IgA LEVELS AFTER ORAL VACCINATION OF MICE WITH SALMONELLA

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ABSTRACT

IgA Levels After Oral Vaccination of Mice with Salmonella

In an attempt to determine the immune status of mice following one oral feeding with various Salmonella strains, we have looked for a correlation between serum and/or intestinal IgA levels and protection against a subsequent challenge with a virulent Salmonella strain (S. typhimurium C5).

The measurement of sIgA presents significant problems due, for example, to such intestinal contents as bile and proteolytic enzymes, and to the relatively low levels of sIgA in samples due to the constant movement and elimination of the gut fluid as well as the dilution necessary in the collection of samples for experimental use. Consequently, we examined the ELISA (enzyme-linked immuno-absorbent assay), in which antibody can be assessed directly against its appropriate antigen in the "solid-phase" and where potentially aberrating components can be washed away. We showed that the ELISA is comparable in sensitivity to the radio-immunoassay without the latter's disadvantages, such as short half-life of principal reagents and hazard in use.

Our results show that those Salmonella strains which persisted in the Peyer's patches of the small intestine of mice following oral immunization afforded excellent protection against a subsequent challenge, and this protection correlated well with relative increases in serum and intestinal sIgA levels.

However, such protection did not correlate with the 'O' somatic antigens of the immunizing and challenging strains. Thus, it seems that the IgA or sIgA level as such is not responsible for the

protection observed, although these levels correlate very well with protection and afford a very good index of the immune status of an animal towards enteric infection.

Finally, this work implies that protection is effected at the cellular level and is dependent upon the persistence of antigen in the Peyer's patches.

STATEMENT

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

P. S. UDOMSANTISUK

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To my Mother, Father and Nibondh.

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CHAPTER 1

General Pathogenesis of Mouse Typhoid

1.1 Typhoid Fever: A Public Health Problem

Typhoid fever is among the most frequent causes of diarrhoeal disease and constitutes a serious public health problem in many regions of the world (see Table 1). In some countries the attack rates are as high as 1 per 1000 population, and in some areas of Egypt rates approaching 2.8% of the population have existed (Messih, 1961). From Table 1, it seems that the disease mainly affects the developing countries and its incidence usually decreases with increased development. In some of the more industrialized countries of North America and Europe and in Japan, typhoid fever has declined significantly and the disease occurs only among travellers returning from the endemic areas. Nevertheless, in a few countries a high incidence of typhoid fever is observed which does not correlate with advanced levels of development.

The factors that maintain the chain of infection derive from a lack of sanitation, the presence of carriers in the population, and, especially, the lack of an effective mechanism of prevention through immunity (= effective vaccine).

That water played a principal role in the spread of typhoid fever was first established by the observations of William Budd (1856, 1873) and has been confirmed by experience in many parts of the world. For example, over 400 cases occurred in a water-borne outbreak in Switzerland in 1963 (Bernard, 1965).

Thus, the incidence of typhoid fever can be decreased by (a) a clean water supply, (b) chemotherapy. The antibiotic treatment gives

Table 1

Typhoid and Paratyphoid Fever, 1975.

Country	Total Reported Cases[a]	Cases/10^6 Population
Australia	17	1
U.S.A.	375	1
U.K.	281	6
Canada	176	7
France	1,045	21
Yugoslavia	1,229	60
Thailand	3,601	105
Hong Kong	591	140
Italy	12,403	225
Colombia	7,600	345
Peru	6,645	474
Chile	6,011	601

a = From the World Health Organization Statistics Report, 1976.

many advantages, such as reduction in fever, enhanced recovery from the infectious syndrome, and the more rapid clearance of Salmonella typhi from the blood and faeces, which can greatly reduce the death rate (e.g., from 10-15% to 0.2-0.8%) in countries with a high incidence of the disease (Salcedo et al., 1978). Despite these advances, there are still some clinical complications due to transmissible drug resistant factors (Thorne and Farrar, 1973; Calderon, 1974) and the inability to cure persistent carriers, so that the morbidity rate, although reduced, remains too high.

Thus, antibiotic treatment per se appears inadequate to break the chain of infection. Consequently, immunization programmes against enteric fever seem to be very worthwhile and may prove important, not only in countries with a high incidence of typhoid fever, but also in countries where the disease is apparently controlled.

1.2 Typhoid Vaccines

Immunization programmes against enteric fever, to date, have not had the same success as some other programmes, notably those directed against small-pox and poliomyelitis.

Numerous attempts have been made to promote resistance to enteric infection by both oral and parenteral immunization techniques, but until 1955 the accumulated evidence for the effectiveness of oral and parenteral vaccines suffered from a lack of controlled experimental techniques. At this time, a series of field trials to evaluate parenteral typhoid vaccines was initiated by the World Health Organization (Cjetanovic et al., 1965). These trials were extended over 12 years, using either acetone inactivated (K-type) or phenol-inactivated (L-type) Salmonella typhi, each administered by

subcutaneous route.

The overall conclusions were that each vaccine afforded some protection, particularly in children (Yugoslav Typhoid Commission, 1964; Hejfec et al., 1966; Ashcroft et al., 1967). Even one dose of the K- or L-type, given to children in Guyana, afforded protection (Ashcroft et al., 1967). Thus, parenteral vaccines have been shown to afford significant protection, especially to persons living in endemic areas (Polish Typhoid Committee, 1965; U.K. Department of Co-operation, 1964). Ashcroft et al. (1967) suggested that in such areas, repeated ingestion of subinfective doses of typhoid bacilli may result in an immunized population and that vaccines enhanced the protection by booster effect. However, the volunteer studies and field trials of the World Health Organization suggested that vaccine-induced resistance by the parenteral route would fail when heavily contaminated food was ingested.

Prior to the interesting report by Wahdan et al. (1982), there was already strong evidence that direct antigenic stimulation of the gastrointestinal tract could provide resistance to typhoid fever (Hornick et al., 1966, 1967; Woodward et al., 1970). The experiments in volunteers thus focussed on the oral route of immunization in order to stimulate resistance of intestinal antibodies or cellular factors in the lamina propria.

Hornick and his colleagues (1970) used two types of oral vaccine containing killed typhoid bacilli in keratinized tablets; one was an acetone killed S. typhi strain Ty 2 (monovalent vaccine), the other one was the heat and acetone killed S. typhi and S. paratyphi A and B bacilli. These vaccines were given to volunteers orally. After ingestion of the challenging dose of viable S. typhi, orally

immunized volunteers showed slightly less resistance to infection than did those vaccinated by parenteral route. There were some trial studies using live mutant strains of S. typhi as oral vaccines (Reitman, 1967; Gilman et al., 1977) which gave a degree of protection and an increase in antibodies to H, O, and Vi antigens after challenging. Moreover, the orally vaccinated group showed a significant reduction in the number of positive stools after challenging with viable S. typhi as compared with the non-vaccinated group.

Recently, the Ty 21a attenuated mutant strain used by Wahdan and his group (1982) showed striking protective efficacy in Alexandria, Egypt. In this well conducted field trial, three doses of the Ty 21a vaccine containing $1\text{-}8 \times 10^6$ live bacteria were given at two-day intervals preceded by sodium bicarbonate tablets. No harmful side effects were noted in persons receiving the vaccine. Stool examination showed that those vaccinated did not excrete the vaccine strain for more than one or two days. Two, of many, comments in a W.H.O. sub-committee report on Live Oral Typhoid Vaccine Ty 21a (1982) were that (1) "Simple, reliable tests are needed for the rapid diagnosis of typhoid fever, particularly in field settings. In addition, more effective tools are needed for measuring the immune response in patients, carriers and vaccinees" and (2) the absence of a simple laboratory or clinical test for verifying the successful "take" following vaccination of an individual was noted.

The work reported in this thesis has aimed at contributing to this end. We have attempted to measure serum and gut IgA levels by a simple, unsophisticated test, namely, the Elisa, and then to determine if such levels correlate (in mice) with protection from a normally lethal dose of Salmonellae.

1.3 Salmonellosis

In man, three clinically distinguishable forms of salmonellosis occur: enteric fever, septicaemia and acute gastroenteritis. Typhoid fever is the name generally used for enteric fever caused by S. typhi. Enteric fever is in essence a septicaemia that is the end of the disease process which starts with malaise, anorexia and headache, followed by fever and then the colonizing of the gastrointestinal tract, causing diarrhoea. Some organisms enter the intestinal lymphatics, from where they are disseminated throughout the body, causing septicaemia.

1.3.1 A mouse model for human typhoid

Salmonellae can be divided into three groups with respect to their host preference. (1) Those which are more or less strictly adapted to humans. (2) Others are adapted to non-human animal hosts, e.g., swine are the primary hosts of S. cholerae-suis, but it occurs in other animals as well as man. (3) A large number of types produce disease equally in man and animals. The reasons for these varying host-parasite interactions are unknown. For example, $3x10^9$ S. typhi Ty 2 strain represents a dose that will infect 95% of orally challenged human volunteers (Hornick et al., 1970). However, higher doses of the same strain, $3x10^{11}$ organisms, are required to infect 75% of orally challenged chimpanzees (Edsall et al., 1960). The disease in the animal was relatively mild and transient compared to that observed in humans.

However, as with other infectious diseases of man, a proper understanding of their pathogenesis depends upon the development of adequate experimental models in animals. To this end, the description

by Orskov et al. (1928) of a typhoid-like disease in mice, caused by Salmonella typhimurium, which produced the salient features of enteric fever in humans, was of extreme importance.

1.3.2 Host resistance to typhoid

The host-parasite relationship represents the overall interplay between the factors controlling the changing virulence of an organism and the immense variability of the host surveillance system. Many factors influence the interaction of the host with Salmonellae, such as the age, genetic predisposition and nutritional state of the host and properties of the Salmonellae, such as the ability to adhere and/or colonize, the ability to produce exotoxin, motility and other invasive properties.

The host factors may be expressed by variations in defense mechanisms, such as (1) natural non-specific immunity (e.g., phagocytosis, alternative complement pathway); (2) specifically acquired immunity (antibody production, cell mediated immunity). Obviously one reason for the peculiar susceptibility of some host species could be due to their failure to provide a rapid effective immunity to Salmonellae. This could be due to a relative immunological tolerance, shown to exist in mice (Rowley and Jenkin, 1962), with regard to a potentially protective antigen of S. typhimurium.

Further, most inbred mouse strains can be divided into two groups, those extremely susceptible or those relatively resistant to Salmonella infection. (However, this resistance or susceptibility does not necessarily extend to other pathogenic organisms.) Consequently, the resistance to Salmonella infection may involve only one or a few dominant genes (Plant and Glynn, 1974), since mouse

strains of intermediate resistance are rare and the progeny of crosses between resistant and susceptible mice are generally resistant (Plant and Glynn, 1974; Robson and Vas, 1972; Von Jeney et al., 1977).

1.3.3 The role of antibody production in Salmonella infection

The role of antibody production in Salmonella infection is difficult to assess. For example, both resistant and susceptible mice develop similar antibody responses to killed Salmonella vaccines (Robson and Vas, 1972), and although the Balb/C strain is less able to produce antibody to the major O-5 antigen of S. typhimurium than is the Swiss strain (Auzins and Rowley, 1969), both are highly susceptible to i.p. challenge. Further, the antibody responses towards enterobacterial common antigen (ECA) do not correlate with Salmonella resistance (Gorzynski et al., 1970).

1.3.4 Cell mediated immunity and Salmonella infection

The lack of correlation between antibody production and resistance to Salmonella infection has led to the concept that cell mediated immunity may be responsible for this resistance. For example, Plant and Glynn (1976) have shown that Salmonella-resistant mouse strains develop better delayed type hypersensitivity (DTH) to Salmonella antigens than do susceptible strains, although the DTH response to other antigens (e.g., oxazolone) was the same in both susceptible and resistant mice. Thus, cell mediated immunity (CMI) may be all important in resistance to Salmonella infection. Further, mice can be protected against S. typhimurium challenge by previously feeding a variety of Salmonella strains which do not need to be antigenically related to the challenge strain in terms of their Kaufman-White O

antigens (Moser et al., 1980).

As a consequence, it appears most likely that resistance to Salmonella infection relies upon the non-specific activation of macrophages, as a result of T-cell mediation, which was observed by Mackeness (1969) to give protection against listeria infections. This activation of local macrophages in ways which enable them to kill the invading pathogens is now known to occur through lymphokines which are released from T-cells which have been previously sensitised to some unidentified antigen of the challenge bacteria.

1.4 Pathogenesis of Salmonella Infection

Following ingestion of Salmonellae, initial foci of infection arise in the Peyer's patches of the small intestine and in the mesenteric lymph nodes; here they multiply and disseminate via the efferent lymphatics, entering the blood stream by the thoracic duct. They are then largely removed from the blood stream by the liver and spleen. Subsequent proliferation in these tissues will produce a systemic bacteraemia, fever and, if unchecked, death.

1.4.1 Gastrointestinal phase

The earliest site of attack following oral administration of Salmonellae to guinea pigs may be in the tonsils (Edsall et al., 1960); this may lead to systemic infection (Gaines et al., 1968; Carter et al., 1975) and infection of the upper respiratory tract (Carter et al., 1975). In mice, temporary infection of the stomach and intestinal tract is observed, with the caecum retaining the greater number of the infecting bacteria for some hours (Carter and Collins, 1974; Ozawa et al., 1973). After about 24 hours when the

animal's gut is largely free from the infecting dose, foci of infection appear in the Peyer's patches (Carter and Collins, 1974). Gaines et al. (1968) have made similar observations in chimpanzees. The Peyer's patches and their draining lymph nodes appear to be the most severely infected intestinal tissues (Carter, 1975), although Salmonella lodging in the caecum may give rise to systemic infection (Ozawa et al., 1973).

1.4.2 Systemic spread from the intestine

Rapid proliferation of Salmonellae in the Peyer's patches is followed by their appearance in the draining lymph nodes of the ileum and caecum (Carter and Collins, 1974), where they continue to multiply before entering the blood stream via the thoracic duct. As they circulate, they are quickly trapped by the cells of the reticulo-endothelial system. Further multiplication in these cells results in a de novo bacteraemia which can cause re-infection of the intestine (Gaines et al., 1968), biliary infection (Edsall et al., 1960) or infection of the upper respiratory tract (Carter et al., 1975).

1.5 Virulence Factors in Salmonellae

As in other organisms, several surface components such as the Vi antigen or smooth LPS increase the virulence of this pathogen by hindering or preventing phagocytosis or complement mediated killing. Further, the presence of pili or other appendages that enable the organism to adhere to mucosal surfaces will promote colonization of the intestinal tract and increase its virulence.

1.6 Homeostatic Mechanisms of the Gut

The innate capacity of the gastrointestinal tract to eliminate large numbers of pathogenic Salmonellae has been frequently demonstrated (Knop and Rowley, 1975; Carter and Collins, 1974). Such elimination is probably due to any or all of the following mechanisms.

1.6.1 Gastric acidity

Once bacteria have been swallowed they are subjected, in the stomach and upper part of the duodenum, to the action of gastric juice with its high acidity. It has long been recognized that the low pH of gastric secretions plays a major role in preventing living bacteria from reaching the intestine (Knop and Rowley, 1975; Arnold and Brody, 1962). The latter workers also showed that a rich duodenal flora could be induced in dogs if sufficient alkali was administered prior to oral feeding with organisms. An important conclusion from such investigations is that periods of starvation predispose an animal to enteric infections (Knop and Rowley, 1975; Tannock and Smith, 1972; Tannock and Savage, 1974; Collins, 1972). Since starvation, even for short periods, results in the rapid removal of water and food from the stomach, via the pyloric sphincter, little acid mediated killing of ingested bacteria could be expected to occur (Mossel, 1976).

1.6.2 Intestinal motility

The rate of movement of intestinal contents must clearly affect the chances of ingested bacteria to colonize the alimentary tract. Indeed, complete inhibition of bowel motility abrogates the ability of the small intestine to move its contents into the caecum. This results in overgrowth in the small intestine of bacteria normally

found only in the lower bowel (Donaldson, 1967). Further, the arrest of peristalsis by dosing with atropine in mice (Knop and Rowley, 1975) or with opiates in guinea pigs (Kent et al., 1966; Takeuchi, 1967) markedly increases the susceptibility of the small intestine to invasion by Salmonellae. In addition, S. enteritidis and Shigellae have been shown to multiply rapidly in the pharmacologically immobilized small bowel (Miller and Bohnhoff, 1962; Formal et al., 1963).

1.6.3 Antibacterial mechanisms

Among the possible bactericidal substances present in the stomach and intestine, lysozyme may be of significance (Goldsworthy and Florey, 1930). Apart from lysozyme, mucus itself may provide an efficient mechanical protective mechanism, as Florey (1933) found that mucous formed a lace-like protective network over the mucosal surface. Indeed, some enteric pathogens possess powerful mucinases. A possible role for specific sIgA is that it forms a strongly hydrophilic coat around the organisms and decreases the likelihood of their adherence to mucosal surfaces (Williams and Gibbons, 1972).

1.6.4 Normal gut flora and enteric infection

A vast number of bacteria constitute the normal flora of the gut and these organisms play an important role in maintaining the natural resistance to enteric infections. Thus, germ-free mice are more susceptible to typhoid than are mice with a conventional flora (Abrams and Bishop, 1966; Collins and Carter, 1978; Tannock and Savage, 1974). Indeed, the LD_{50} for S. enteritidis by oral challenge in normal mice is about 5×10^6 organisms, but for germ-free mice only about 5 organisms constitute an LD_{50}.

Also, mice show much greater susceptibility to oral infection with Salmonellae following oral administration of antibiotics (Meynell and Subbaiah, 1963; Collins, 1970). Freter (1955, 1956) used streptomycin to remove the normal gut flora and thus made guinea pigs susceptible to oral infection with Shigella flexneri and V. cholerae. Their normal insusceptibility could be restored by feeding the animals E. coli.

It therefore seems that the normal flora is required to maintain the resistance of the gut and is able to reduce the chance of invasion by Salmonellae (1) by producing substances toxic for Salmonellae, e.g., organic acids (Meynell, 1963; Bohnhoff et al., 1964) or bacteriocins; (2) by stimulating bowel motility (Savage, 1972); (3) by dispossessing the invaders of the niches in which, and the cell surfaces to which, they can adhere so that their ability to colonize is affected (Savage, 1969).

It therefore seems quite clear that the ultimate determinant of Salmonella infection is the ability of the pathogen to grow in and colonize the small intestine.

The barriers operating against such intestinal infection in normal animals are formidable. Consequently, the relatively high levels of enteric disease apparent in many countries of the world must reflect the degree of malnutrition and lack of hygiene and the high bacterial antigenic load which exists in these areas.

CHAPTER 2

The Mucosal Immune System

The interface between the body and its environment may be divided into two very different areas, the skin and the mucosae. The former is nearly impermeable to potential invaders from the outside; in contrast, innumerable environmental agents gain access to the animal host via the interface between the environment and the mucous membranes of the gastrointestinal and respiratory tracts and, unlike the skin, mucous membranes do not have the physical protection of a keratin sheath.

The gastrointestinal tract, a single layer of epithelial cells in direct contact with the external environment, must provide the "first line of defense" against penetration of a variety of potentially noxious substances which reside in the intestinal lumen. These intraluminal substances, such as microorganisms, dietary antigens, toxins, enzymes, and intestinal breakdown products, may penetrate the mucosal epithelial barrier under pathologic conditions and gain access to the interstitial tissue space of the intestine or be taken up into the systemic circulation. Penetration of the mucosal barrier may, in turn, result in clinical disease manifested by infection, allergy or autoimmune states.

In order to combat antigens which cross the mucosal barrier, the animal host has created an elaborate system of defense mechanisms within the intestinal lumen and on the luminal mucosal surface which act to control and maintain the epithelium as a barrier to the uptake of macromolecular antigens.

In this chapter we will emphasize the immunological defenses of

the host in terms of the local immunologic system. We will outline the structure and function of the gastrointestinal immune system and discuss the nature and function of mucosal antibody. But, first, a short acknowledgement of those who pioneered this field and whose efforts have provided the foundations for our current knowledge and direction.

2.1 Historical Perspective: The Demonstration of Mucosal Antibody and its Nature

In 1927, Professor A. Besredka of the Pasteur Institute published a monograph entitled "Local Immunization". Among the many experiments reported, he discussed the protection of rabbits following oral immunization when subsequently challenged i.v. with Shigella bacilli. He also pioneered the concept of local immunity separate from systemic immunity. However, as Tomasi (1969) observes, even Besredka's work was anticipated to a degree by the neurologist Chvostek and by Shiga, both of whom carried out, in 1908-1909, very similar protection experiments.

Although several workers, for example, Davies (1922), showed certain antibodies to be present in various secretions, when none could be detected in the serum, perhaps the first clear demonstration of a possible role for local antibody was made by Burrows in the late 1940's (Burrows et al., 1947; Burrows and Havens, 1948). Again, Burrows showed that copro antibody existed independently of serum antibody. The role of local immunity in the respiratory tract was subsequently pursued by Fazekas de St Groth and his colleagues in the 1950's (Fazekas de St Groth et al., 1951; Fazekas de St Groth, 1951). Their studies on experimentally induced influenza virus infections

in mice clearly showed the association of local antibody with the mucosal cells of the respiratory tract and its stimulation by aerosol deposition of antigen.

However, little attention was, as yet, being given to the concept of local immunity. Indeed, a full appreciation of the importance of a local tissue resistance could not have developed in the absence of studies on the fundamental structure of antibodies. In this regard the work of Heremans (1960), in which he described the IgA class of antibodies in the serum, proved of the greatest importance. Further observations showed that IgA was the predominant immunoglobulin in milk (Hanson, 1961), and it was also found to be the predominant antibody in secretions bathing mucous surfaces (Tomasi and Zigelbaum, 1963; Cebra and Robbins, 1966; Heremans and Vaerman, 1971), although it constituted only a minor fraction of the total serum immunoglobulin.

Such observations led ultimately to the concept of a secretory immune system, and immunofluorescent techniques were subsequently used to demonstrate the marked predominance of IgA producing cells locally in secretory tissues, first in parotid tissues (Tomasi et al., 1965; Gelzayd et al., 1967; Rossen et al., 1967), and later in the tissues of the respiratory tract (Brandtzaeg et al., 1967; Rossen et al., 1967) and gastrointestinal tract (Brandtzaeg et al., 1967; Jeffries and Sleisenger, 1965; Crabbé and Heremans, 1966; Crabbé et al., 1965; Rubin et al., 1965; Crabbé, 1967). In 1965, Tomasi et al. demonstrated that the unique characteristics of IgA, which facilitated its transfer through the epithelial barrier, were due to the presence of a polypeptide which they called transport piece and now called Secretory Component (SC). This secretory immunoglobulin (sIgA) was

synthesized in the plasma cells of the local mucosa (Tomasi and Bienenstock, 1968).

