuckle, 1970; Smith and Linggood, 1971) has been demonstrated. Jones and Rutter (1972) showed that K88 antigen was synthesized by a K88-positive enteropathogenic strain of *E. coli* in the small intestine of piglets where it functioned as an adhesin enabling the bacteria to adhere and colonize the mucosa. The adhesive properties of *Streptococcus pyogenes* to buccal epithelial cells has been attributed to the M-

protein (Ellen and Gibbons, 1972). Observations by Kellog et al. (1968) and Swanson et al. (1971) suggest that pili might correlate with virulence of gonococci through an influence on interactions between the gonococcal surface and host epithelial cells. The association of V. cholerae to the intestinal mucosa appears necessary for its pathogenesis (Freter, 1969). The nature of the attachment sites, however, has not been defined.

Inhibition of bacterial attachment by antibody followed by mechanical removal of the organisms could be an important mechanism to prevent mucosal colonization and penetration by enteropathogenic organisms. Freter (1969) was the first to show that the numbers of viable V. cholerae adsorbed to the intestinal mucosa could be reduced by antibody. He suggested that prevention of adsorption could be an important antibacterial defence mechanism on mucous surfaces. However, it is not clear how antibody inhibits the association of the vibrios with the mucosa.

Several authors have suggested that antibody acts by blocking specific attachment sites on the bacterial surface. Such a function of antibody has been proposed in protection against E. coli enteritis in piglets (Rutter and Jones, 1973; Smith, 1972) and in streptococcal infections (Williams and Gibbons, 1972).

Our results, however, indicate that antibody prevents adsorption primarily by agglutination and/or the accompanying inhibition of motility of the bacteria. Microscopic examination of the epithelial cells and the bacterial population at protective antibody concentrations revealed that bacterial aggregates were present containing 100 to 1000 bacteria or more. Agglutination would, therefore, reduce the challenge dose of organisms at the epithelial surface 100 to 1000 fold.

In a recent paper Steele et al. demonstrated by using antibody fragments that protective antibody must in all cases have cross-linking potential for full activity and this must be taken into account whether thinking in terms of inhibition of motility or of agglutination as the effector mechanism.

Although it is reasonable to assume that cross-linking could be a protective mechanism against mucosal infections in general, it may be that it will not be sufficient in situations where the pathogen exerts a particularly strong association with the mucosa. In such situations neutralization of attachment sites and other factors which hinder attachment may become of increasing importance.

8.4 Hypothesis: Synergistic action of antibody-mediated prevention of adsorption and unspecific killing

Bacterial growth rates on mucosal surfaces are determined by multiplication of the organisms on one side and death and mechanical loss on the other. In the adult mouse killing and mechanical loss exceeded multiplication. In the infant mouse local defence mechanisms reduced the number of living organisms to some extent initially but later on bacterial multiplication overwhelmed these mechanisms. However, antibody increased the efficiency of the anti-bacterial factors (mechanical removal and killing) and thus prevented colonization and multiplication. In the antibody-treated infant mice the total number of living organisms recovered from the whole gastro-intestinal tract was reduced. This reduction is not due to fecal excretion as demonstrated by Chaicumpa (1972) in mice with blocked ani but is clearly caused by killing. This observation is consistant with the results of the experiment in which antibody not only enhanced the mechanical removal of bacteria by prevention of adsorption but also increased the killing rate. Increased killing was demonstrated by a higher specific activity of the organisms recovered after 360 minutes from the small as well as from the large in-testine (Fig. 4.3 and 4.4) of antibody-treated infant mice. This, however, contradicts the findings, as discussed above (8.3), that antibody does not enhance a bactericidal me-chanism in this model. These apparently contradictory

findings may be explained as follows:
As shown in Chapters 4 and 6 the bactericidal mechanism of the mucosa in infant mice is quite weak and unable to kill all the attached organisms. These bacteria multiply and eventually produce their pathogenic effect. Antibody by preventing adsorption reduces the bacterial load of the mucosa and allows the killing mechanisms to completely kill the few remaining organisms. Thus, antibody indirectly enhances the efficiency of the killing system in the small intestine.

The majority of the living organisms are removed into and killed in the large intestine (probably unspecific killing by the normal flora). Accumulation of fluid in the large intestine with a continuous supply of living organisms from the small intestine in mice suffering from an infection would inhibit this unspecific killing (see Chapter 4.4).

Shedlofsky and Freter (1974) recently demonstrated in the large intestine of monocontaminated adult mice that local antibacterial immunity was considerably more effective when it operated in conjunction with bacterial antagonism. They proposed that antibody mediated inhibition of adhesion and the antagonistic action of the normal flora form a synergistic system.

Our results in infant mice support this proposal to some extent although mainly for the large intestine. In the small intestine which is the affected part of the intestine in cholera, a synergism between non-specific mucosa-associated killing and antibody-mediated inhibition of adsorption reduces the number of living organisms in the intestine and prevents colonization of the mucosa by bacterial pathogens.

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