The sIgA molecule (Fig. 2.1) is grossly hydrophilic, unlike IgG, and it appears that one of its major functions is to specifically coat pathogens, allergens and other environmental antigens, thus preventing their association with epithelial cells and subsequent invasion of, or insult to, the body of the host.

2.2 The Histology and Ultrastructure of the Gastrointestinal Immune System

The main function of the intestinal tract is the absorption of nutrients. However, due to its continuity with the external environment, it is also required to exclude microorganisms, toxins and other deleterious environmental agents.

The intestinal surface is constituted of epithelial cells tightly joined at their apices and covered by rigid, closely packed microvilli forming a mechanical barrier to intestinal organisms (Owen, 1981). It has been suggested that the mucosal immune system evolved as an adjunct to this mechanical barrier and modulates and controls the movement of molecules and particles through and from the lumen (Waksman and Ozer, 1976).

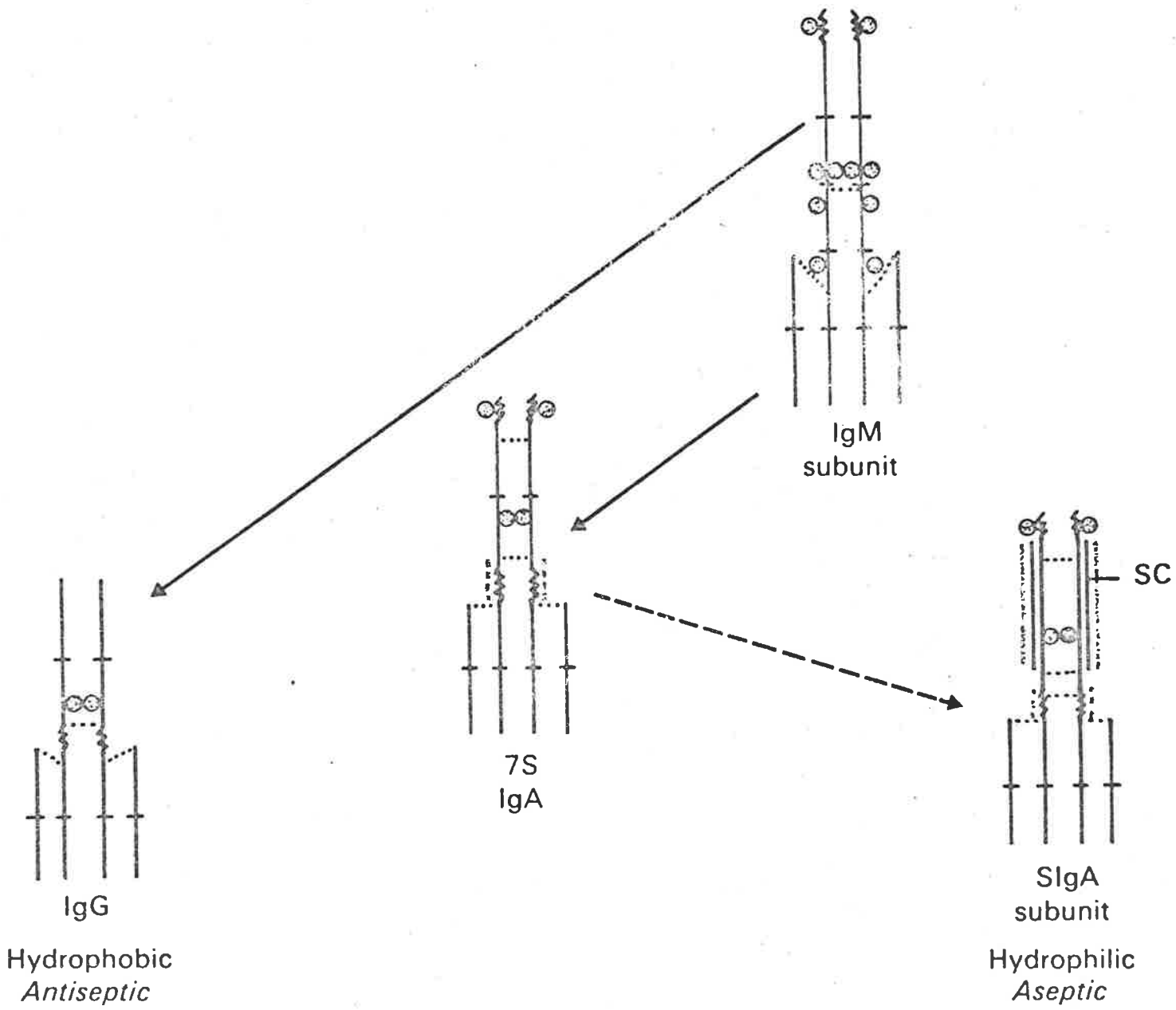
Lymphoid tissue is organized along the intestinal tract into structures which optimize the sampling of intestinal antigens (Owen and Nemanie, 1978). Although discrete lymphoid nodules occur scattered throughout the intestine, the most prominent of these lymphoid organs are the tonsils, the Peyer's patches and the appendix.

During foetal development, gut associated lymphoid tissue (GALT) is organized into follicles made up of macrophages and lymphocytes.

Figure 2.1

Development and carbohydrate location of the different immunoglobulin classes. Carbohydrate shown as solid balls, with the exception of the hinge region of IgA and sIgA where, due to lack of space, five points are drawn. The carbohydrate of the SC has not been localized precisely and is shown as a superficial line.

[From "Cell Adhesion and Motility" (eds. Curtis, A. S. G., and Pitts, J. D.). The Third Symposium of the British Society for Cell Biology, Cambridge University Press, 1980.]


IgM
subunit
7S
IgA
SC
SIgA
subunit
IgG
Hydrophobic
Antiseptic
Hydrophilic
Aseptic

At birth, when these follicles are exposed to antigens, germinal centres develop where replicating lymphocytes replenish migrating cells which pass via the lymphatics to the circulation.

Owen and Nemanie (1978) state that the epithelium of all follicles is modified to enhance the local uptake of luminal particles and microorganisms. This consequent localized decrease in the intestinal barrier is, in part, compensated by the containment of particles and organisms by the macrophages, which, together with T and B lymphocytes, populate the domes beneath the follicle epithelium and which participate in the initiation of the immune response.

2.3 Gut Lymphoid Tissue

2.3.1 Histology of Peyer's patches

The gastrointestinal tract is replete with lymphoid tissue capable of mounting an immunologic response to prevent the penetration of the epithelial barrier by antigens. Lymphocytes and macrophages are present in abundance as aggregates in the Peyer's patches of the small intestine or as a diffuse population of cells in the lamina propria of the small and large intestine, where they co-exist with immunoglobulin secreting plasma cells. The Peyer's patches which are formed by groups of lymphoid follicles are also distributed throughout the ileum and appendix. Their distribution in humans increases distally from a few follicles to more than 900 in the terminal ileum (Cornes, 1965); there is a similar increase in bacterial concentration. In mice or rats, patches of roughly uniform size occur throughout the small intestine and usually consist of 3 to 9 follicles (Abe and Ito, 1977). In mice, Peyer's patches appear as white bulges on the serosal surface. The active follicles form mounds, pushing aside the villi

and projecting into the lumen (Fig. 2.2). In Balb/C mice, the follicles are large and round with rapidly replicating lymphocytes. Some mouse strains, such as C57, have smaller, flatter follicles with a less active lymphocyte population.

Owen (1981) gives the following description of Peyer's patches in mice. The surface of the Peyer's patch follicle consists of columnar cells, covered by closely knit microvilli and intervening M cells whose surfaces are roughened and irregular. The M cell provides a reduced barrier to particles, and large molecules and the membrane-like attenuation of M cell cytoplasm facilitates the approach of underlying lymphocytes (mainly T-cells) and macrophages to the intestinal lumen (Fig. 2.3).

Macromolecules are transported by M cells from the lumen, by vesicles, into the space between cells surrounding the migrating lymphocytes (Owen, 1977). Beneath the dome epithelium is an area populated largely by macrophages and lymphocytes (Waksman and Ozer, 1976) which seem to be in constant transit in and out of the epithelium and into the lymphatics.

Beneath this traffic area lie the germinal centres consisting largely of B cells and macrophages containing cellular and non-cellular debris (Owen and Nemanie, 1978). Germinal centres lie deeper in the patch tissues and these develop only when the surfaces of the patches are exposed to luminal antigens (Owen, 1981).

2.3.2 Antigen uptake by GALT

Particulate antigens which enter Peyer's patches via the M cells are degraded by macrophages; they then pass to the mesenteric nodes via the lymphatics, or enter the portal circulation to be taken up

Figure 2.2

Diagram of Peyer's patches structure in the mouse. GC - germinal centre; TDA - thymus-dependent area.

[From Parrott, D. M. V. (1976). The gut as a lymphoid organ. Clin. Gastroenterology, 5(2), 211.]

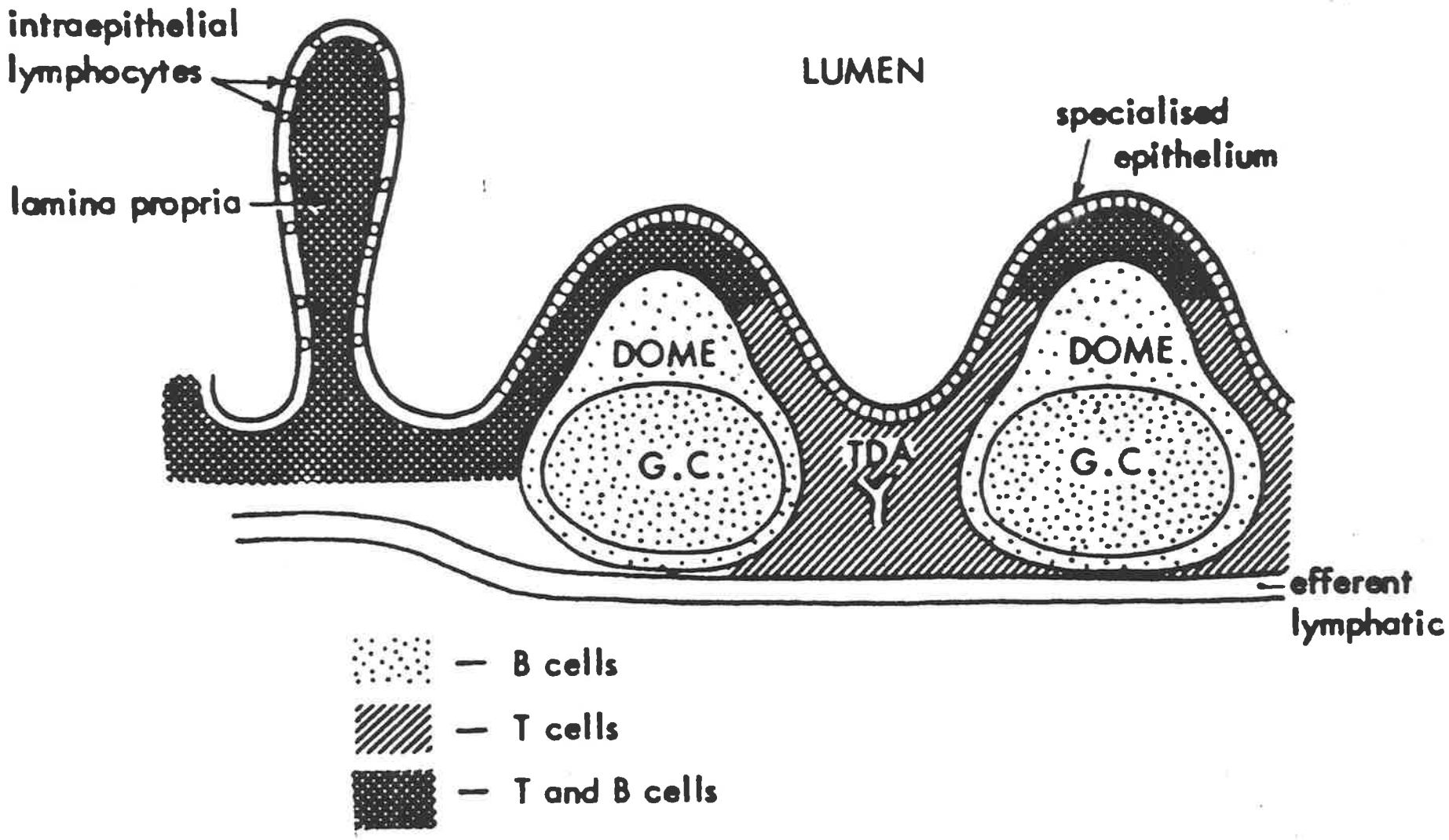
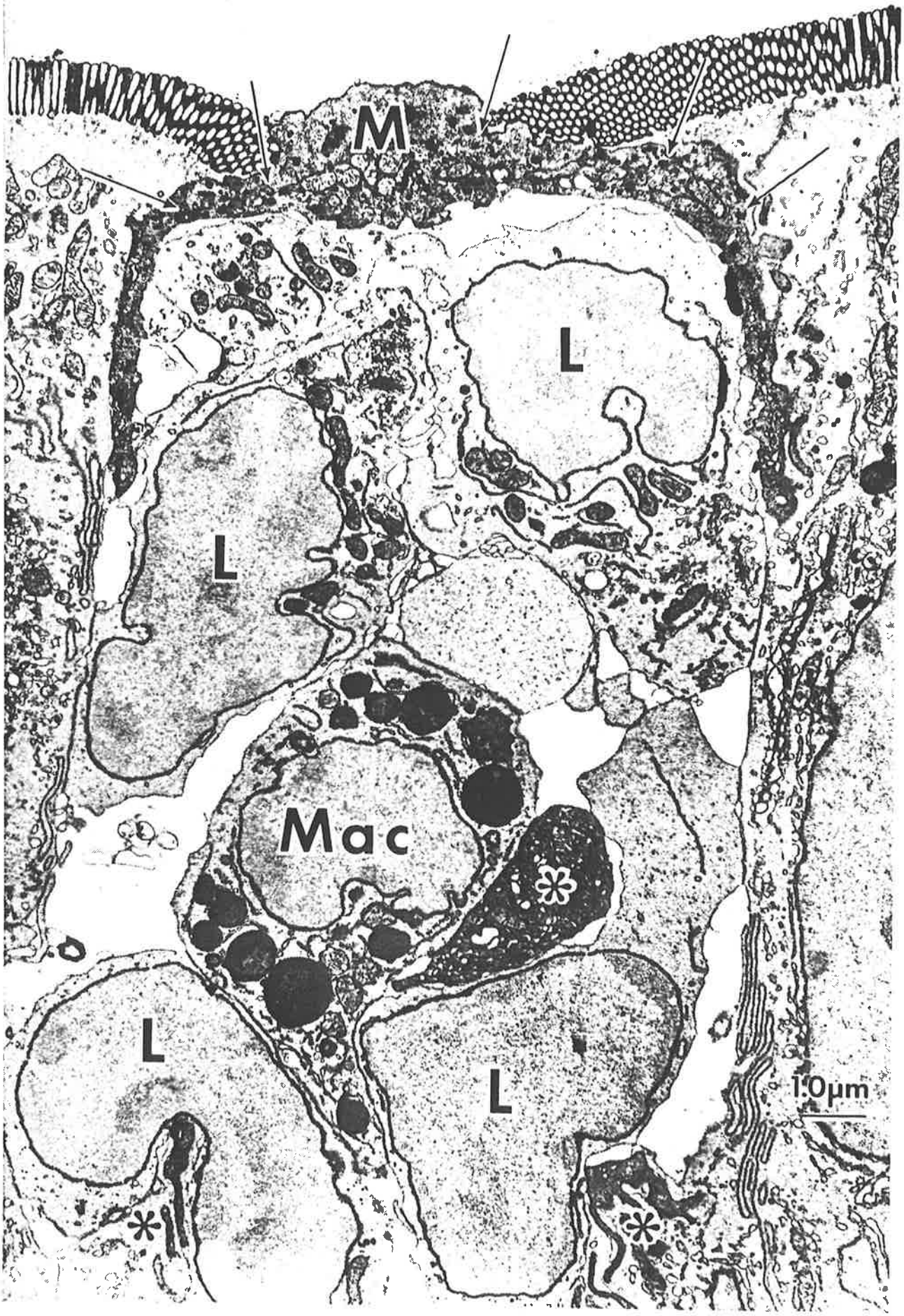

intraepithelial
lymphocytes
lamina propria
LUMEN
specialised
epithelium
DOME
G.C
IDA
DOME
G.C
efferent
lymphatic
— B cells
— T cells
— T and B cells

Figure 2.3

Darkly stained cytoplasm of a mouse M cell (M) stretched between two microvillus-covered columnar cells (C) like a membrane separating wandering lymphocytes (L) and a macrophage (Mac) from the lumen above. Vesicles (arrows) are transporting exogenously administered horseradish peroxidase from lumen to enfolded lymphoid cells.

[From Report of the 81st Ross Conference on Pediatric Research, published by Ross Laboratory, Columbus, Ohio, 1980.]


M
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L
1·0μm

by Kupffer cells (Owen, 1977; Carter and Collins, 1974). Passage through the specialized epithelium of lymphoid follicles, rather than leakage into intracellular spaces, may determine whether a secretory IgA response will result rather than an IgG response.

Although normal antigen uptake from the lumen is concentrated at areas above the lymphoid follicles, the effector areas of the immune response are distributed throughout the intestine. Lymphocytes, after replication, distribute along the intestinal epithelium, lodging between columnar cells and in the lamina propria (Ottaway and Parrot, 1979). Some of these lymphocytes differentiate into plasma cells beneath the basal lamina and others appear to migrate into the intestinal lumen (McDermott et al., 1980). (Fig 2.4).

Macrophages are found both in organized mucosal lymphoid tissue such as GALT and also in the lamina propria (Bienenstock and Dolezel, 1971). Only very recently has the role of the mucosal macrophage received attention (Lefevre et al., 1979). Carageenan in the diet can cause mucosal ulceration and is found in mucosal macrophages (Abraham et al., 1974). The concentration of environmental noxious agents in these cells is obviously of great biological importance. It is possible that mucosal macrophages have a selective migration pattern and traffic between mucosal tissues. Further examination of this aspect should help us understand how we balance our contact with environmental agents.

In mice, the existence of T blasts in organized lymphoid tissue may be of two types, one that is circulating and has a marked tendency to home to the gut, and a second that does not home to the gut and may be sessile (Guy-Grand et al., 1974). T lymphocytes, after circulating, return to the lamina propria and the intra-epithelial

cells of the intestine, may mature into mast cells in the intestinal wall (Guy-Grand et al., 1978). Goblet cells have been considered important in mucosal infection for many years (Ackert et al., 1939; Wells, 1963). Miller and Nawa (1979a, b) have reported goblet cell hyperplasia following intestinal nematode infection in rats and shown that this is a thymus-dependent phenomenon. Furthermore, the release of mucous from goblet cells can be stimulated by immune complexes (Walker et al., 1977) and by antigens given orally (Lake et al., 1979). The possible importance of mucous secretion is further suggested as it is promoted by the IgE-mediated intestinal anaphylactic reaction (Lake et al., 1980).

In response to certain antigens, polymorphonuclear cells, i.e., mucosal mast cells, lymphocytes, macrophages and goblet cells, which play a role in non-specific resistance mechanisms of mucosal immunity, pass into the lumen via the tonsils, the intestine and the appendix, suggesting that a specific function is being carried out and that such migration is not just a means of eliminating senescent cells (Bellamy and Nielsen, 1974). These cells are sometimes so numerous that they appear as an exudate covering the surface of the lumen. Owen (1981) concludes that "Even though the lumen lies outside the body proper, we must now revise our evaluation and recognise that it lies at least within the sphere of influence of the intestinal immune system".

2.4 Liver and the Secretory Immune System

Experiments in the rat have shown that sIgA is the major immunoglobulin in bile and that ligation of the bile duct causes the prompt and selective appearance of sIgA in serum (Lemaitre-Coelho et al.,

1977, 1978a). Indeed, the liver actively transports circulating polymeric IgA into the bile by a secretory component mediated transport system (Jackson et al., 1978; Orlans et al., 1978; Mullock et al., 1979; Birbeck et al., 1979; Fisher et al., 1979; Socken et al., 1979). Further, IgA antibodies directed against antigens introduced into the gut lumen or Peyer's patches appear in the bile (Hall et al., 1979; Lemaitre-Coelho et al., 1978b). It therefore seems likely that circulating IgA can be removed from serum and be transferred as sIgA into the upper gut via the bile. Thus, the surface of the intestine is coated not only with antibodies locally synthesized and secreted, but also by those synthesized elsewhere and secreted via bile into the intestine. The liver itself has been shown to contain very few IgA-forming plasma cells (Brown et al., 1980).

2.5 Cellular Immunity

The existence of local cellular immunity associated with the mucosal surface has been recently recognized. Like humoral immunity, it seems to function, at least partially, independently of systemic cell-mediated immunity (Galindo and Myrvik, 1970). Most investigations into local cell-mediated immunity have focussed on lung tissue; Henney and Waldman (1970) have reported that splenic lymphocytes from animals immunized subcutaneously inhibited the migration of normal guinea pig macrophages in the presence of antigens, but cells obtained from the bronchial washings of these animals exhibited no inhibition of macrophage migration. On the other hand, the splenic lymphocytes from animals immunized locally showed no inhibition of macrophage migration, but bronchial lymphocytes strongly inhibited macrophage migration in the presence of antigen. This study points

to the existence of a respiratory tract (local) cell mediated immunity which is stimulated to a greater extent by local immunization than by parenteral immunization and may be independent of systemic cell mediated immunity although the differences observed may be purely qualitative. The independent existence of local cell-mediated immunity in the respiratory tract against M. tuberculosis antigens has been demonstrated in humans (Jurgenson et al., 1973). The work of Yamamoto et al. (1970) and Barclay et al. (1973) showed that this local cell mediated immunity correlated with protection in vivo as aerosol immunization gave protection against an M. tuberculosis aerosol challenge, whereas parenteral immunization did not.

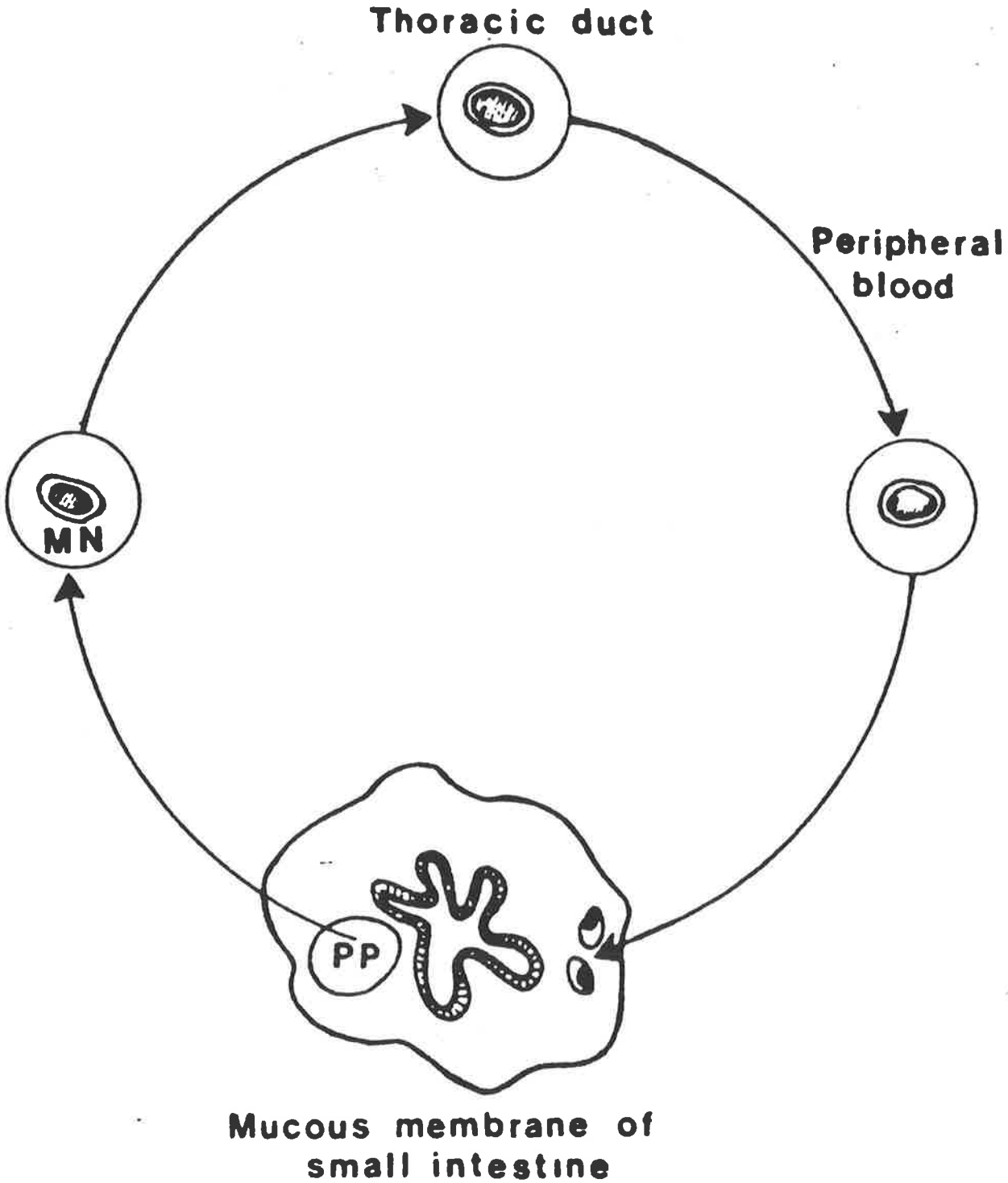
Although most investigations of local cell mediated immunity have involved lung tissues, the studies reported by Müller-Schoop and Good (1975) indicate that lymphocytes committed to cell mediated immunity were present in the Peyer's patches of the ileum and were capable of responding to antigens present within the intestinal lumen.

It has also been shown that some protein antigens immunize when given parenterally but tolerize when given orally, and this latter effect is mediated by T cells (Richman et al., 1978). Such findings suggest a divergence in systemic and local responses. Gowans and Knight (1964) and Parrott and Fergusson (1974) have shown that the gut mucosae are populated by T and B immunoblasts. All cells entering the gut mucosae are in the form of these activated immunoblasts that pass from the mesenteric lymph node and proceed via the thoracic duct to the circulation and then home to the gut (Fig. 2.4) (Griscelli et al., 1969; Parrott et al., 1975; Hall et al., 1972; Guy-Grand et al., 1974). These T lymphoblasts have been found

Figure 2.4

Scheme for the IgA cell cycle. Precursor cells in organized gut-associated lymphoid tissue, such as Peyer's patches (PP), travel by lymphatics to mesenteric lymph nodes (MN), where they divide and differentiate. From the MN, cells proceed via the thoracic duct to the blood, whence they preferentially lodge in mucous membranes, principally in the intestine, to become IgA plasma cells.

[From Report of the 81st Ross Conference on Pediatric Research, published by Ross Laboratories, Columbus, Ohio, 1980.]


Thoracic duct
Peripheral blood
MN
PP
Mucous membrane of small intestine

selectively lodging between intra-epithelial cells and in the lamina propria (Guy-Grand et al., 1974), and some of them become mucosal mast cells (Guy-Grand et al., 1978). While B blasts contain and secrete antibody and differentiate into antibody-secreting plasma cells, T blasts differentiate into cytotoxic killer cells, or revert to small lymphocytes which exert effector functions in cell-mediated immunity by their effects upon other cell types including macrophages, monocytes, eosinophils, etc.

Oral immunization can lead to a systemic delayed hypersensitivity reaction (Perrotto et al., 1974) or the generation of cytotoxic T lymphocytes in the Peyer's patches and extra-intestinal sites (Kagnoff, 1978). Nelson et al. (1976) also have shown the cytotoxic effector cell function in organized lymphoid tissue of the gut (GALT) in guinea pig. Levin et al. (1976) studied the sequence of development of cell-mediated immunity in the Peyer's patches of rats after local infection with Trichinella spiralis. Furthermore, it was observed that the cell-mediated immunity in the Peyer's patches was only transient.

In addition, administration of antigen to the gastrointestinal tract or respiratory tract can lead to the appearance of cells capable of releasing macrophage migration inhibition factor (MIF) (Frederick and Bohl, 1976; Huntley et al., 1979; Heuney and Waldman, 1970; Nash and Holle, 1973). Further mice fed orally with Salmonellae developed delayed type hypersensitivity (DTH) to Salmonella antigens which was concomitant with the development of intestinal and serum antibodies (Moser et al., 1980).

2.6 The Chemical Structure of IgA and sIgA and a Comparison with Other Immunoglobulins

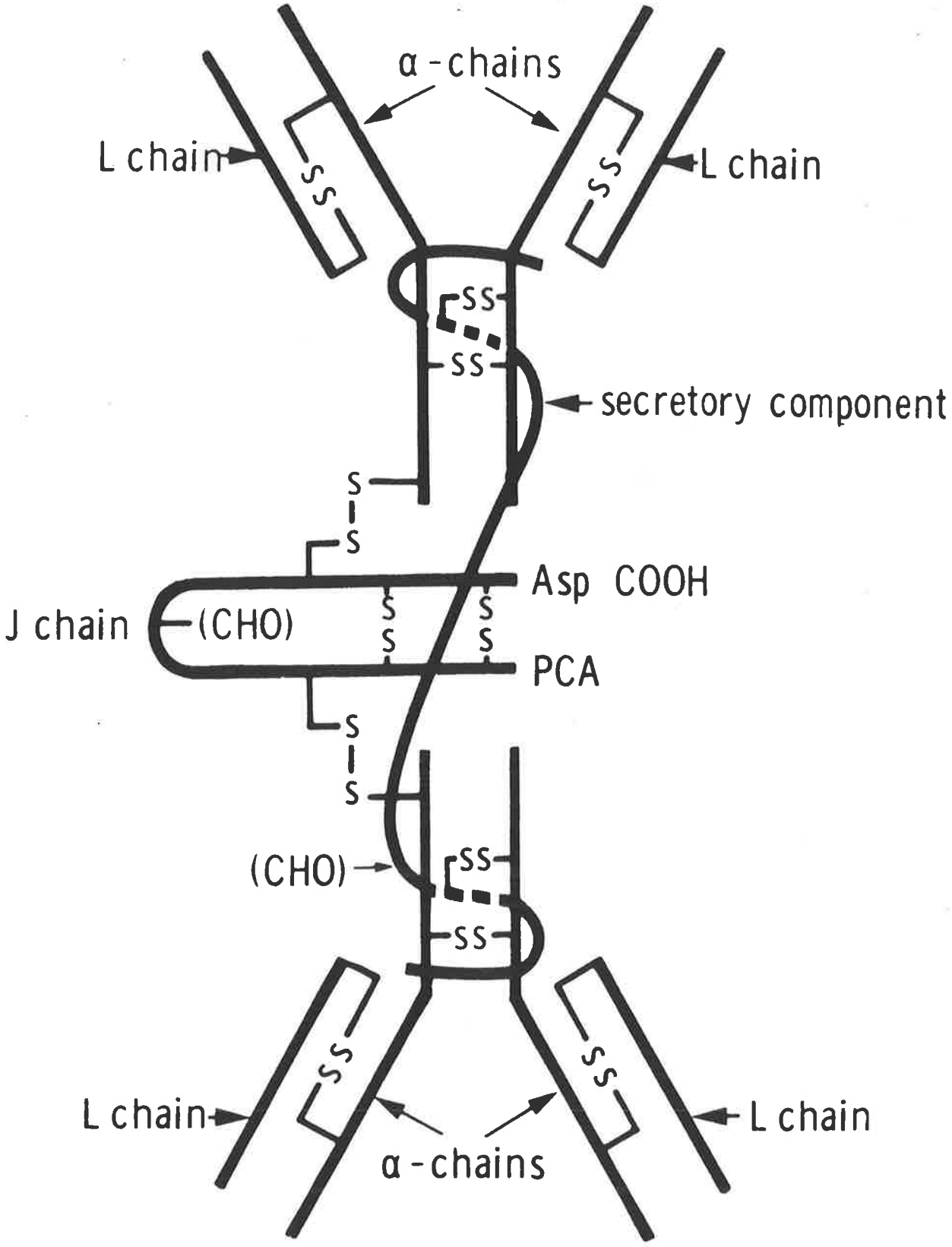
In 1963, Chodirker and Tomasi reported that IgA was the predominant immunoglobulin in secretions. The majority of IgA molecules in external secretions exist as dimers, composed of two 7s IgA monomers, plus two other proteins, J chains (15,000 d) and a secretory component (SC) of 70,000 d. This sIgA molecule is depicted in Fig. 2.5, which shows the two IgA monomers, linked via disulphide bridges between the heavy chains, plus two additional proteins. It is not clear how the various peptide chains are linked together. However, the J chain is known to be the product of the plasma cells which synthesize the IgA molecule and that it is added just before secretion. It is also known that the J chain is attached to the penultimate half-cystine of the α-chain (Mestecky et al., 1974) and is shown in an internal location between the carboxyl terminal ends of the two sets of chains.

In contrast to the J chain, the secretory component is not synthesized by plasma cells but by mucosal epithelial cells of exocrine glands. A secretory component is a single polypeptide chain containing carbohydrate, approximately 9% (Tomasi and Bienenstock, 1968; Halpern and Koshland, 1970) which becomes covalently bonded to α-chain in the Fc region of 80% of sIgA molecules (Halpern and Koshland, 1970). Strong non-covalent interactions also help to stabilize the molecule (Tomasi and Bienenstock, 1968; Newcomb et al., 1968). The secretory component is shown (Fig. 2.5) coiled about the Fc ends of the two IgA monomers (Svehg and Bloth, 1970). The molecular weight of sIgA is about 390,000 daltons and its sedimentation coefficient about 11s (Tomasi et al., 1965).

Figure 2.5

Schematic diagram of human secretory IgA showing the possible arrangement of IgA monomers, secretory component, and J chain.

[From "Immunochemistry: An Advanced Textbook" (eds. Glynn, L. E., and Steward, M. W.). Wiley, 1977.]


α-chains
L chain
L chain
SS
SS
SS
SS
secretory component
S
S
Asp COOH
J chain
(CHO)
S
S
S
S
PCA
S
S
(CHO)
SS
SS
SS
SS
L chain
L chain
α-chains

The secretory component is extremely rich in carbohydrate (22.8%) according to Lindh (1975), and since the secretory component binding requires protein-protein interaction with disulphide bridge formation (Halpern and Koshland, 1970), this probably leads to a superficial location of the carbohydrate moieties of the secretory component (SC), creating a strongly hydrophilic molecule.

Differences among the various classes of immunoglobulins reside in the primary structure of their heavy chains which are designated γ (IgG), α (IgA), μ (IgM), δ (IgD) and ε (IgE), as shown diagrammatically in Fig. 2.1; two kappa or lambda light chains are found on a certain percentage of the monomers of each class. Thus, anti-light chain serum will cross react with all of these Ig species, but anti-α serum will interact specifically with IgA, anti-γ serum with IgG and anti-μ serum with IgM, etc. The gross chemical composition of the different immunoglobulin classes shows the greatest differences with respect to carbohydrate content; IgM, sIgA and IgE show concentrations of 11-15%, whereas IgG has only 3%, as shown in Table 2.1 (Heremans, 1974; Beale and Feinstein, 1976; Bennich and von Bahr-Lindström, 1974).

In IgM there are five carbohydrate moieties associated with each μ chain and most of these are located on the outside of the molecule. Thus, sIgA and IgM could confer an hydrophilic character on particles to which they bind. Further, since IgM can fix complement (C), it also has the potential to produce an hydrophobic coat due to the bound, non-polar C3b component.

2.7 The Synthesis and Secretion of sIgA

sIgA is produced by plasma cells located in the lamina propria

Table 2.1

Presence of carbohydrate in immunoglobulins.

Immunoglobulin Class	Concentration (%)	Location
IgM	12	Surface (a)
IgG	3	Inside (a)
IgA	7.5	Mainly surface (a)
sIgA	11-15	Surface (a)
IgE	12	Mainly surface (b)

(a) Beale and Feinstein (1976).

(b) Bennich and von Bahr-Lindström (1974).

of the gastrointestinal tract (see IgA cell cycle, section 2.8) (Tomasi and Bienenstock, 1968) as a dimer containing an additional polypeptide J chain (Koshland, 1975) that has been shown to be localized to those plasma cells, but the SC is not; this is localized in the mucosal epithelium, in the intracellular spaces of the mucosa and on the surface of the epithelial cells lining the lumen of the secretory glands (Brandtzaeg, 1974, 1978; Crago et al., 1978).

Biosynthetic, histologic and immunofluorescent studies by several groups, notably Brandtzaeg (1974), Poger and Lamm (1974) and Tourville et al. (1969), have differentiated between free and bound SC and suggest the following sequence for the assembly of sIgA:

(1) The IgA dimer with the J chain is formed intracellularly in plasma cells of the lamina propria and is secreted into the intestinal fluid via epithelial cells.

(2) Most of the dimeric IgA then moves towards the lumen through the basement membranes and between the basement membranes, and through the epithelial cells.

(3) The covalent and non-covalent interactions between these dimeric IgA molecules and SC produced by mucosal epithelial cells occurs as the dimers pass between and through the epithelial cells. This complex of sIgA is transported across the epithelial cell in vesicles to be released at the apical surface into the intestinal lumen (Allen et al., 1973; Nagura et al., 1979).

2.8 The "IgA Cell Cycle"

In section 2.2 it was noted that the surface M cells of Peyer's patches provide a reduced barrier to particulate antigens and macromolecules; this is also true of the follicles aggregated into the

appendix and cecal patches.

Antigenic stimulation is initiated in these organized lymphoid tissues with the production of lymphoblasts. These lymphoblasts proceed circuitously from the intestine via lymphatics to the mesenteric lymph nodes, and via the lymphatics to the thoracic duct, and then 'home' to the gut-associated lymphoid tissues, including the intestine (Gowans and Knight, 1964; Hall et al., 1972).

While studying the recirculation of lymphocytes, Gowans and Knight (1964) observed that the large lymphocytes in the thoracic duct lymph of rats behaved differently from the bulk of the small lymphocytes. The large cells would incorporate a pulse of H^3-thymidine, and instead of continually recirculating to various peripheral lymphoid organs, they went to the gut in a particular anatomical location; they went primarily and rapidly to the intestinal lymphoid tissues, especially the lamina propria. Many developed into immunoglobulin secreting plasma cells (Hall et al., 1972). According to the evidence obtained in many laboratories (Gowans and Knight, 1964; Griscelli et al., 1969; Hall and Smith, 1970; Guy-Grand et al., 1974; Parrott and Ferguson, 1974; McWilliams et al., 1975), it is now well known that blast cells derived from the mesenteric lymph node or thoracic duct have a predilection for the small intestine. These workers have all studied the migration pathways of the cells obtained from different lymphoid tissues by allowing these cells to incorporate a radioactive label, injecting them intravenously and observing where they went. The principal site of migration depends on the source of the cells. When they are obtained from mesenteric lymph nodes or thoracic duct lymph, they 'home' to gut-associated lymphoid tissue, including mesenteric lymph nodes, Peyer's patches and lamina propria

of the intestines. In contrast, cells from peripheral lymph nodes tend to migrate to peripheral nodes.

Twenty-four hours after adoptive transfer these cells are predominantly of the IgA isotype. To study the sources of precursors of these IgA positive cells, Guy-Grand et al. (1974), Jones and Cebra (1974), Jensenius and Williams (1974), and Williams and Gowans (1975) have shown that the percentage of cells in the gut-associated lymphoid tissue and thoracic duct lymph, which bear and contain α chains, is greater than in peripheral lymph nodes and lymph. The frequency of blast cells is highest in Peyer's patches and lowest in thoracic duct lymph. IgA is the predominant surface immunoglobulin, especially in the case of thoracic duct blasts, where it occurs on about 85% of those bearing immunoglobulin. Intracellular IgA is infrequent in Peyer's patch blasts, whereas in mesenteric lymph nodes, and even more in thoracic duct blasts, it is relatively common. All blasts which contain IgA have IgA on their surfaces, as shown by a double immunofluorescent technique. Such blasts are found in mesenteric lymph nodes and in thoracic duct lymph, but not in Peyer's patches. The observations of Guy-Grand et al. (1974) in mice make it likely that the 'homing' cells are precommitted to IgA production, and the Peyer's patch blast cells which lack intracellular IgA, unlike the mesenteric node blast cells and the thoracic duct lymph blast cells, do not 'home'. The studies of Craig and Cebra (1971, 1975) and Rudzik et al. (1975) in rabbits have shown that Peyer's patches and appendix are fertile sources of the precursors of IgA plasma cells.

It should perhaps be stressed that T as well as B blasts can 'home' to the gut-associated lymphoid tissues (Guy-Grand et al., 1974;

McWilliams et al., 1975) and that there are likely to be multiple mechanisms by which 'homing' can occur.

To summarize, evidence has been accumulating in the several laboratories noted above suggesting that lymphoblasts in the thoracic duct lymph include many cells already committed to IgA; these cells have their origins in the gut-associated lymphoid tissue, especially the Peyer's patches, and have entered the thoracic duct via the abdominal lymphatics and mesenteric lymph nodes; from the thoracic duct lymph they reach the lamina propria of the intestine via the blood stream, and soon after they reach the intestine where they mature into IgA-secreting plasma cells. A scheme for the IgA cell cycle is shown in Fig. 2.6.

2.9 Route of Administration and Antibody Species

It now seems certain that the oral route of administration of antigens gives the highest level of luminal sIgA. This was foreshadowed by the work of Crabbé et al. (1969). They used ferritin as an antigen, administered subcutaneously or intraperitoneally into germ-free adult mice. Plasma cells producing ferritin antibody were subsequently located in lymph nodes and spleen, but few were found in the gut lamina propria. The extra-intestinal plasma cells were secreting IgM after the first stimulation and IgG after multiple stimulation. The relatively few plasma cells found in the gut were producing IgA. The circulating antibodies, after repeated immunizations, were essentially of the IgG class.

Alternatively, mice immunized orally had relatively large numbers of plasma cells in the gut mucosa, less in the mesenteric lymph nodes, and a few in the lymphoid tissues. Further, whatever

their location, these cells all produced IgA and the circulating antibody was found to be predominantly IgA. Thus, this early experiment highlights the role of the gut in IgA synthesis.

Later, Pierce and Gowans (1975), using cholera toxoid as antigen in rats, found that the highest number of antitoxin-containing cells was obtained in the lamina propria following intraperitoneal priming and subsequent intraduodenal challenge.

Bloom and Rowley (1979a), using various immunization schedules with V. cholerae in mice, found that they could obtain good primary and secondary plaque forming cell (PFC) responses in the spleen following intravenous administration. However, the local response for both IgM and IgA classes of antibody was negligible. Indeed, the best local response was obtained by oral priming followed by intravenous boosting.

Obviously, there are important factors associated with the nature of the antigen which may be of crucial influence in determining the magnitude of the local response. One such factor appears to be the adherence of the antigen and its subsequent penetration into local lymphoid tissue, including the Peyer's patches of the intestines (Bloom and Rowley, 1979b). They also found that intravenous doses of live V. cholerae did not prime the gut lymphoid tissues but stimulated a strong secondary response at this site. Also of potential practical importance, V. cholerae antigens introduced systemically must cross the vascular epithelium into the lamina propria where they can provoke a secondary response; but they do not appear capable of priming the local lymphocytes and this defect must be presumed to lie in the method of presentation. The selective intestinal binding of cholera exotoxin may explain its ability to

prime the intestine after parenteral administration (Pierce and Gowans, 1975).

2.10 The Physico-chemical Characteristics of sIgA

At least two physical characteristics of the sIgA molecule seem admirably suited to its role as an antagonist of bacteria invading mucosal surfaces: (1) It is clearly relatively resistant to the proteolytic enzymes that pervade the intestinal lumen and which would destroy other immunoglobulin species (Wilson and Williams, 1969; Shuster, 1971; Horsfall et al., 1978). (2) As mentioned earlier, sIgA has a high carbohydrate content, 11-15% (Heremans, 1974; Beale and Feinstein, 1976). The location of carbohydrate in serum IgA is similar to that of IgG (Beale and Feinstein, 1976), but there are five additional carbohydrate sites in the hinge region (Feinstein et al., 1971; Beale and Feinstein, 1976). Moreover, the binding to dimeric IgA of the secretory component, which is extremely rich in carbohydrate (22.8%; Lindh, 1975), leads to superficial location of the carbohydrate moieties of the secretory component, since the binding requires protein-protein interaction with disulphide bridge formation. In consequence, the molecule is intensely hydrophilic.

2.11 The Antibacterial Activity of sIgA

sIgA antibodies bind antigens efficiently and can neutralize viruses and bacterial enterotoxins (Hanson and Brandtzaeg, 1980). They agglutinate bacteria well, possibly aided by their 4-valencies. These capacities probably explain the ability of sIgA to prevent attachment of bacteria to epithelial cells, which seems to be important for colonization by these mucosal infecting organisms

(Williams and Gibbons, 1972; Fubara and Freter, 1973; Hanson et al., 1978; Svenborg-Eden and Svennerholm, 1978).

However, whether IgA and sIgA antibodies can mediate any secondary effector functions following the union of their combining sites with antigen seems doubtful.

2.11.1 Viral neutralization

There is evidence that IgA antibodies have viral neutralizing activity and that neutralization can occur in the absence of complement fixation (Hanson and Brandtzaeg, 1980).

2.11.2 Complement fixation

Native sIgA does not activate complement via the classical or alternate pathway (Adinolfi et al., 1966; Heremans et al., 1963; Rawson and Abelson, 1964; Vaerman and Heremans, 1968), although aggregated IgA does (Hanson and Brandtzaeg, 1980; Colten and Bienenstock, 1974). However, in one series of experiments (Colton and Bienenstock, 1974), the combination of sIgA antibodies with antigens on the surface of red blood cells failed to activate the alternative pathway. sIgA antibodies have been reported to interact with complement and lysozyme together so as to bring about bacteriolysis (Adinolfi et al., 1966; Hill and Porter, 1974). Further, Steele et al. (1974) showed that IgA promoted negligible complement mediated *Vibriacidal* activity.

2.11.3 Opsonization

Opsonization is a phenomenon that can be mediated directly by the Fc portion of an antibody or indirectly via complement. In the

case of IgA antibodies, the ability to opsonize is controversial. Although parotid secretions do promote the ingestion of streptococci by leucocytes in vitro (Shklair et al., 1969), there is no good evidence that this activity is due to the bacterial coating with salivary IgA. From the study of Eddie et al. (1971), purified IgG and IgA anti S. typhimurium anti 'O' antibodies were isolated from rabbit intestinal fluid and colostrum and their specific activities measured on a molar basis. Serum and exocrine IgG were essentially identical in complement-dependent bactericidal opsonization, while exocrine IgA had little or no activity in any of these assays. Steele et al. (1974) observed a lack of opsonic activity with rabbit IgA directed against V. cholerae. Indeed, studies in the mouse suggest specific receptors on macrophages for the cytophilic attachment of IgG, IgG_2, IgG_3 and IgM, but not for IgA. These data would be consistent with the lack of opsonization of IgA antibody (Unanue et al., 1971). Another possible biological function of the sIgA system may be in limiting absorption and/or inactivating antigen which are inhaled or ingested. It has been demonstrated that deliberate immunization of the intestinal tract can prevent the absorption of foreign proteins and that antigen-antibody complexes are not absorbed (Walker et al., 1972; André et al., 1974).

It has also been demonstrated that sIgA antibodies can prevent bacteria from adhering to epithelial cells (Williams and Gibbons, 1972). Magnusson et al. (1979) have shown that binding of sIgA to bacteria surfaces has hydrophilic (see 2.10) and anti-adhesive effect which may serve to exclude antigen from mucosal surfaces.

That T lymphocytes have been demonstrated to bear Fc receptors for IgA (Strober et al., 1978; Lum et al., 1979) would appear to be

a significant development. Similarly, the existence of IgA receptors on neutrophils is undoubtedly significant in the function of this cell type (Van Epps and Williams, 1976; Van Epps et al., 1978).

Although the role for sIgA antibodies in resistance to viral infections seems certain, its role in protecting against bacterial infection is far less certain. It seems unlikely that the classical defense mechanisms associated with IgG and IgM antibodies are manifested by IgA or sIgA. Since IgA does not fix complement, as discussed above, and is also intensely hydrophilic, it can neither promote the serum bactericidal reaction nor phagocytosis.

However, bacteria coated with sIgA may become sufficiently hydrophilic to militate against their adherence to mucosal cells and this, per se, presumably prevents colonization of the mucosal surface, leading to some degree of protection of the host, although ultimate protection is presumably manifested by cellular mechanisms (Marneerushapisal and Rowley, 1981).

Although the role of IgA antibodies in the protection of the host is in some doubt, the work reported in this thesis has aimed at looking for a correlation between the IgA levels obtained in both serum and intestinal secretions of mice, following oral priming with various Salmonella strains and the level of protection afforded to a subsequent oral challenge with a smooth strain of S. typhimurium, the causative agent of mouse typhoid.

If such correlation can be shown to exist, the determination of serum IgA levels may prove both a useful and rapid method for estimating human resistance to various enteric infections.

CHAPTER 3

The Enzyme-Linked Immunoabsorbent Assay ("ELISA")

3.1 Introduction

One reason for the exquisite sensitivity of assays which employ enzyme, radio-isotope, fluorescent or chemiluminescent markers is that they each measure the primary interaction between antigen and antibody and are therefore quite independent of secondary reactions such as agglutination or complement fixation.

In this chapter we will elaborate on the enzyme-linked immunoabsorbent assay ("ELISA"). This system employs enzyme markers conjugated either to antigens or antibodies. "ELISA" techniques require antigens or antibodies to be linked to an insoluble carrier surface and these, in turn, are then interacted with the appropriate antibodies or antigens in the test system. The insoluble antigen-antibody complexes are then detected and estimated by means of an enzyme-labelled antigen or antibody. Quantitation is in terms of the extinction value of some coloured degradation product of a suitable enzyme substrate.

Although enzyme/antibody conjugates have been used histochemically to detect antigens in tissue sections (Avrameas, 1969), the "ELISA" was developed independently by Engvall and Perlmann (1971) and by Van Weemen and Schuurs (1971). "ELISA" techniques are now widely used as an alternative or substitute for other immunologically based assay as:

(1) The "ELISA" is simple and easy to perform.

(2) It can be adapted to simple field screening procedures.

(3) Many suitable enzymes are available and most are relatively

inexpensive.

(4) The assays can be performed with very small amounts of material in microtitration plastic plates manually or in automated systems.

(5) The enzyme conjugates have a long shelf-life, often retaining their activity for years.

(6) Health hazards during labelling and performing the test are negligible.

(7) The assay is extremely sensitive and its class specificity and applicability is similar to that of the radio-immunoassay ("RIA").

(8) It overcomes the difficulties of measuring antibodies associated with gastric juice (Horsfall and Rowley, 1979), as interfering juice components are washed away and its high sensitivity compensates for the unavoidable dilution when collecting juice samples.

Thus, "ELISA" becomes the method of choice for many assays carried out in laboratories with either sophisticated or limited facilities.

Because of their close relationship, the advantages and disadvantages of "ELISA", "RIA" and "IF" (immunofluorescence test) are compared in Table 3.1.

3.2 Methodology

The "ELISA" depends upon two important factors. Firstly, that the antigen or antibody can be linked or adsorbed to the surface of a solid carrier and retain activity; secondly, that an enzyme marker can be coupled to an antibody or antigen with retention of both

Table 3.1

Summary of advantages and disadvantages of ELISA and other indirect antiglobulin tests (RIA and IF).

Reproduced from World Health Organization (1976).[b]

Criterion	ELISA	RIA	IF
Sensitivity	High	High	Lower
Specificity	Depends on Ag preparation	Depends on Ag preparation	High
Reproducibility	Acceptable	Acceptable	Acceptable
Reading	Objective	Objective	Subjective
Antigen preparation	Can be complicated	Can be complicated	Easy
Feasibility of performance under field conditions	Easy	Not easy	Intermediate
Relative cost per test	Low	High	High
Shelf-life of reagents [a]	Long	Short	Long
Health hazards for laboratory personnel [a]	None or minor	Present	None or minor

a Should be regarded as critical factors.

b Bull. Wld Hlth Org., 54, 129.

immunological and enzyme activity. In the next section, therefore, the solid phase support and the choice of enzyme will be discussed.

3.2.1 Solid phase support

The solid phase can be beads, tubes, or plates. Leininger et al. (1961) found that most proteins could adsorb to a plastic polymer surface. Polystyrene forms a suitable surface; indeed, this was first used by Catt and Tregear (1967) as the solid phase support "coated tube" in a radio-immunoassay of human placental lactogen and human growth hormone. Engvall and Perlmann (1971, 1972) also used such "coated tubes" in their studies, as did many other workers.

Polystyrene, in the form of microplates, was first used in the "ELISA" by Voller et al. (1974). They are suitable for use on a large scale because they are cheap, simple to use and small quantities of reagents can be employed. There are many other polymers that have been used as well as polystyrene, such as polyvinyl (Herrmann and Collins, 1976; Zollinger et al., 1976; Frankel and Gerhard, 1979), polyallomer (a co-polymer of ethylene and propylene), cellulose nitrate (Herrmann and Collins, 1976), nylon (Lehtonen and Viljanen, 1980), and polypropylene (Catt and Tregear, 1967).

Herrmann and Collins (1976) stated that polystyrene, polyvinyl and polyallomer were able to adsorb protein (IgG) to approximately the same degree (about 79% to 97% adsorbed protein of 1 µg/ml protein after 18 hours incubation). However, cellulose nitrate adsorbed IgG poorly.

Moreover, glycoprotein, such as carcino-embyronic antigen (Hammarström et al., 1975), bacterial lipopolysaccharide (Carlsson et al., 1972, 1975, 1976), V. cholera enterotoxin (Holmgren and

Table 3.2

The binding of chicken anti-bovine serum albumin to cuvette surfaces.

Support	Protein concn in coupling solution (µg/ml)	Protein surface concn [a]	
		Mean (ng/cm²)	S.D [b]
Polystyrene [c]	100	66	15
	100	150	20
Nylon	10	150	11
	100	210	5.2

a Five parallel determinations.

b Standard deviation.

c Two batches of cuvettes.

Svennerholm, 1973), polysaccharides (Branefors-Helander and Dahlberg, 1975) and nucleic acids (Pesce et al., 1974) all bind to the plastic surfaces. Thus, virtually all varieties of antigens can bind to the plastic surfaces, as do immunoglobulins which bind strongly (Engvall and Perlmann, 1971).

Antigens and antibodies are adsorbed to plastic polymer surfaces by weak, mainly physical forces (Van Oss and Singer, 1966). At present, it is not known what part of these molecules is preferentially bound to the surfaces but the binding is almost certainly due to hydrophobic interactions. The extent of such binding will clearly depend on the orientation of the molecules in the solution.

Consequently, the resultant surface coating will depend upon the size of the tubes, the volume, concentration, and the pH of the coating solution, and the time and temperature of coating (Engvall et al., 1971; Engvall and Perlmann, 1972; Carlsson et al., 1972; Herrmann and Collins, 1976).

Another factor that should be borne in mind is that coating of the solid surface seems to depend on the quality and the type of plastic surfaces. Even in the same plastic product different batches may give different binding of antigens or antibodies.

Although it would appear from the work of Herrmann and Collins (1976) that polystyrene, polyvinyl and polyallomer gave approximately the same degree of adsorption of various proteins, the work of Lehtonen and Viljanen (1980) shows that considerable variation in adsorption can occur depending upon the support used. Further, they showed that different batches of polystyrene gave different surface binding concentrations for chicken anti-bovine serum albumin, as shown in Table 3.2.

Although the enzyme immunoassay systems are facilitated by the fact that plastic plates normally provide an adequate support surface, little is known about the forces involved in this passive binding and consequently no perfect system has, as yet, been produced. Thus, desorption or "leakage" from the surface may occur (Engvall et al., 1971). This will obviously lower the precision and affect the sensitivity of the assays to some extent. Such criticism, of course, applies to any solid-phase immunoassay system.

3.2.2 Choice of enzymes and conjugation

The sensitivity of the enzyme immunoassay in general depends on the preparation of enzyme-antigen or enzyme-antibody conjugates. As mentioned in section 3.2.1, the enzymes used in these immunoassays must, when attached to antibody or antigen, form a complex which retains both enzymic and immunological activity. In addition, the enzyme should be stable and be commercially available in a purified form, have a high turnover number, and the hydrolysed substrate product should have a high molar extinction coefficient that can be measured by a simple spectrophotometric technique.

The most commonly used enzymes are alkaline phosphatase (from calf intestine) and horseradish peroxidase (Engvall, 1977). The determination using alkaline phosphatase is a very simple and sensitive assay; its substrate is stable and is hydrolysed to give a stable coloured product, which can be measured by spectrophotometric or visual reading. The peroxidase system is cheaper; however, it is a multistep redox reaction and the substrate (H_2O_2) is relatively unstable. Several other enzymes have been used, such as glucose oxidase (Masseyeff et al., 1973; Maiolini and Masseyeff, 1975),

β-D-galactosidase (Kato et al., 1975; Lauer and Erlanger, 1974), lactate dehydrogenase (Casu and Avrameas, 1969) and lactoperoxidase (Pesce et al., 1976).

Any of these enzymes can be coupled to antigens or antibodies by a variety of cross-linking agents. The cross-linking agent reacts via its active groups with the functional groups present in the enzymes and immunoglobulins or protein antigens, so such cross-linking must occur through a bifunctional reagent; glutaraldehyde is the most commonly employed coupling agent. Some advantages are that it reacts rapidly and irreversibly in aqueous solution and that it is stable over a wide range of pH conditions.

Avrameas (1969) has coupled enzymes to antibodies and to antigens with glutaraldehyde for the sensitive detection of either antigens or antibodies in cell preparations. The amplifying mechanism for detection occurs via the enzymic activity. The conjugates were found to retain more of their enzymatic and immunological activities when cross-linked with glutaraldehyde than with other bifunctional reagents. Glutaraldehyde coupling can be performed as a single-step reaction where antigens or antibodies are mixed with enzyme and glutaraldehyde to be reacted at the same time (Avrameas and Ternynek, 1971). However, a two-step reaction in which the enzyme or antigens or antibodies are reacted with excess glutaraldehyde followed by reaction with the other component after the removal of excess glutaraldehyde (Avrameas, 1969; Avrameas et al., 1978; Avrameas and Ternynck, 1971) is a clear advantage if one component of the conjugate system is relatively sensitive to gluraraldehyde.

In the case of enzyme-antibody conjugates, little or no free antibody was found to be present after glutaraldehyde coupling

(Avrameas, 1969; Boorsma and Streefkerk, 1976; Pesce et al., 1976). The enzymic activity after coupling was about 60-70% of the initial activity (Boorsma and Streefkerk, 1976). However, Engvall's group have shown that alkaline phosphatase conjugates prepared with the single-step glutaraldehyde procedure were quite adequate. This method for the preparation of conjugates was used exclusively in the work reported in this thesis and was found to be most satisfactory.

CHAPTER 4

Materials and Methods

4.1 Bacterial Strains

4.1.1 Description of strains

These are summarized in Table 4.1.

4.1.2 Strain maintenance

All these strains were from the stocks of this department and were stored as lyophylized cultures at 4^{o}.

The two hybrid strains, F885 and F1142, were originally received from Dr G. Schmidt of the Max Planck Institute, Freiburg, Federal Republic of Germany.

For routine use, all lyophylized cultures were grown on nutrient agar plates, and a single colony was transferred and kept in semi-solid agar stock bottles for 2-3 weeks at 4^{o}.

4.1.3 Strain propagation

Cultures were transferred from semi-solid agar stock bottles to nutrient broth at 37^{o} for 18 hours. These cultures were diluted 1:10 with fresh nutrient broth media and incubated as a shaking culture for 3 hours. The ultimate number of bacteria in such cultures was about $2x10^{9}$/ml.

In some cases where such concentrations were adequate, bacterial cultures were used without further processing or suitably diluted. Where higher concentrations were required, the cultures were sedimented by centrifugation and the bacterial pellets were resuspended to the required concentration.

Table 4.1

Bacterial strains. [g]

Strains	Serotype [f]	
	O-antigen	H-antigen(s)
S. typhimurium F885 [a]	8	i:1,2
E. coli F1142 [b]	4,5,12	NM [e]
S. bonariensis	6,8	i:e,n,x
S. salford	16	l,v:e,n,x
S. stanley	1,4,5,12	d:1,2
S. strasbourg	9,46	d:1,7
S. kirkee	17	b:1,2
S. enteritidis 11RX [c]	1,9,12	g,m
S. budapest	1,4,12	g,t
S. typhimurium M206 [d]	1,4,5,12	i:1,2
S. fridenau	13,22	d:1,6
S. chester	4,5,12	e,h:e,n,x
S. derby	1,4,5,12	f,g
S. humber	53	z4,z24
S. typhimurium C5 [d]	1,4,5,12	i:1,2

a Hybrid strains: SF1591 x E. coli Hfr459. Specific name is arbitrary and does not necessarily reflect serotype.

b Hybrid strains: S. typhimurium Hfr K9 x E. coli F492 his (Schmidt, 1972).

c Ushiba et al. (1959).

d Rowley and Whitby (1959).

e NM = non-motile.

f These serotypes were kindly rechecked by the Institute of Medical and Veterinary Science, Adelaide.

g Kauffman (1966).

4.1.4 Media

Nutrient broth, 16 grams per litre of Difco nutrient broth (Detroit, Michigan) with the addition of 0.5% NaCl.

Semi-solid agar, 0.7% of nutrient agar (Difco, Detroit, Michigan) in nutrient broth.

Nutrient agar plate (Difco, Detroit, Michigan).

4.2 Mice

Specific-pathogen-free LAC strain mice (Medical Research Council Laboratory Animals Centre, Carshalton, Surrey, England) bred as a closed colony were used in this study.

Mice were used when 6-8 weeks of age.

4.3 LD_{50} Determination

The lethal dose of organisms is expressed as an LD_{50} value which is calculated using the formulae described by Reed and Muench (1938). Doses are usually graded by 1 $\log_{10}$ and each dose level is given orally to 5-10 mice. The LD_{50} of S. typhimurium C5 when given orally is 6.8×10^4 organisms.

4.4 Immunization

4.4.1 Oral immunization and oral infection

Following feeding with 0.2 ml of a 50% sodium bicarbonate in saline, mice were orally immunized with the required dose of organisms, 0.2 ml of suspension. At this strength of sodium bicarbonate, the stomach acid was neutralized (Horsfall, 1977). The oral dose was administered to the mice by means of a syringe and blunt-tipped hypodermic needle (19 g).

4.5 Collection and Storage of Serum

Blood was obtained from the retro-orbital plexus of mice. The blood was allowed to clot at room temperature for 1-2 hours and the sera were separated after 15 minutes at 37°. The sera were dispensed in small aliquots and then stored at -20° without adding any preservative.

4.6 Collection and Storage of Intestinal Fluid Washings

Mice were killed by cervical dislocation and the intestines, from pylorus to the ileo-caecal junction, were immediately removed and their exterior surface rinsed thoroughly in two ice-cold saline baths. The intestines were flushed with 1 ml of ice-cold saline from the distal end. The saline was introduced by means of a syringe and blunt-end hypodermic needle (19 g) and its passage facilitated by gentle massage with the fingers. The washings from a group of immunized mice (20-25 mice) and non-immunized mice were collected in the 50 ml plastic centrifuge tubes on ice, and then homogenized (Ultraturrax, Jake and Kunkel KG, West Germany) and clarified by centrifugation (Sorval, 12,000 x g, 15 minutes, 4°). The supernatant was then dispensed into 2 ml aliquots and stored at -20°. No tryptic inhibitor was used.

4.7 Recovery and Enumeration of Bacteria from the Peyer's Patches

At specified times, groups containing 5-6 mice were killed by cervical dislocation. The small intestines were removed, separated and freed from the surrounding mesentery. Ten ml of saline was passed through each intestine to get rid of the intestinal contents and then rinsed with saline. The Peyer's patches from each intestine

were then excised and placed in the 2 ml ice-cold saline. The Peyer's patches from each intestine were homogenized with the Ultra-turrax homogenizer (Janke and Kunkel, West Germany). Each sample of homogenized patches was plated out on nutrient agar (Difco) and incubated for 18 hours at 37°. The recovered colonies were counted, and the number of organisms recovered from the homogenized patches from each mouse was recorded and the group average obtained.

4.8 Protein Estimation

4.8.1 Extinction coefficients

Protein estimations of mouse immunoglobulin fractions were made using a Hitachi double-beam spectrophotometer. The extinction coefficients used were those described for rabbit immunoglobulins by Eddie et al. (1971), viz., $E^{1\%}_{280\ nm}$ of 13.6 for IgG, 13.5 for IgA and 12.8 for IgM.

4.8.2 Quantitative Folin assay

For complex mixtures of proteins such as biological fluids and antigenic extracts, the sensitive Lowry modification of the Folin-Ciocalteu reaction was used (Lowry et al., 1951). This was performed as outlined in Williams and Chase (1968). Standard dilutions of bovine serum albumin were routinely included.

4.9 Determination of Protein Purity

4.9.1 Double radial immunodiffusion (Ouchterlony)

The method used in this thesis was based on that of Ouchterlony and Nilsson (1973).

4.9.2 Radio-immunoprecipitation (RIP)

In addition, to provide a quantitative measure of anti-isotype activity, we used precipitation by I^{125} labelled antigen as this is far more sensitive than the immunodiffusion method. The method used in this study was adopted from Leonore A. Herzenberg and Herzenberg (1973).

Briefly, 50 µl of labelled IgG1, IgG2a, IgG2b, IgM or IgA in 3% BSA-tris (i.e., 0.05 M tris buffer, final pH 7.6, containing 3% bovine serum albumin [BSA]), diluted to contain approximately $2x10^5$ cpm/50 µl, was placed in a 6x50 mm culture tube. To this was added, with rapid mixing, 50 µl of the rabbit anti-mouse Fab antiserum or the goat anti-mouse α-chain antiserum serially diluted in S-dil (i.e., 9 parts of 3% BSA plus 1 part normal rabbit serum). Before incubation, 50 µl of S-dil was added to every tube, including the control (50 µl of BSA-dil, 50 µl of S-dil and 50 µl of labelled Ig(s)). The tubes were incubated at 37° for 3 hours and followed by 4° overnight, then centrifuged in the cold at 10,000 g for 10 minutes; 110 µl of the supernatant was carefully removed and placed in a disposable counting tube. Samples were counted in a gamma counter.

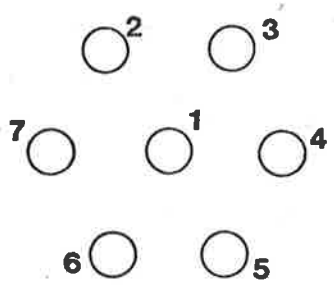
4.10 Preparation and Purification of Antisera for Enzyme Conjugates

4.10.1 Rabbit anti-mouse Fab antiserum

This antiserum was kindly donated by Dr P. L. Ey of this department. It was prepared by immunizing rabbits with mouse $(Fab')_2$ fragments of whole mouse IgG sub-classes. The resulting hyperimmune serum was purified by passing through a $(Fab')_2$ sepharose 4B affinity column. Fig. 4.1 shows an Ouchterlony double immuno-diffusion gel of the rabbit anti-mouse Fab antiserum against mouse

Figure 4.1

Immunodiffusion reaction (Ouchterlony) of the rabbit anti-mouse Fab antiserum and mouse immunoglobulin classes.



1 = Rabbit anti-mouse Fab antiserum.

2 = IgG1.

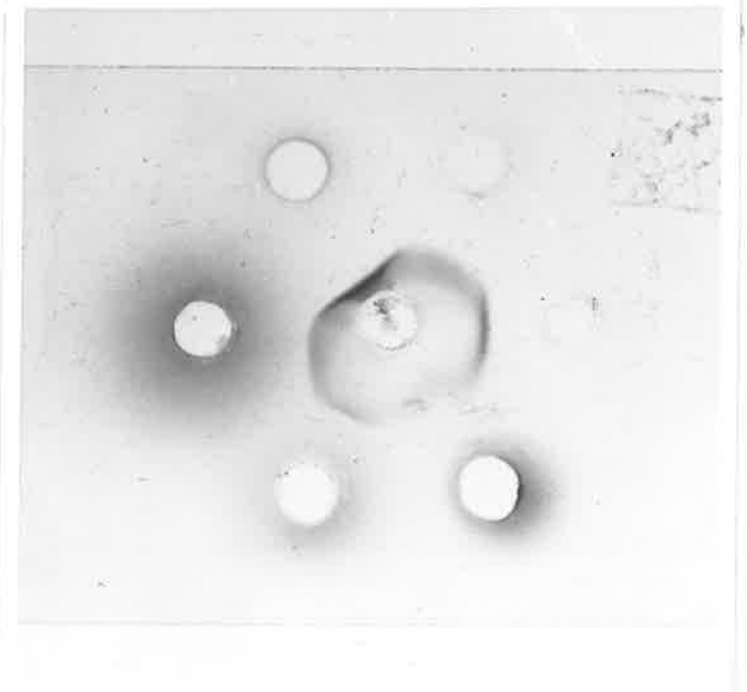
3 = IgG2a.

4 = IgG2b.

5 = IgA.

6 = IgM.

7 = Normal mouse serum.



immunoglobulin sub-classes. Further, the mouse anti-allotype antibody of the rabbit anti-mouse Fab was determined by radio-immunoprecipitation (RIP) using I^{125}-IgG1, I^{125}-IgG2a, I^{125}-IgG2b, I^{125}-IgM, and I^{125}-IgA from the mouse. This rabbit anti-mouse Fab antiserum showed degrees of precipitation with all classes of mouse immunoglobulins.

4.10.2 Goat anti-mouse α-chain antiserum

This was obtained by further purification of a goat anti-mouse sIgA prepared originally by Dr D. J. Horsfall (1977) and kindly donated for this work.

This antiserum was passed down the sepharose 4B columns, to which were coupled mouse immunoglobulin sub-classes, IgG1, IgG2a, IgG2b and IgM, respectively. These were originally prepared by Dr P. L. Ey.

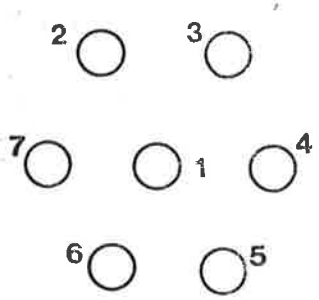
The purified goat anti-mouse α-chain was obtained at a concentration of 1.3 mg/ml. Examination of this preparation by Ouchterlony showed only a single precipitation band directed against mouse IgA (Fig. 4.2). Further RIP technique using I^{125}-labelled mouse IgG1, IgG2a, IgG2b, IgM and IgA with this antiserum showed degrees of precipitation only with I^{125}-IgA.

4.11 Method of Coupling Antibodies to Enzyme

The technique described by Engvall and Perlmann (1972) was employed with some modification of the method of Avrameas (1969). The enzyme used was alkaline phosphatase Sigma Type VII-S. Alkaline phosphatase containing 1.5 mg protein was added to purified goat anti-mouse α-chain containing 0.5 mg protein and mixed well. The volume of the mixture was about 2.5 ml. Then the protein mixture was subjected to dialysis against 2 L of PBS buffer containing

Figure 4.2

Immunodiffusion reaction (Ouchterlony) of the goat anti-mouse α-chain antiserum and mouse immunoglobulin classes.



1 = Goat anti-mouse α-chain antiserum.

2 = IgG1.

3 = IgG2a.

4 = IgG2b.

5 = IgA.

6 = IgM.

7 = Normal mouse serum.

Mg^{++} and Zn^{++} (PBS-Mg^{++}-Zn^{++}) overnight in the cold, with two changes of buffer. After dialysis the dialysing bag was rinsed with 1 ml saline. Five per cent. glutaraldehyde freshly prepared in phosphate buffer pH 7.4 was added to the dialysed protein mixture solution while it was stirred to give 0.2% final concentration of glutaraldehyde. The coupling reaction was allowed to proceed for 2 hours at room temperature. After coupling 0.1 ml of 1 M lysine pH 7.0 was added to and it was dialysed again against 2 L of PBS-Mg^{++}-Zn^{++} buffer overnight in the cold, with two changes of buffer. It was centrifuged at 20,000 rpm for 30 minutes in an L-8 Beckman using a 60 Ti rotor. The conjugate in the supernatant was separated and 0.1 volume of 20 mg/ml BSA was added. After dilution to 10 ml with enzyme diluent, 10 ml of glycerol was added. The conjugate preparation was then dispensed into 1 ml aliquots and stored at 4^{o}. An analogous procedure was used to coupling alkaline phosphatase to rabbit anti-mouse Fab antiserum.

4.12 Determination of the Conjugates Activities

After coupling the conjugates were tested for their activities by using the direct ELISA method. It was done as follows:

(1) Mouse immunoglobulin (mouse IgG1 for the rabbit anti-mouse conjugate, mouse IgA for the goat anti-mouse α-chain-alkaline phosphatase conjugate) was used to coat the polystyrene microtitre plates (5 µg/ml, 200 µl). The plates were incubated for 3 hours at 37^{o}, then washed 3 times with PBS-Tw.

(2) The serial dilutions of the conjugates diluted in enzyme-diluent (200 µl) were added to the wells (each determination was in duplicate). The plates were incubated at 25^{o} for 18 hours. The plates were washed before adding the enzyme substrate.

Fig. 4.3 shows the activities of the rabbit anti-mouse Fab-alkaline phosphatase conjugate and the goat anti-mouse α-chain-alkaline phosphatase conjugate.

4.13 Preparations of Antigens

4.13.1 Lipopolysaccharides (LPS)

The lipopolysaccharides of the Salmonella strains used in this thesis were extracted by the phenol-water method originally described by Westphal et al. (1952).

After the extraction the lipopolysaccharide containing some protein was dissolved in distilled water containing magnesium ions and then treated with RNAase and DNAase. The cleaned lipopolysaccharide was then washed and pelleted by ultra-centrifugation. The pellet was resuspended in distilled water and preserved with 0.02% sodium azide and stored at 4^{o}.

Protein contamination was determined by Folin-Ciocalteu assay. All prepared lipopolysaccharides contained less than 0.5% protein.

4.13.2 Outer membrane protein

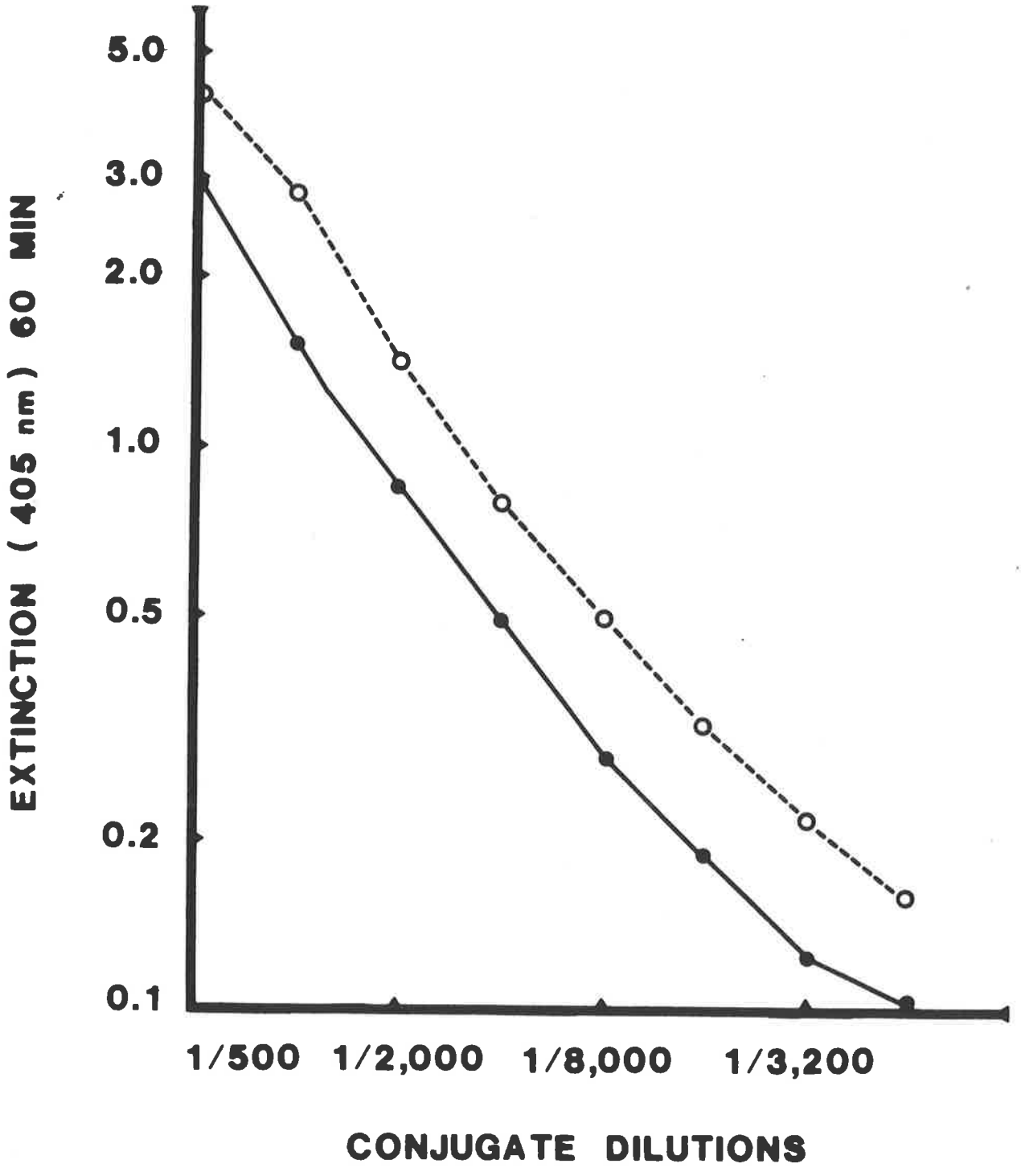
The outer membrane protein of S. typhimurium C5 was prepared following the method of Schnaitman (1974). Briefly, the bacterial cells were broken and the envelope fraction obtained, after treatment with RNAase, DNAase and lysozyme, by centrifugation at 78,000xg for 60 minutes. The envelope fraction was then extracted twice with 2% Triton X-100 in 10 mM HEPES buffer at pH 7.4. The pellet obtained by centrifugation at 78,000xg for 60 minutes was further treated with lysozyme, and after further centrifugation the pellet was extracted twice with 2% Triton X-100 in HEPES buffer (pH 7.4) containing 5 mM

Figure 4.3

The activities of conjugates.

The rabbit anti-mouse Fab-alkaline phosphatase conjugate (●—●) was tested against mouse IgG1 (MOPC-21) coated plates, and the goat anti-mouse α-chain-alkaline phosphatase conjugate (○···○) was tested against mouse IgA (S107) coated plates.

FIGURE 4.3


EXTINCTION (405 nm) 60 MIN
5.0
3.0
2.0
1.0
0.5
0.2
0.1
1/500
1/2,000
1/8,000
1/3,200
CONJUGATE DILUTIONS

EDTA. The supernatants from these extractions were pooled, mixed with twice their volume of ice-cold ethanol and allowed to stand overnight in the cold. The precipitated outer-membrane protein was obtained by centrifugation at 20,000xg.

4.14 Coupling of LPS to Methylated Bovine Serum Albumin

The method of Vos et al. (1979) was used. Methylated bovine serum albumin (Sigma) 0.05 ml, at a concentration of 10 mg/ml in distilled water, was added to 0.5 mg LPS contained in 1 ml of sodium carbonate buffer (0.1 M, pH 9.6) with continual stirring and the mixture was left to stand at 25° for 20 minutes.

To coat the polystyrene plates, 200 µl of methylated BSA-LPS complex, at 5 µg/ml in sodium carbonate buffer, was added to each well. The plate was then incubated at 37° for 3 hours in a humid chamber and finally left at 4° overnight before use.

4.15 Radio-iodination

The chloramine-T method used in this study was modified from that of Greenwood, Hunter and Glover (1963). A 0.5-1.0 mCi (5-10 µl) aliquot of Na ^{125}I solution was dispensed into a polystyrene tube and 10 µl of 0.25 M phosphate buffer, pH 7.5, was added to buffer the iodide solution. The following were added in rapid succession while continuously agitating the reaction mixture with a small glass-covered magnetic stirrer: (a) 10 µl (2-5 µg) of protein solution, (b) 10 µl (50 µg) of chloramine-T solution, (c) 100 µl (120 µg) of sodium metabisulphite solution. The volume of the solution was made up to 1.0 ml with KI solution. The free iodide was separated by chromatography on sephadex G-25.

4.16 Solid-phase Radio-immunoassay

This procedure was modified from the method described by Horsfall and Rowley (1979). The Salmonella LPS complexed with methylated-BSA was used as an antigen for coating the polystyrene plate. After washing the plate with PBS-Tw, 200 µl of serial two-fold dilutions of mouse anti-Salmonella serum was added to the sensitized plate. After 6 hours incubation at 25° and washing, the I^{125} -anti-mouse immunoglobulin (5000 cpm) was added. After washing with PBS-Tw the radioactivity was measured by a Packard Auto-gamma counter. The end-point titre was the reciprocal of the highest Ab dilution which bound more than 500 cpm (10% binding limit).

4.17 Microtitre Plates

The polystyrene microtitre plates "Cook Microtitre System" M24A (Sterilin Product, U.K.) were used as the solid phase support in the ELISA.

4.18 Enzyme-linked Immunosorbent Assay (ELISA)

4.18.1 Elisa solutions

1 Coating buffer: 0.1 M sodium carbonate buffer pH 9.6 and 0.02% sodium azide.

2 Washing buffer (PBS-Tw): PBS containing 0.05% Tween 20 and 0.02% sodium azide.

3 Sample diluting buffer (PBS-BSA-Tw): PBS containing 2% BSA, 0.05% Tween 20 and 0.02% sodium azide.

4 Enzyme diluent (for diluting the conjugates):

8 gm NaCl in 800 ml water.

50 ml 0.25 M triethanolamine, pH 7.6*.

10 ml 100xMg/Zn**.

10 ml 20 mg/ml BSA.

10 ml 10% sodium azide.

Distilled water to 1000 ml.

* 37.5 g triethanolamine neutralized with 2.5 M HCl to pH 7.6.

** 100 mM $MgCl_2$/0.25 mM $ZnCl_2$.

5 Substrate diluent: 10% diethanolamine buffer containing 1 mM $MgCl_2$.

4.18.2 ELISA methods (Voller et al., 1976)

The "Indirect ELISA" method was mainly used in this thesis for detecting antibodies in serum and intestinal fluid washings. The steps of the assay are described in detail below.

(1) Round bottomed polystyrene microtitre plates were coated with a Salmonella LPS complex with methylated BSA (200 µl of 5 µg/ml) and incubated for 3 hours at 37°, followed by 18 hours at 4°. The plates were then washed 3 times with PBS-Tw.

(2) Serum or intestinal fluid samples were serially diluted, 2-fold, in PBS-BSA-Tw, and 200 µl were added to the corresponding test sample wells and to their corresponding control wells. The plates were incubated at 25° for 6 hours, and after the incubation the plates were washed 3 times with PBS-Tw.

(3) The enzyme-anti-immunoglobulin conjugates were added (200 µl of a given working dilution) to the wells and incubated for 18 hours at 25°. In this study the rabbit anti-mouse Fab-alkaline phosphatase conjugate 1:500 was used in order to assess total antibodies in serum samples, and the goat anti-mouse α-chain-alkaline phosphatase conjugate 1:1000 was used in order to assess IgA antibodies

in serum samples and intestinal fluid washing samples. The conjugates were added only to the test sample wells and not to the sample controls.

(4) After overnight incubation the plates were washed 3 times, and then the enzyme activity was measured by adding p-nitrophenyl phosphate (200 µl of 1 mg/ml) to every well. All were then incubated at 37° for 180 minutes prior to spectrophotometric analysis at 405 nm (Titertek Multiskan® MC).

(5) Substrate and conjugate controls were carried out in addition to sample controls.

Sample control = LPS coated plate + test sample dilutions + substrate.

Conjugate control = LPS coated plate + conjugate + substrate.

Substrate control = LPS coated plate + substrate (= blank).

These controls were carried out under the same conditions as the samples.

Sandwich Assay

This technique was used for titrating the standard immunoglobulins for the standard curves in this study. The details of the technique was described in Chapter 5. It is mentioned briefly as follows.

(1) The microtitre plates were coated with pure antibody (200 µl, 5 µg/ml) and incubated at 37° for 3 hours. The plates were washed 3 times with PBS-Tw.

(2) The samples containing antigen specific for coated antibody were serially diluted out in 2-fold dilution with PBS-BSA-Tw, and 200 µl of these dilutions were added to their corresponding wells. The

plates were incubated at 25° for 6 hours. After incubation the plates were again washed 3 times with PBS-Tw (it was carried out in duplicate).

(3) The enzyme-antibody (specific for test antigen) conjugate was added to the wells and the plates were incubated for 18 hours at 25°. The plates were washed before adding the enzyme substrate.

CHAPTER 5

The Indirect ELISA for Detecting Mouse Anti-Salmonella Antibody in the Intestinal Fluid and Serum

5.1 Introduction

Serological methods are playing an increasingly important role in the diagnosis of infectious diseases, in studies of the epidemiology of diseases and in preventive medicine. The diagnosis of infectious diseases relies on the identification of the infectious agents. However, the infecting organisms are often difficult to characterize directly and so an increase in the antibody titre of various animal fluids, if unequivocally measured, is a most useful diagnostic of a particular infection.

Further, when the infecting bacterium has disappeared from the blood, stools or urine, the determination of antibody level against a particular microorganism provides a most useful diagnostic tool. A great variety of methods have been used for the immunological diagnosis of infectious disease. These rely on either antigen or antibody determination, as in, for example, simple tube agglutination, passive haemagglutination, gel precipitation, complement fixation and neutralization. Such techniques depend upon the secondary manifestation of antigen-antibody reactions, such as precipitation, etc., and, in consequence, relatively large amounts of antigen or antibody are usually required by such tests.

Further, such techniques may utilize the characteristics of only certain classes of immunoglobulins. For example, IgA does not activate complement and sub-classes of IgG also vary in this respect. Consequently, a sensitive technique which directly measures the

degree of antigen-antibody interaction, irrespective of immunoglobulin class, has distinct advantages.

As previously discussed in Chapter 3, the ELISA appears to meet these requirements as it measures the primary antigen-antibody interaction with high specificity and sensitivity. This objective assay is certainly preferable to the subjective immunofluorescent method and is obviously less complicated, less hazardous and cheaper than radioimmunoassay.

Historically, Nakane and Pierce (1967) demonstrated the feasibility of an indirect immuno-enzyme assay (analagous to the indirect immunofluorescent assay), and subsequently Engvall and his colleagues (1971, 1972) developed the enzyme-immunoassays by linking soluble antigens or antibodies to an insoluble solid phase so that the reactivity of the immunological component was retained. Such was the basis for the development of techniques known as ELISA (Enzyme-Linked Immunoabsorbent Assay).

The simple performance and easy determination of the ELISA permit its use in the absence of sophisticated apparatus and demanding conditions. Indeed, it can be readily adapted for use in the field or at the patient's bedside.

5.2 Principle of the Indirect Method

The indirect ELISA is widely used to detect antibody in biological fluids such as serum, cerebrospinal fluid, synovial joint fluid and intestinal fluid. The antibodies can be qualitated or quantitated as follows. The homologous antigen (e.g., bacteria or bacterial wall components) is coupled to a solid phase support and the biological fluid thought to contain antibody is incubated with

this system. Excess fluid containing the unreacted components are washed away prior to the addition of an enzyme-labelled anti-immunoglobulin conjugate. The amount of such conjugate which subsequently attaches to the antibody bound to the carrier surface can then be measured by the level of degradation of added enzyme substrate.

Thus, the total amount of all classes of specific antibody in biological fluids can be determined using an enzyme-labelled anti-Fab immunoglobulin and class-specific antibodies may be detected using enzyme-labelled antisera to the heavy chains of IgG, IgM or IgA.

5.3 A Summary of the Steps of Quantitation of Antibodies

1. COATING OF CARRIER SURFACE WITH ANTIGEN : WASH

2. INCUBATE COATED CARRIER SURFACE WITH ANTISERUM : WASH

3. INCUBATE WITH ANTIIMMUNOGLOBULIN ENZYME CONJUGATE : WASH

4. ESTIMATE BOUND ENZYME CONJUGATE BY ADDING SUBSTRATE

5.4 Investigation of Practical Aspects: Experiments and Results

5.4.1 Carrier surfaces

A great variety of macromolecules can be attached to the solid plastic surfaces of tubes, beads or microtitre plates. In the work reported here all assays have been carried out using microtitre plates, as defined in Chapter 3.

5.4.2 Antigens used for coating plates

The ELISA was first used for the quantitation of antibodies against Salmonella O antigens by Carlsson et al. (1972). Infections caused by gram-negative bacteria give rise to antibodies directed mainly against outer membrane structures such as O and K antigens. In Salmonella, the O antigenic specificity is found in the polysaccharide moiety of the lipopolysaccharide (LPS). Consequently, LPS and outer membrane proteins were used as antigens for coating the plates. These were obtained and purified, as discussed in Chapter 4, from 14 strains of Salmonella.

5.4.3 (i) Coupling of LPS to the carrier surfaces

The concentration of LPS, the pH and temperature, and the time allowed for coupling are all important factors (Carlsson and Lindberg, 1978). The nature and uniformity of the plastic carriers is also of vital importance.

In general, LPS has been found to couple easily and directly to plastic surfaces (Carlsson et al., 1972; Holmgren and Svennerholm, 1973; Carlsson et al., 1976; Lamb et al., 1979; Keren, 1979). However, with the microtitre plates available to us, we found that Salmonella LPS, as such, coupled poorly (Fig. 5.1). In consequence,

we were required to interact the LPS with methylated bovine serum albumin (MBSA) (Vos et al., 1979) and this complex coupled well to our brand of microtitre plates. These techniques are described in Chapter 4.

5.4.3 (ii) Determination of the optimal concentration of LPS for coating the polystyrene plates

Two hundred µl each of varying concentrations of the complexed LPS in carbonate buffer (0.1 M, pH 9.6) was added to the plate, which was then incubated at 37° for 3 hours followed by 4° overnight. The plate was then washed three times with PBS-Tw and tested with a dilution of homologous antiserum. Maximum absorption of the complex was obtained at 5-10 µg/ml for the LPS from each of the 14 strains investigated. Typical results are shown in Figs. 5.1 and 5.2. LPS per se was relatively poorly adsorbed to the plastic microtitre plates; however, much better adsorption was obtained with the LPS-MBSA complex.

In future work it was decided to coat the plates using the LPS-MBSA complex at a concentration of 5 µg/ml.

5.4.4 Sera and fluids

Pooled sera and intestinal fluids containing antibodies directed against a particular Salmonella strain were obtained from groups of immunized mice as described in Chapter 4.

Figure 5.1

Determination of the optimal concentration of LPS-MBSA for coating polystyrene plates.

Varying amounts of Salmonella LPS from S. bonariensis and the hybrid strain S. typhimurium F885 were used to coat the plates and then tested with their homologous mouse anti-Salmonella antiserum at 1:5000 dilution. Rabbit anti-mouse Fab-alkaline phosphatase conjugate was used at 1:500 dilution. Substrate incubation time was 90 minutes.

- O F885 LPS alone.
- ● F885 (1,4,5,12) LPS-MBSA.
- ▲ S. bonariensis (6,8) LPS-MBSA.

FIGURE 5.1

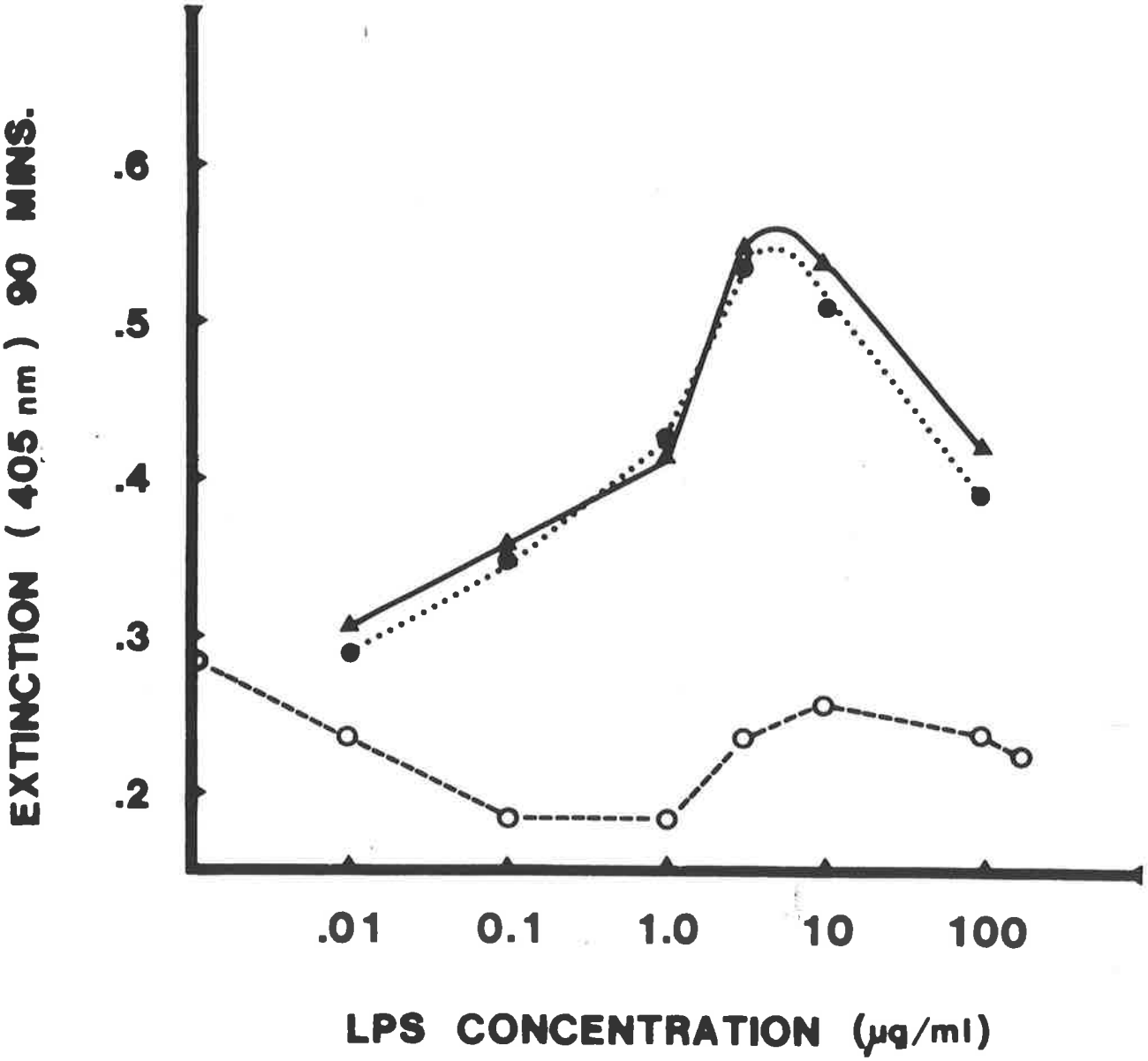

EXTINCTION (405 nm) 90 MINS.
.6
.5
.4
.3
.2
.01
0.1
1.0
10
100
LPS CONCENTRATION (µg/ml)

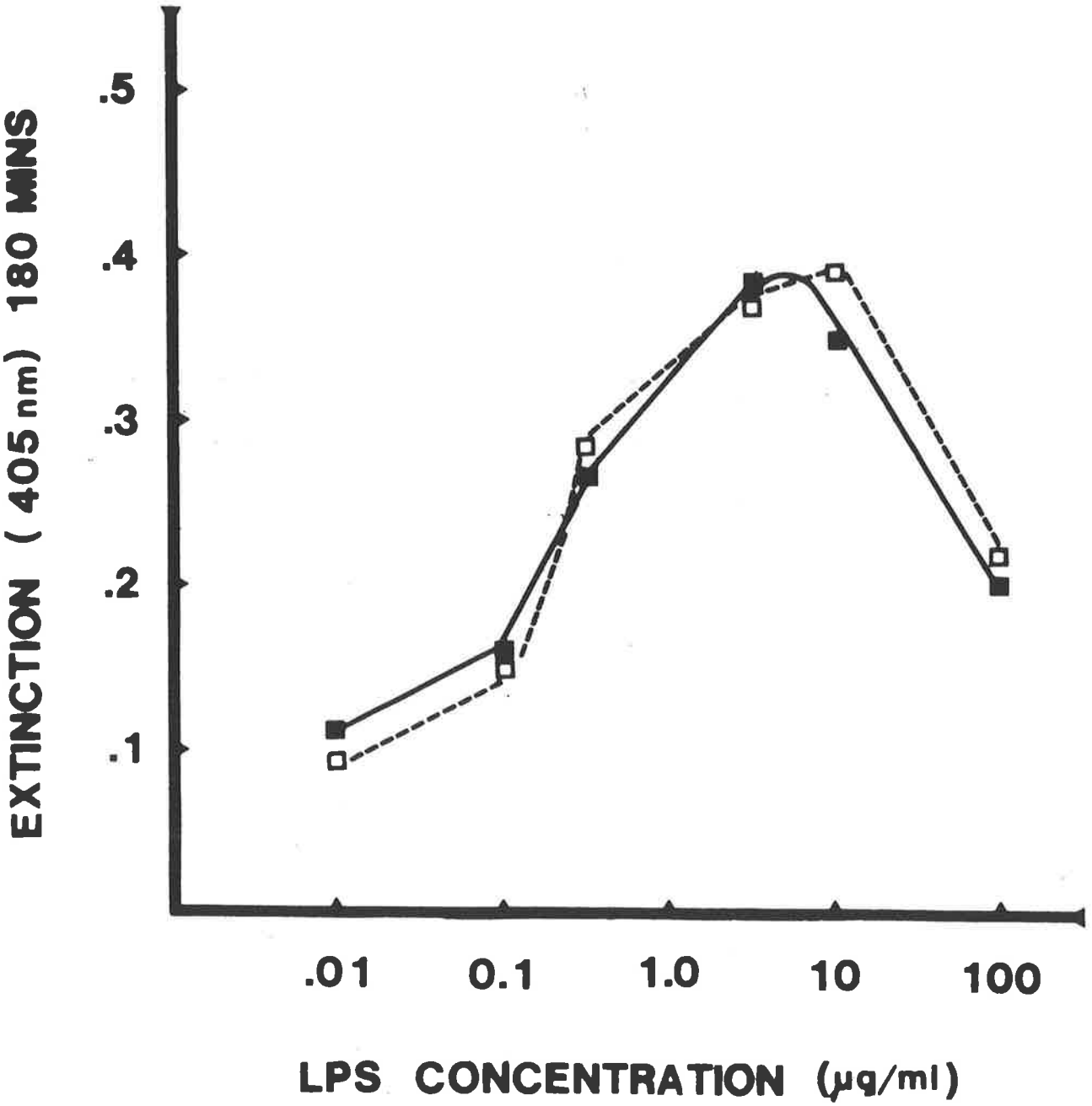
Figure 5.2

Determination of the optimal concentration of LPS-MBSA for coating polystyrene plates.

Varying amounts of LPSs from S. humber and hybrid strain E. coli F1142 were used to coat the plates and then tested with their homologous mouse antiserum at 1:2000 dilution. Rabbit anti-mouse Fab-alkaline phosphatase was used at 1:500 dilution. Substrate incubation time was 180 minutes.

- ■ E. coli F1142 (0,8) LPS-MBSA.
- □ S. humber (53) LPS-MBSA.

FIGURE 5.2


EXTINCTION (405 nm) 180 MINS
.5
.4
.3
.2
.1
.01
0.1
1.0
10
100
LPS CONCENTRATION (µg/ml)

5.4.5 The activities and working dilutions of rabbit anti-mouse Fab and of goat anti-mouse α-chain alkaline phosphatase conjugates

Rabbit anti-mouse Fab and goat anti-mouse α-chain were purified by the methods described in Chapter 4. These antisera were then coupled to alkaline phosphatase as described in Chapter 4. The activities and specificities of these conjugates were assessed by reacting each with purified mouse myeloma IgA (S107) and purified mouse myeloma IgG1 (MOPC21), IgG2a (ID45), IgG2b (MPC11) and IgM (E1-M) adsorbed to polystyrene plates. The mouse myelomas were kindly donated by Dr P. L. Ey of this department.

The data presented in Fig. 5.3 and Fig. 5.4 demonstrate the degrees of interaction of the anti-mouse Fab-alkaline phosphatase complex and of the anti-mouse α-chain alkaline phosphatase complex, respectively, with various immunoglobulin classes. Thus, polystyrene plates were coated, in duplicate, with either 200 µL of IgG1, IgG2a, IgG2b or IgA (all at 5 µg/ml) or with IgM (at 2 µg/ml). The plates were then incubated for 3 hours at 37° before washing three times with PBS-Tween buffer. Suitable dilutions of conjugate were added to one or other of the duplicately prepared plates and allowed to interact at room temperature for 18 hours. Following washing the p-nitrophenol substrate (200 µL at 1 mg/ml) was added to each well, and the absorbance at 405 nm was read after standing at room temperature for 3 hours. The anti-mouse Fab enzyme conjugate reacted with all classes of immunoglobulin to about the same degree, whereas the anti-mouse α-chain enzyme conjugate was specific only for IgA and exquisitely sensitive.

The data presented in Fig. 5.5 and Fig. 5.6 summarize experiments

Figure 5.3

The activity of rabbit anti-mouse Fab enzyme conjugate.

Serial dilutions of the conjugate were added to polystyrene plates previously sensitized with either IgG1, IgG2a, IgG2b or IgA (200 µL at 5 µg/ml) or IgM (200 µL at 2 µg/ml). The plates were incubated at 37° for 3 hours, washed 3 times with PBS-Tween buffer prior to the addition (200 µL) of suitable dilutions of conjugate. After 18 hours incubation at room temperature the chromogenic substrate was added to each well of the washed plates. All assays were carried out in duplicate.

- ● IgG1 sensitized wells.
- △ IgG2a sensitized wells.
- □ IgG2b sensitized wells.
- ■ IgM sensitized wells.
- ○ IgA sensitised wells.
- ▲ Conjugate control.

FIGURE 5.3

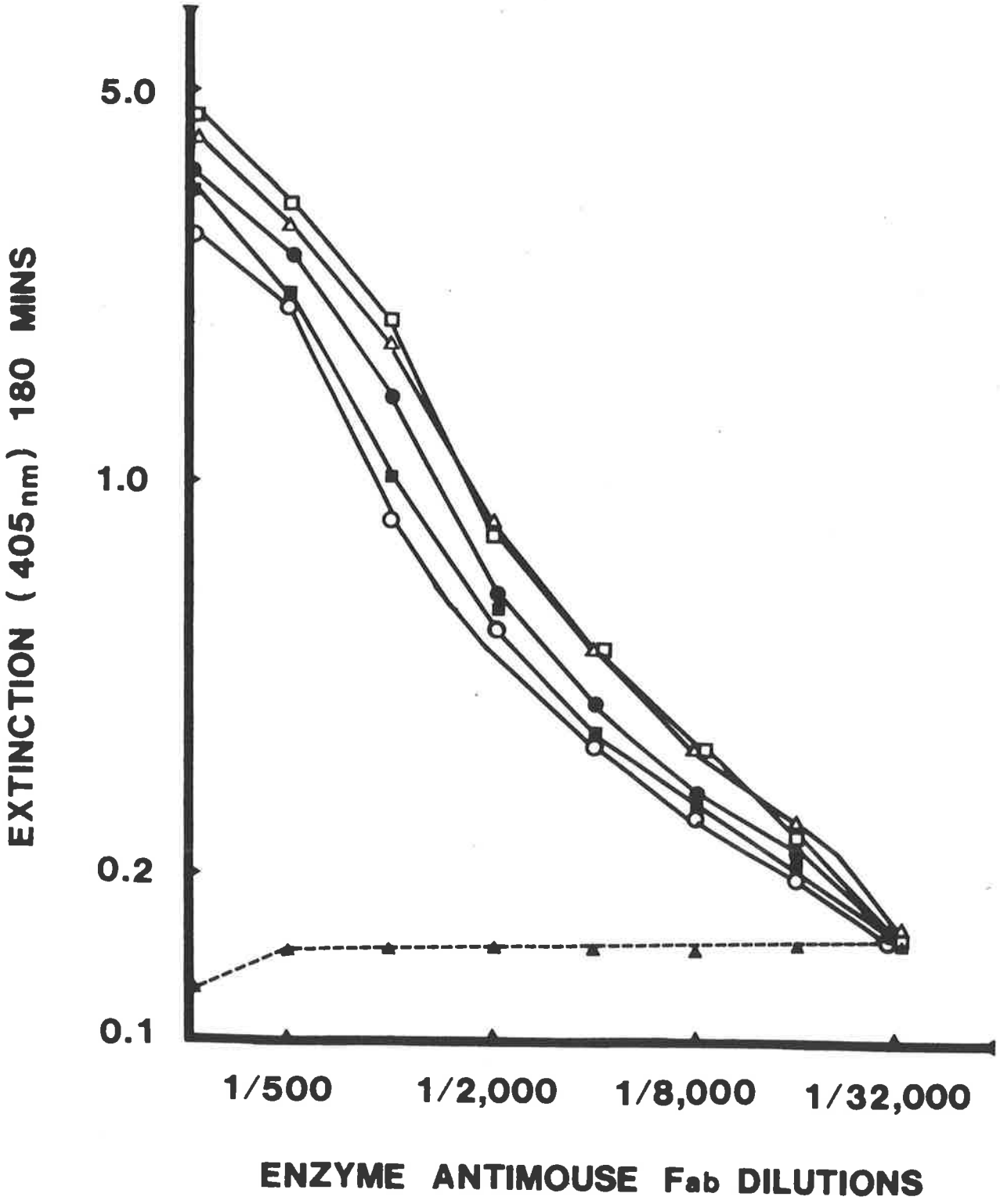

5.0
1.0
0.2
0.1
EXTINCTION (405nm) 180 MINS
1/500
1/2,000
1/8,000
1/32,000
ENZYME ANTIMOUSE Fab DILUTIONS

Figure 5.4

The activity of goat anti-mouse α-chain enzyme conjugate.

Serial dilutions of the conjugate were added to polystyrene plates previously sensitized with either IgG1, IgG2a, IgG2b or IgA (200 µL at 5 µg/ml) or IgM (200 µL at 2 µg/ml). The plates were incubated at 37° for 3 hours, washed 3 times with PBS-Tween buffer prior to the addition (200 µL) of suitable dilutions of conjugate. After 18 hours incubation at room temperature the chromogenic substrate was added to each well of the washed plates. All assays were carried out in duplicate.

- ○ IgG1.
- △ IgG2a.
- □ IgG2b.
- ■ IgM.
- ● IgA.
- ▲ Conjugate control.

FIGURE 5.4

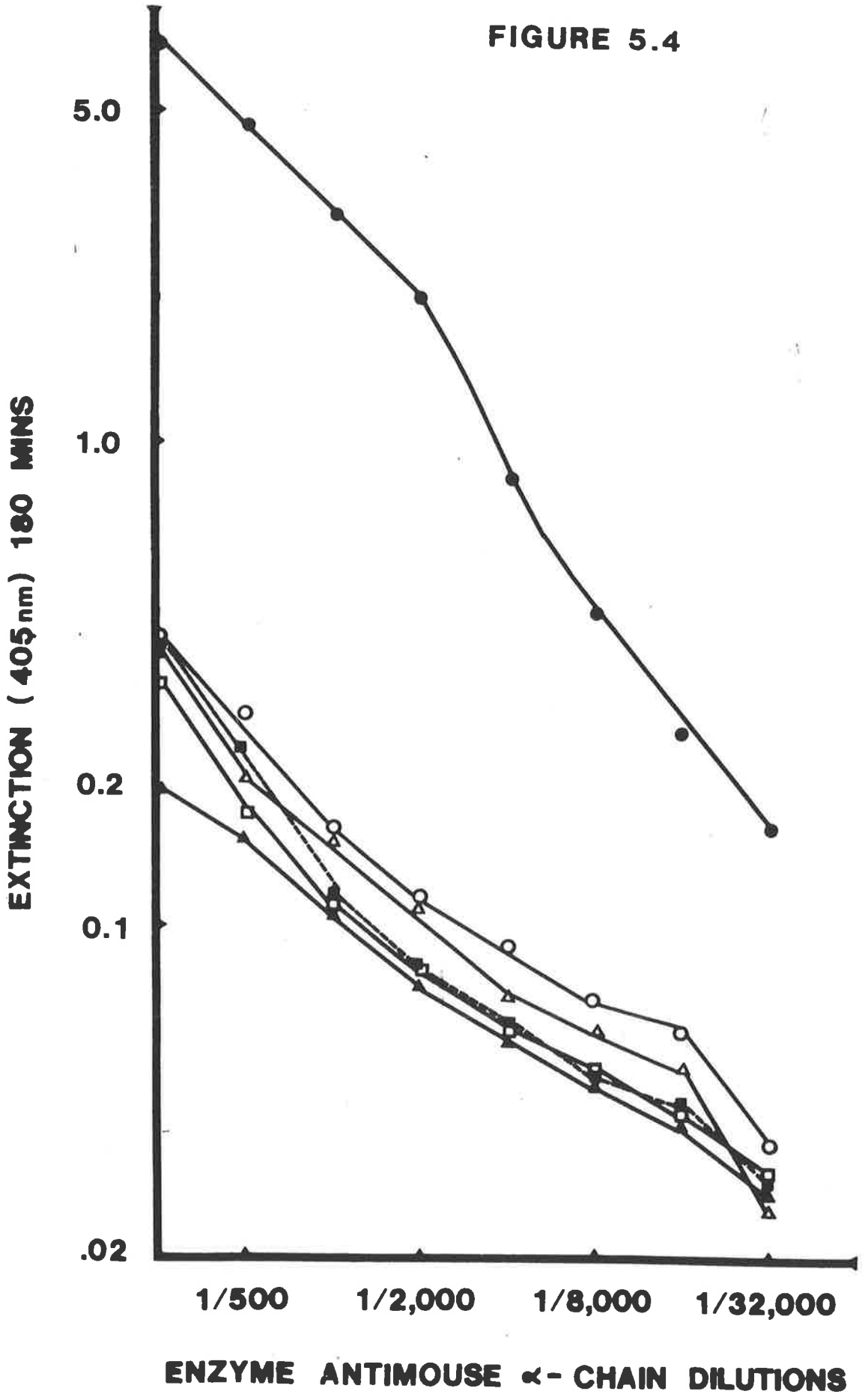

5.0
1.0
0.2
0.1
.02
EXTINCTION (405nm) 180 MINS
1/500
1/2,000
1/8,000
1/32,000
ENZYME ANTIMOUSE ∝- CHAIN DILUTIONS

Figure 5.5

Determination of working strength of the rabbit anti-mouse Fab enzyme conjugate.

An indirect microplate ELISA was employed (see 4.18.2) using 4 conjugate dilutions,, 1:500, 1:1000, 1:2000 and 1:4000, diluted in enzyme diluent. Salmonella F885 LPS antigen was used for coating the plates. A positive mouse anti-F885 antiserum and normal mouse serum were tested with each series of dilutions. The closed symbols represent the F885 serum and the open symbols represent the normal mouse serum.

Conjugate diluted 1:500. ○ ●

Conjugate diluted 1:1000. △ ▲

Conjugate diluted 1:2000. □ ■

Conjugate diluted 1:4000. ▽ ▼

Conjugate control (1:500). ⊙

Conjugate control (1:1000). ⊙

Conjugate control (1:2000). ⊙

Conjugate control (1:4000). ⊙

FIGURE 5.5

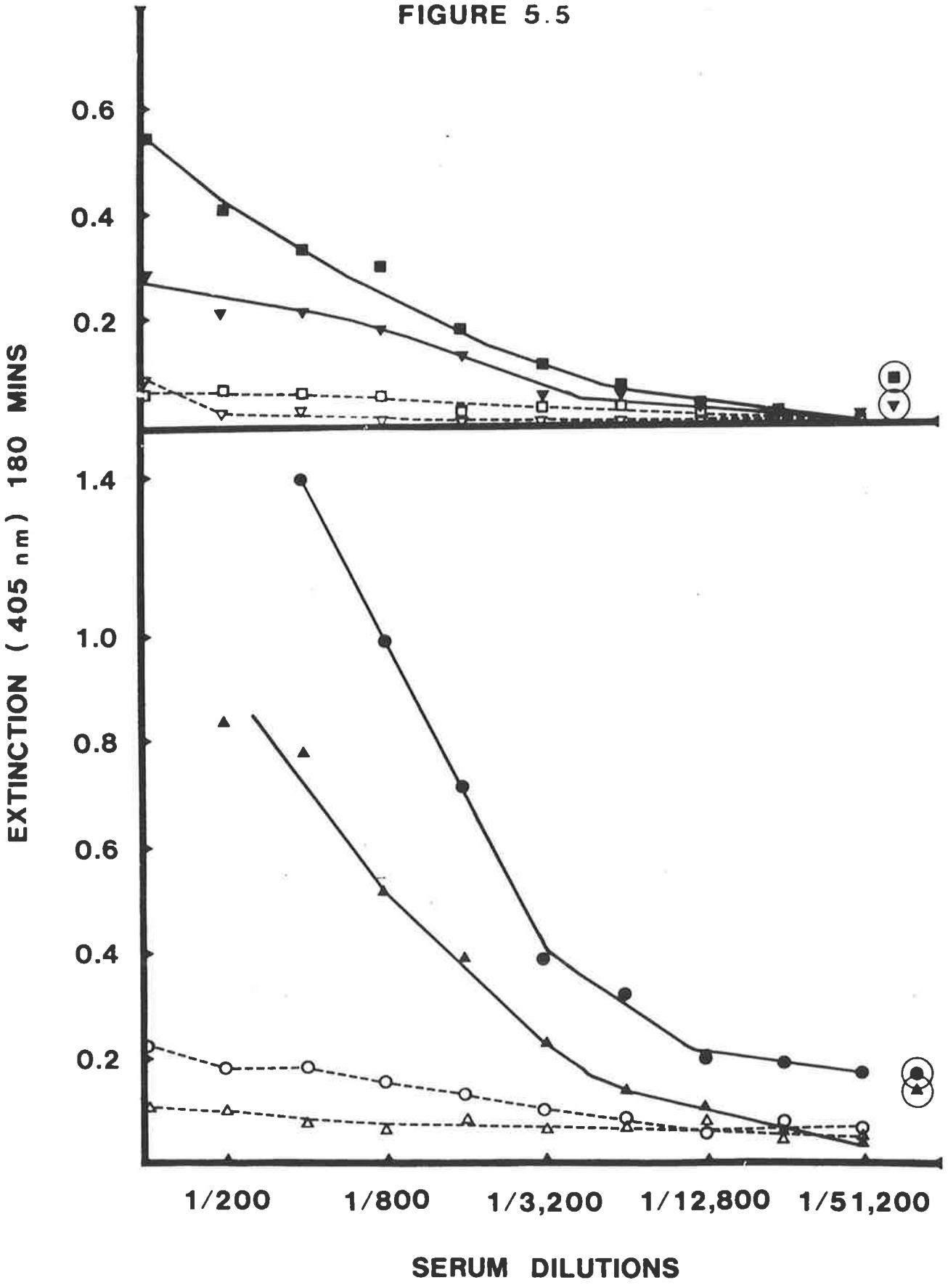

0.6
0.4
0.2
1.4
1.0
0.8
0.6
0.4
0.2
EXTINCTION (405 nm) 180 MINS
1/200
1/800
1/3,200
1/12,800
1/51,200
SERUM DILUTIONS

Figure 5.6

Determination of working strength of the goat anti-mouse α-chain enzyme conjugate.

An indirect microplate ELISA was employed (see 4.18.2) using 4 conjugate dilutions, 1:500, 1:1000, 1:2000 and 1:4000, diluted in enzyme diluent. Salmonella F885 LPS antigen was used for coating the plates. A positive mouse anti-F885 antiserum and normal mouse serum were tested with each series of dilutions. The closed symbols represent the positive F885 serum and the open symbols represent the negative normal serum.

○ ● Conjugate diluted 1:500.

△ ▲ Conjugate diluted 1:1000.

□ ■ Conjugate diluted 1:2000.

▽ ▼ Conjugate diluted 1:4000.

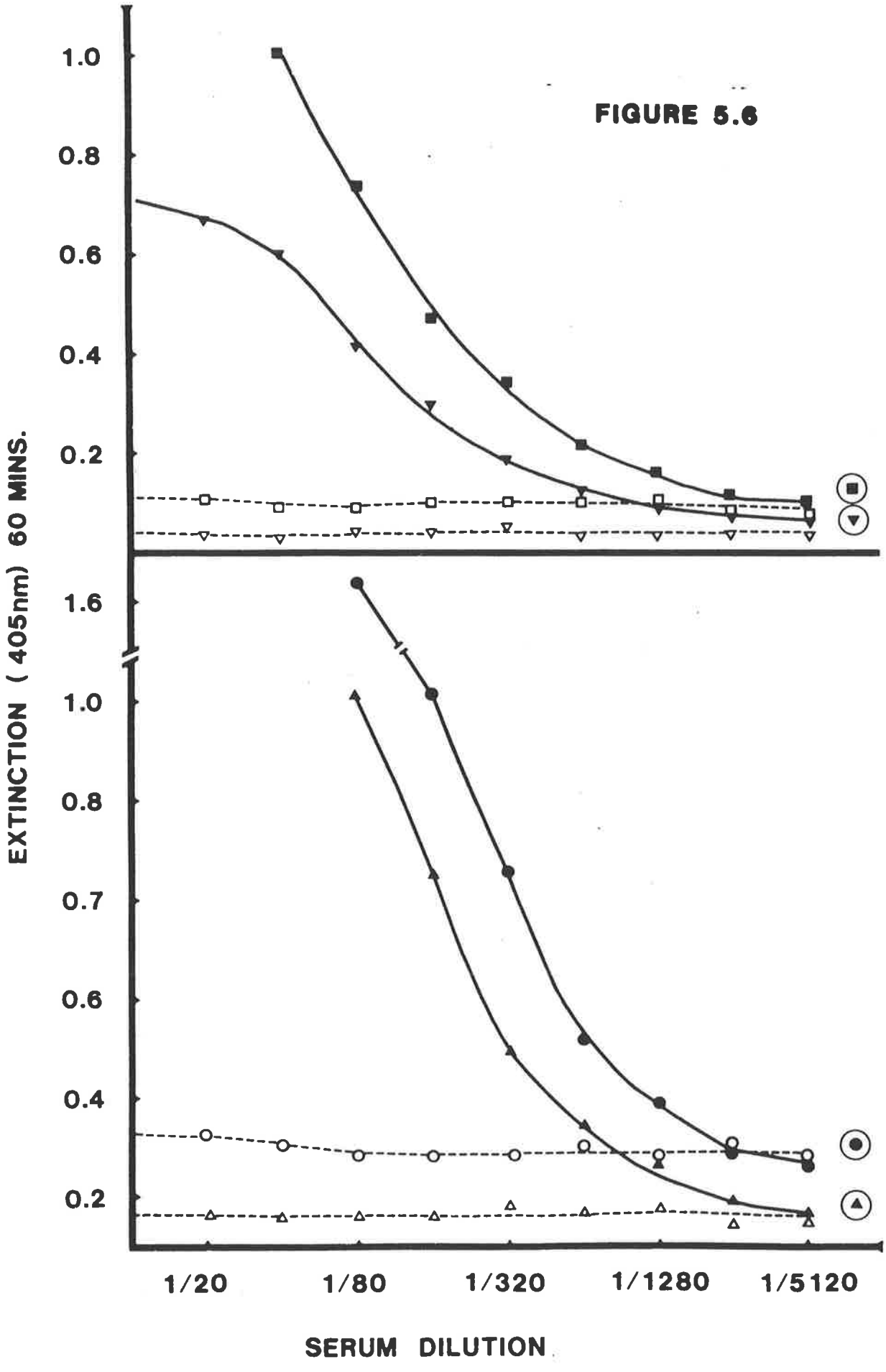
◉ Conjugate control (1:500).

▲ Conjugate control (1:1000).

■ Conjugate control (1:2000).

▼ Conjugate control (1:4000).

FIGURE 5.6


1.0
0.8
0.6
0.4
0.2
1.6
1.0
0.8
0.7
0.6
0.4
0.2
EXTINCTION (405nm) 60 MINS.
1/20
1/80
1/320
1/1280
1/5120
SERUM DILUTION

designed to determine the optimal concentrations of anti-Fab enzyme conjugate and anti-α-chain enzyme conjugate, respectively, for use in the assay systems. An indirect ELISA was performed using dilutions of each of the two conjugates at 1:500, 1:1000, 1:2000 and 1:4000 in enzyme diluent. Salmonella F885 LPS coated plates were incubated at room temperature with serial dilutions of anti-Salmonella F885 mouse serum, or with normal mouse serum, for 6 hours. The plates were then washed and incubated with the various dilutions of either enzyme conjugate, as shown above. After a further incubation of 18 hours at room temperature the washed plates were then incubated with the substrate to determine the activity of bound enzyme. Such experiments showed that increases in conjugate concentration result in increased sensitivity, i.e., antibodies can be detected at very low concentration. It was decided to use the anti-Fab enzyme conjugate at 1:500 dilution and the anti-α-chain enzyme conjugate at 1:1000 dilution in further work, since these levels gave a high sensitivity with relatively low background due to non-specific binding of conjugate.

5.4.6 Sensitivities and specificities of the conjugates

The specificity and sensitivity of the goat anti-mouse α-chain alkaline phosphatase conjugate was assessed by a double antibody technique or sandwich method described in detail in Chapter 4. Briefly, plates were coated with rabbit anti-mouse Fab; varying amounts of either IgG1, IgA or IgM were added to the wells, which were then stood at room temperature for 6 hours. After washing, conjugate was added to the plates which were incubated and then washed prior to incubation with substrate. As shown in Fig. 5.7, the anti-mouse

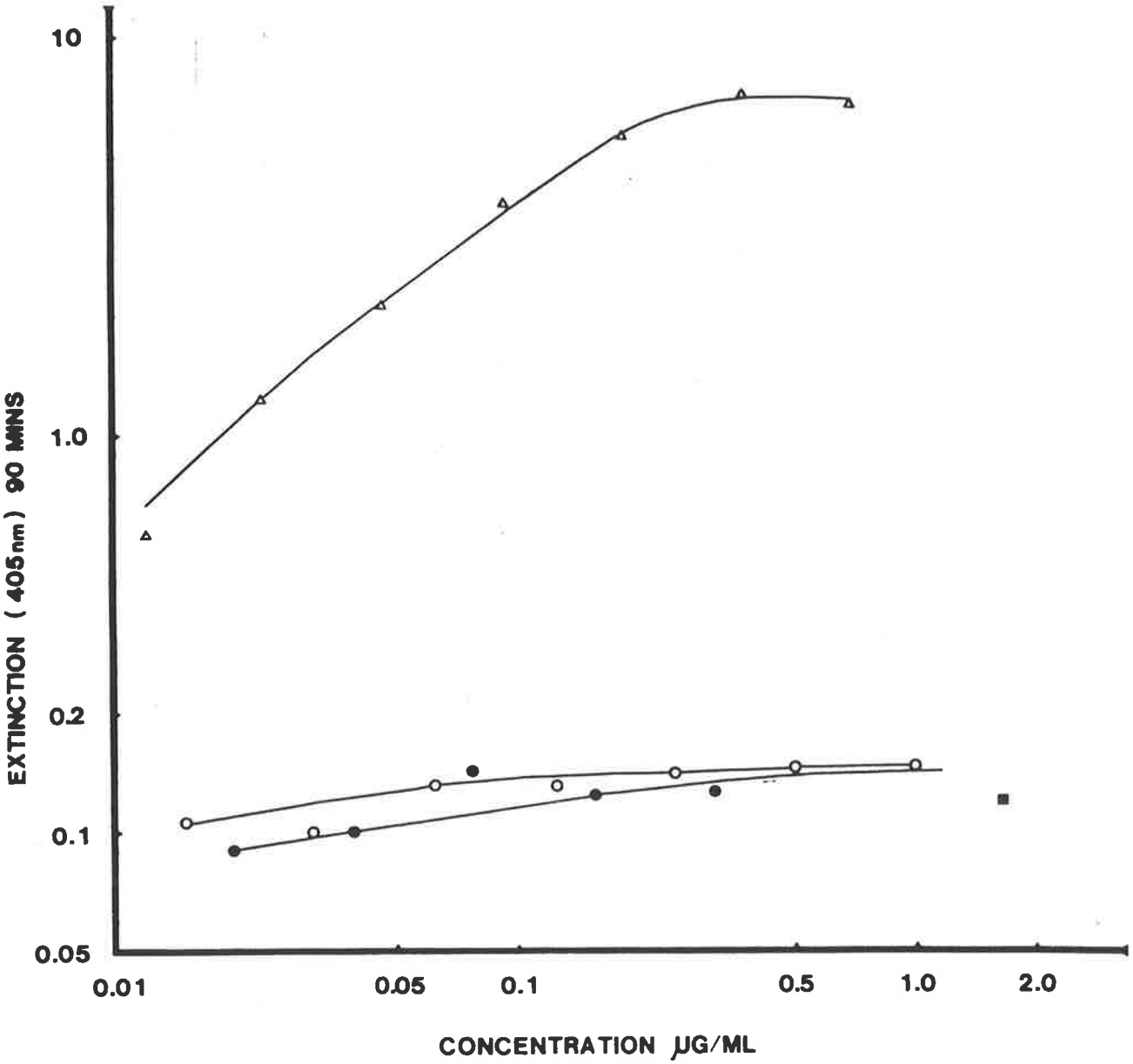
Figure 5.7

Specificity of the enzyme anti-mouse α-chain conjugate.

The enzyme anti-mouse α-chain conjugate 1:1000 dilution was tested with varying amounts of mouse myeloma IgG1, IgM and IgA initially reacted with rabbit anti-mouse Fab adsorbed to the plate.

- △ IgA sensitized wells.
- ● IgG1 sensitized wells.
- ○ IgM sensitized wells.
- ■ Conjugate control.

FIGURE 5.7


10
1.0
0.2
0.1
0.05
EXTINCTION (405nm) 90 MINS
0.01
0.05
0.1
0.5
1.0
2.0
CONCENTRATION µG/ML

α-chain preparation detected only IgA; there was negligible cross-reaction with IgG1 or IgM. Subsequent examination (Fig. 6.4a) showed that this technique can detect IgA at a concentration of approximately 45 ng/ml at the limiting O.D. 405, 0.2. Similar procedures were used to assess the specificity and sensitivity of the rabbit anti-mouse Fab conjugate. The data in Fig. 5.8 shows that the conjugate could readily detect all three major classes of immunoglobulins. Subsequent examination (Fig. 6.4b, c, d) showed that about 30 ng/ml of IgG1, IgG2a and IgG2b, 250 ng/ml of IgA and 50 ng/ml of IgM can be detected by this system at the limiting O.D. 405, 0.2.

5.4.7 Optimal temperatures and times for various incubations

The rate of interaction of the enzyme with its substrate is clearly temperature and time dependent (in the presence of excess substrate). The time and temperature of such interaction will clearly influence the sensitivity of the ELISA.

However, prior to these considerations, one needs to know the conditions necessary for optimal binding of antibody to the antigen initially adsorbed to the plate.

(a) **The incubation of antibody with LPS-coated plates:** The polystyrene plates were initially coated with Salmonella F885 LPS using a concentration of 5 μg/ml; the LPS was allowed to adsorb for 3 hours at 37°. After washing, the plates were incubated, at various temperatures, for different times with mouse anti-F885 serum at two dilutions (1:1000 and 1:10,000). For the second stage antibody conjugate a dilution of 1/500 was used.

The results (Fig. 5.9) show that the interaction of serum antibodies with LPS was virtually complete after one hour at all

Figure 5.8

Specificity of the enzyme anti-mouse Fab conjugate.

1:500 dilution of the enzyme anti-mouse Fab conjugate was tested with varying amounts of mouse myeloma IgG1, IgA and IgM initially reacted with rabbit anti-mouse Fab adsorbed to the plate.

IgG1 sensitized wells. ●

IgM sensitized wells. ○

IgA sensitized wells. △

Conjugate control. ■

FIGURE 5.8

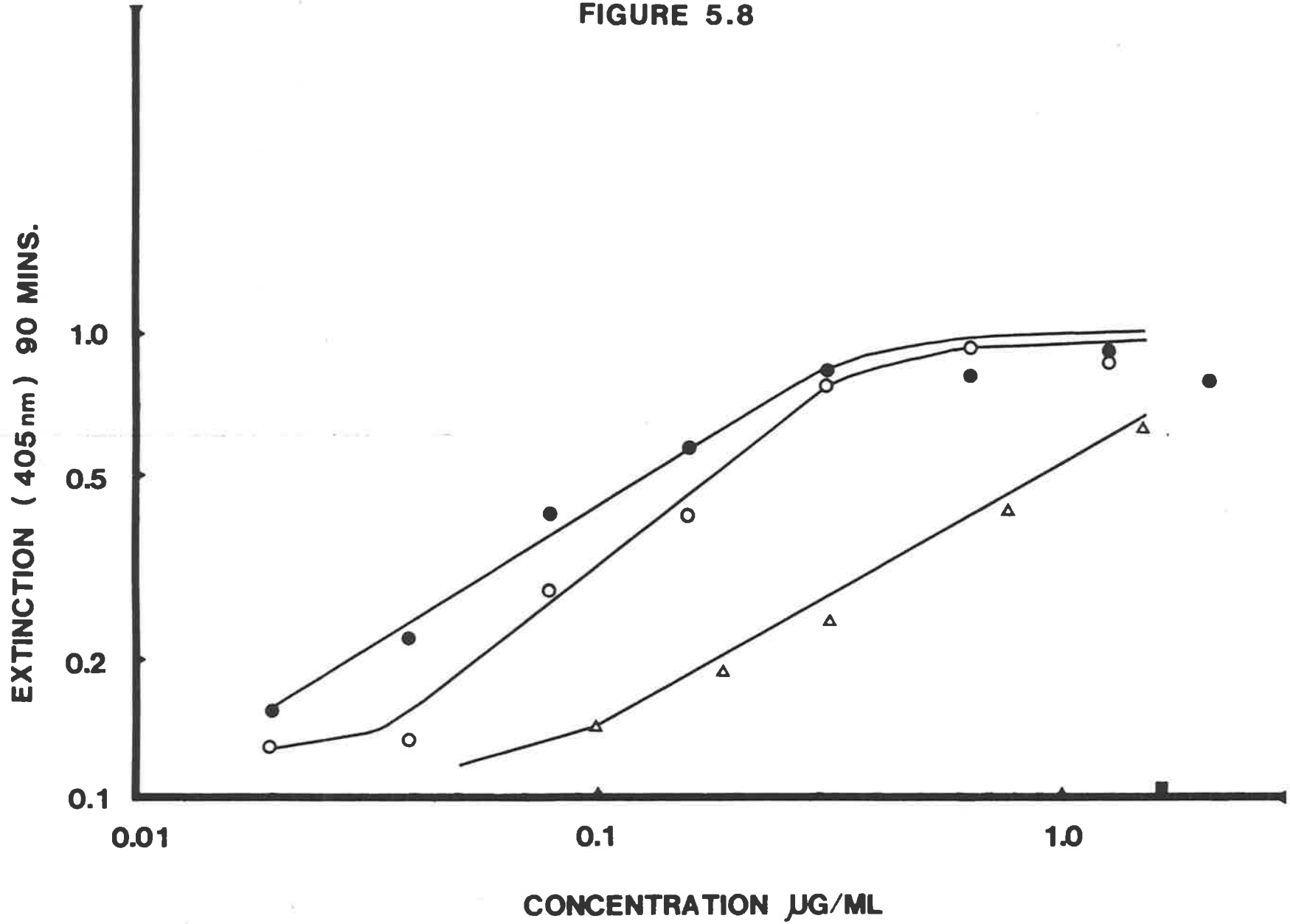

EXTINCTION (405nm) 90 MINS.
1.0
0.5
0.2
0.1
0.01
0.1
1.0
CONCENTRATION µG/ML

Figure 5.9

Kinetics of binding of mouse antiserum.

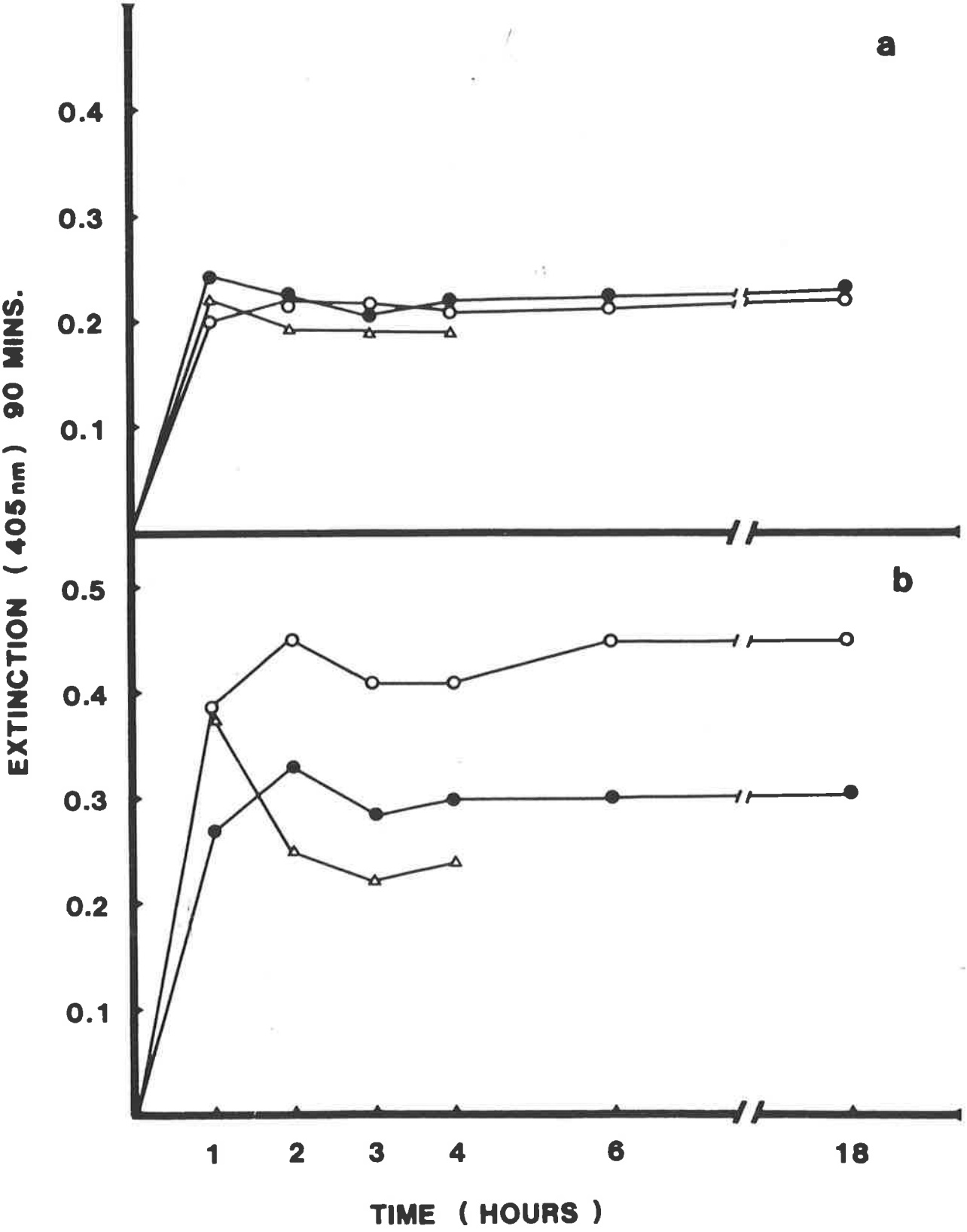
The microtitre plates coated with F885 LPS, 5 µg/ml (3 hours at 37°), were incubated with mouse anti-F885 diluted 1:10,000 (a) or 1:1000 (b) at 4°, 25° (room temperature) and 37° for different periods of time. The antibody binding was estimated as bound enzyme rabbit anti-mouse Fab conjugate after 18 hours incubation at 25°.

● Incubation at 4°.

O Incubation at 25°.

Δ Incubation at 37°.

FIGURE 5.9


a
b
0.4
0.3
0.2
0.1
0.5
0.4
0.3
0.2
0.1
EXTINCTION (405nm) 90 MINS.
1
2
3
4
6
18
TIME (HOURS)

temperatures tested. However, the decrease which occurred at 37° was probably due to desorption of LPS from the plates, possibly under the influence of antibody. Similar investigations were carried out using higher concentrations of the intestinal juice (1 in 800 and 1 in 200) from Salmonella F885 immunized mice. The results, shown in Fig. 5.10, show that incubation at 37° apparently results in less binding. This may well be due to interference, at this higher temperature, by the various proteolytic enzymes in the intestinal fluids.

(b) **Incubation with conjugates:** Optimal conditions for the interaction of conjugates with their homologous immunoglobulins was determined as shown in Figs. 5.11 and 5.12. Either anti-mouse Fab conjugate or anti-mouse α-chain conjugate, at fixed concentration, were incubated for varying times with the homologous antibodies contained in serum or intestinal fluid from immunized mice which had previously been fixed to plates via adsorbed homologous LPS. The maximum binding of either conjugate required about 2 hours at 37° whether serum or intestinal fluid was used as the primary antibody source. At lower temperatures, however, longer times may be needed (up to 8 hours or more) to effect maximum binding.

(c) **Kinetics of the substrate reaction:** The interaction of alkaline phosphatase with substrate, p-nitrophenyl phosphate, liberates p-nitrophenol, which has an absorbance maximum at 405 nm. Temperature and time of reaction and conjugate concentration will all clearly influence this hydrolytic event.

To investigate these aspects, the sandwich (double antibody ELISA) was employed. Briefly, plates were coated with anti-mouse Fab

Figure 5.10

Kinetics of binding of mouse antibody-positive intestinal fluids.

The microtitre plates coated with F885 LPS, 5 µg/ml (3 hours at 37°), were incubated with mouse anti-F885 intestinal fluid diluted 1:800 (a) or 1:200 (b) at 4°, 25° (room temperature) and 37° for different periods of time. The antibody binding was estimated as bound enzyme goat anti-mouse α-chain conjugate after 18 hours incubation at 25°.

- ● Incubation at 4°.
- ○ Incubation at 25°.
- △ Incubation at 37°.

FIGURE 5.10

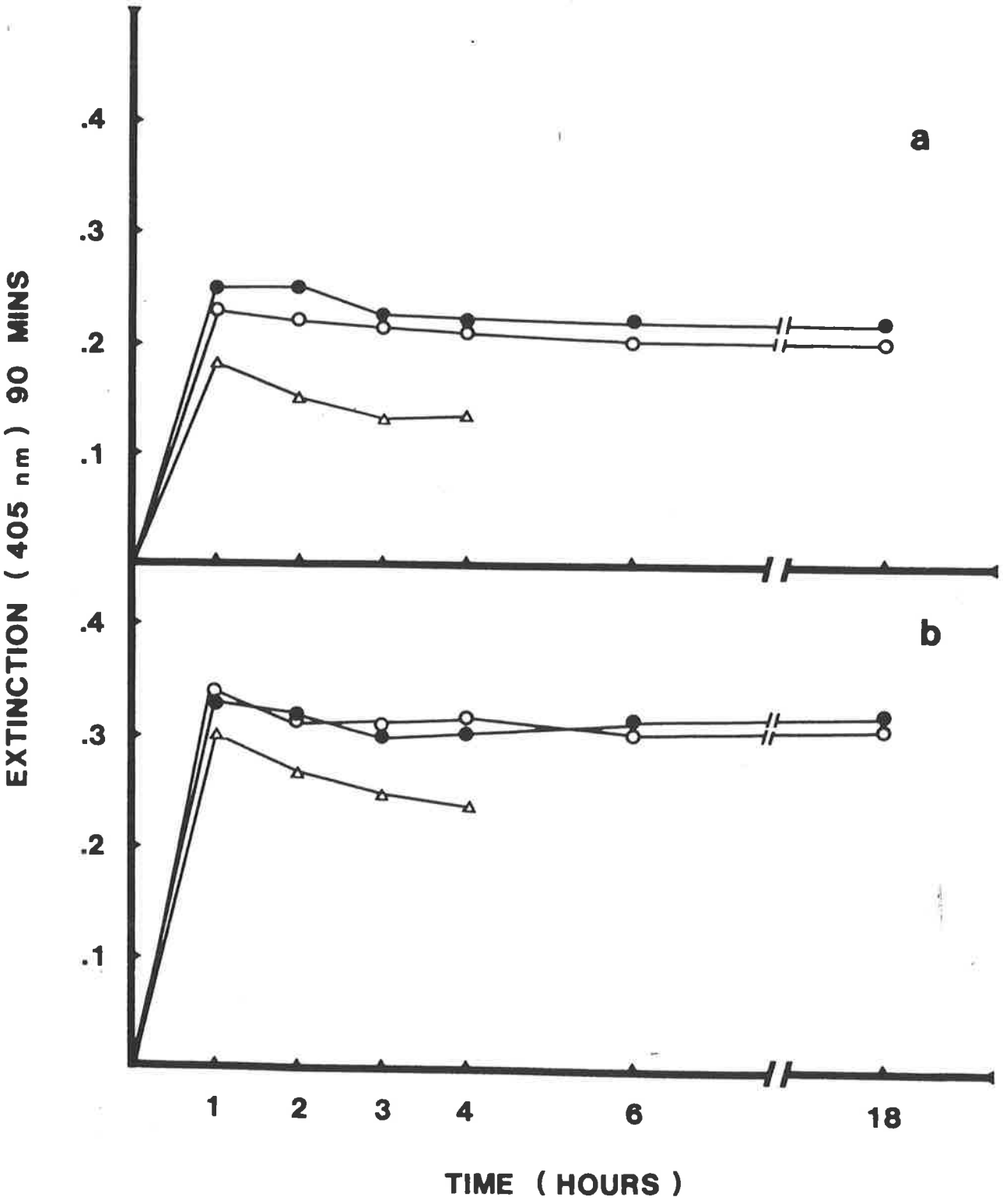


Figure 5.11

Kinetics of the rabbit anti-mouse Fab-alkaline phosphatase conjugate binding.

Microtitre plates were coated with F885 LPS, 5 µg/ml (3 hours at 37°) and then incubated with mouse anti-F885 serum at two dilutions, 1:10,000 (a) or 1:1000 (b), at 25° for 6 hours. After washing the plates were incubated with the enzyme anti-mouse Fab conjugate diluted 1:500 at 4°, 25° (room temperature) and 37° for different periods of time. Bound conjugate was estimated as enzyme activity after 90 minute substrate incubation.

- ● Incubation at 25°.
- O Incubation at 4°.
- △ Incubation at 37°.

FIGURE 5.11

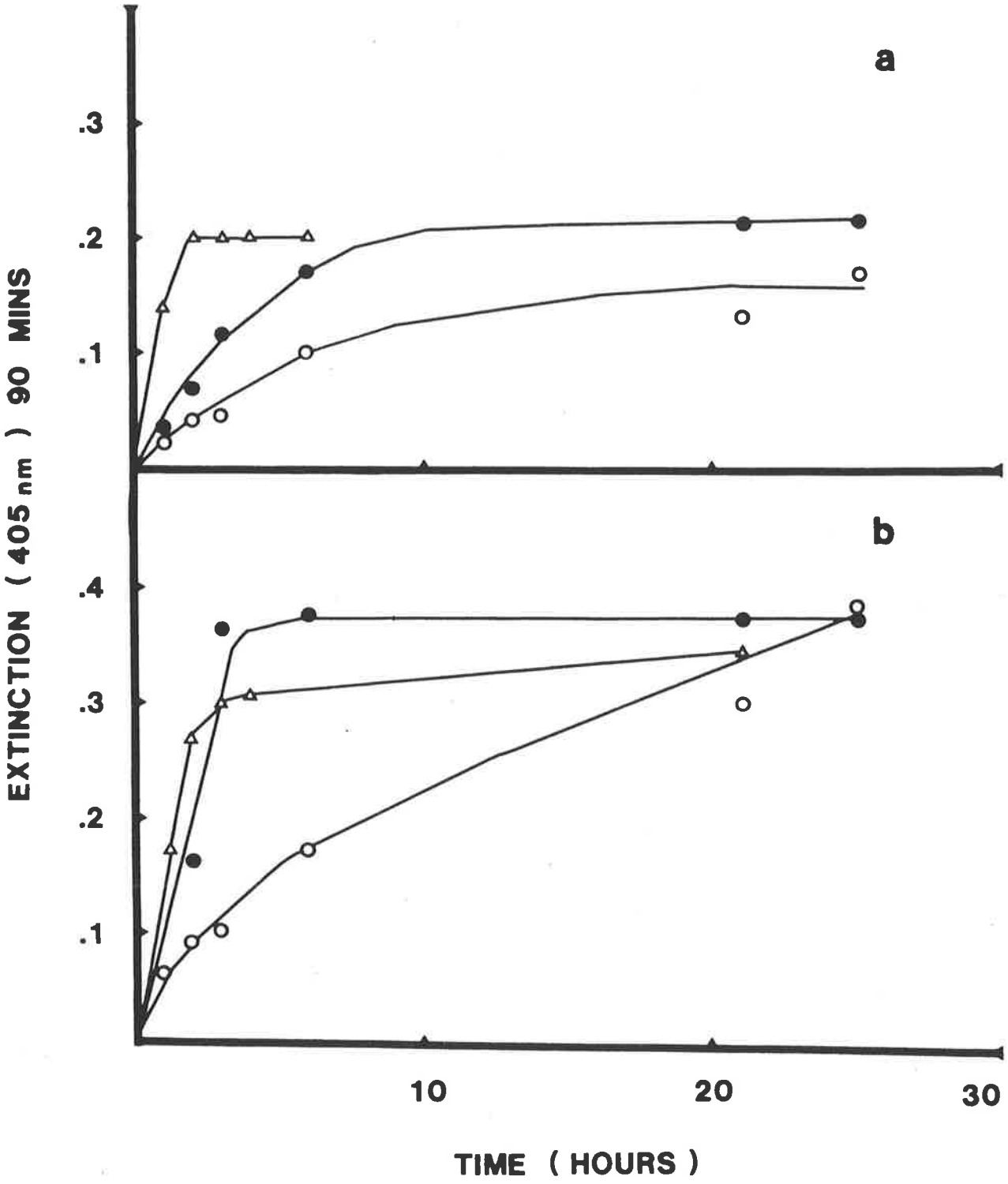


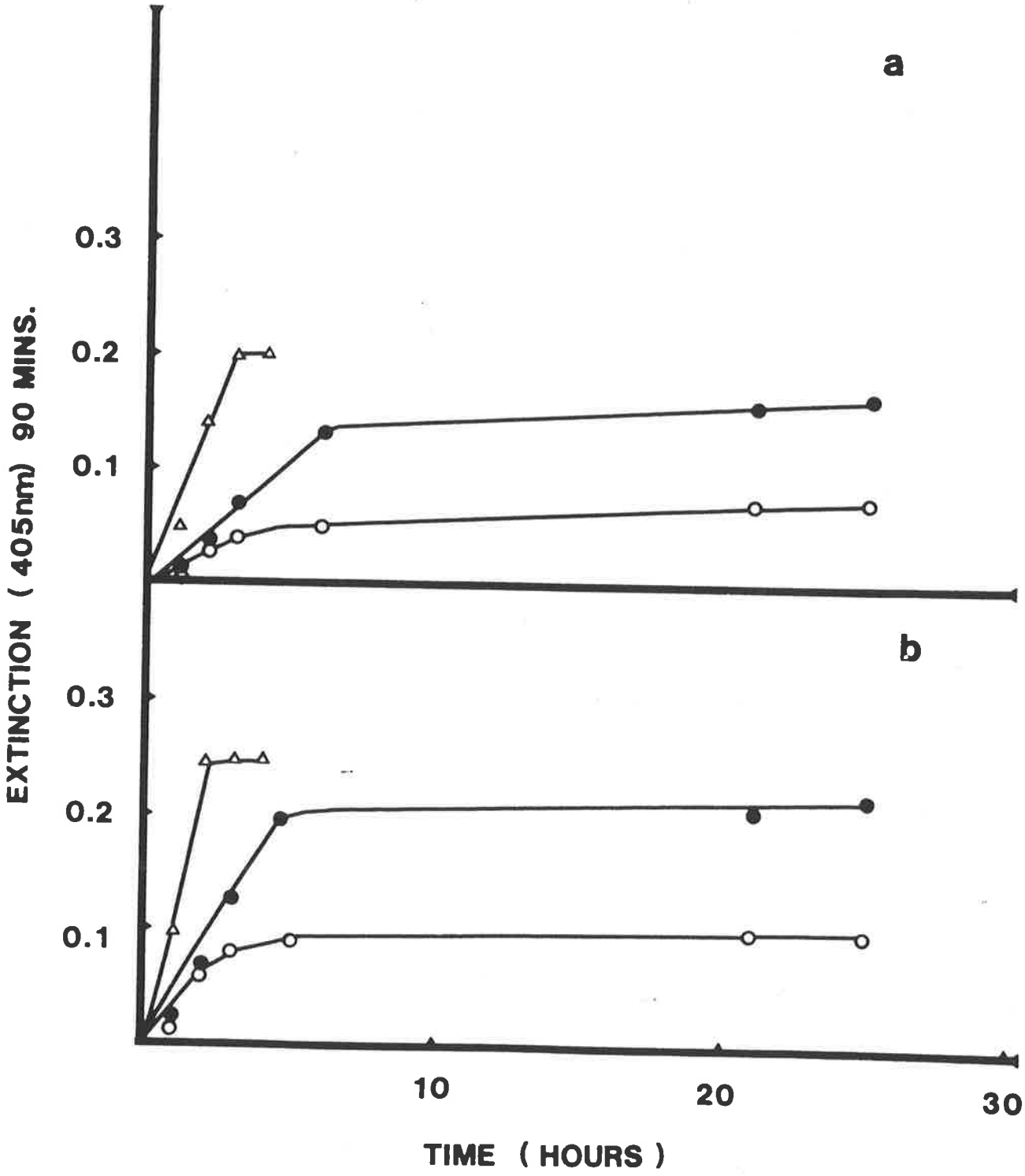
Figure 5.12

Kinetics of the goat anti-mouse α-chain-alkaline phosphatase conjugate binding.

Microtitre plates were coated with F885 LPS, 5 µg/ml (3 hours at 37°) and then incubated with mouse anti-F885 intestinal fluids at two dilutions, 1:800 (a) or 1:200 (b), at 25° for 6 hours. After washing the plates were incubated with the enzyme goat anti-mouse α-chain conjugate diluted 1:1000 at 4°, 25° (room temperature) and 37° for different periods of time. Bound conjugate was estimated as enzyme activity after 90 minute substrate incubation.

- ● Incubation at 25°.
- O Incubation at 4°.
- Δ Incubation at 37°.

FIGURE 5.12


a
b
0.3
0.2
0.1
0.3
0.2
0.1
EXTINCTION (405nm) 90 MINS.
10
20
30
TIME (HOURS)

in the usual manner and these were then incubated with varying concentrations of mouse IgG1 for 6 hours at 25°, washed and then incubated with anti-mouse Fab-alkaline phosphatase conjugate.

Following 18 hours incubation at 25°, the bound enzyme conjugate was estimated by the degree of hydrolysis of the substrate occurring after varying periods of incubation. The data shown in Fig. 5.13a demonstrates, as expected, that shorter incubation times decrease the sensitivity of the assay. At the limiting O.D. 405 reading of 0.2, incubation for 60 minutes detected about 80 ng/ml of IgG1. Although incubation for 120 minutes detected about 20 ng/ml, the longer incubation time gave twice the background control and militated against performing the assay in a single day.

Fig. 5.13b demonstrates that the relationship between time of incubation and substrate hydrolysed is linear over at least a 300 minute period at 37° when the system was such as to give about the limiting O.D. 405 reading of 0.2 at 60 minutes.

5.4.8 Non-specific binding

Since the ELISA depends upon the binding of molecules of large molecular weight to plastic plates, there is clearly a tendency for molecules to become non-specifically attached to areas unoccupied by the initially adsorbed antigen. This is particularly the case when body fluids are investigated by the indirect ELISA. Aberrations (i.e., false positives) can occur when alkaline phosphatase conjugates are used to determine immunoglobulins in intestinal juice, as this is a source of non-specifically adsorbing alkaline phosphatase. However, such anomalies may be significantly reduced, if not eliminated, by diluting test samples into a buffer of relatively high salt

Figure 5.13(a)

Kinetics of substrate reaction.

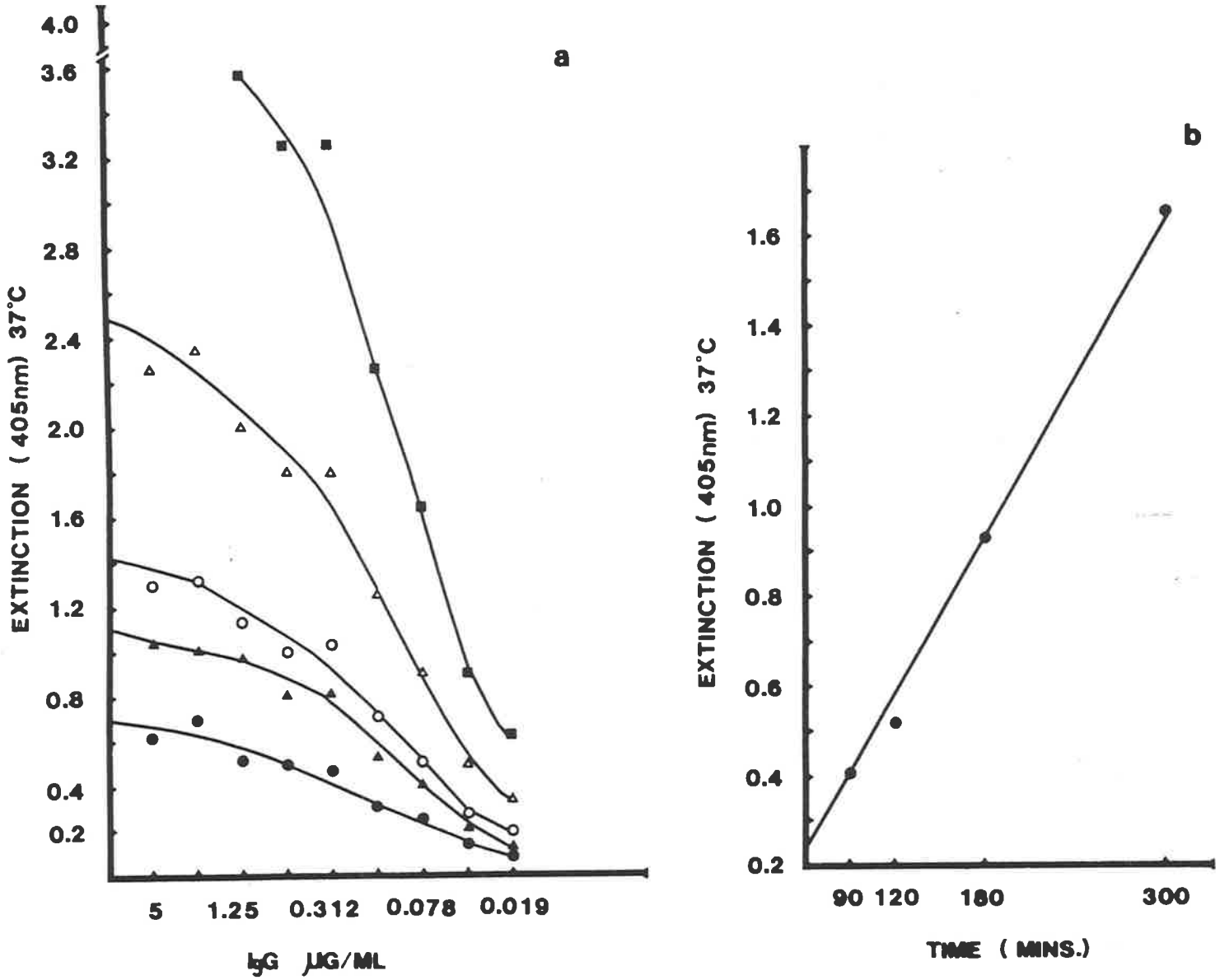
Varying concentrations of mouse IgG1 were incubated with rabbit anti-mouse Fab coated plates (6 hours at 25°). 1:500 rabbit anti-mouse Fab-alkaline phosphatase conjugate was added to each well. After 18 hours incubation at 25° the bound enzyme conjugate was estimated by the addition of p-nitro-phenyl phosphate. After various incubation times at 37° the optical density was measured at 405 nm.

- ■ 5 hours incubation; conjugate control = 0.301 O.D.
- Δ 180 minutes incubation; conjugate control = 0.171 O.D.
- O 120 minutes incubation; conjugate control = 0.117 O.D.
- ▲ 90 minutes incubation; conjugate control = 0.085 O.D.
- ● 60 minutes incubation; conjugate control = 0.051 O.D.

Figure 5.13(b)

At 0.08 µg/ml of IgG1 used in the sandwich method, the time of the substrate incubation was plotted against its optical density.

FIGURE 5.13


a
4.0
3.6
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0.2
EXTINCTION (405nm) 37°C
5
1.25
0.312
0.078
0.019
IgG µG/ML
b
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
EXTINCTION (405nm) 37°C
90 120
180
300
TIME (MINS.)

concentration (e.g., PBS) containing a mild, non-ionic detergent such as Tween 20. The addition of protein (e.g., albumin) reduced non-specific binding to an even greater extent. Fig. 5.14 shows the relative effects of PBS + Tween and PBS + Tween + BSA20 on the ELISA; this latter diluent appears to increase the sensitivity to a significant extent. The results are in agreement with Labroy (1979), who used a similar technique. Initial saturation of the plates with various levels of albumin, as used by Ruitenberg et al. (1974), appeared to greatly reduce the specific binding of antibody (Figs. 5.15 and 5.16).

5.4.9 Controls

In all of the experiments discussed above which use the indirect ELISA for the estimation of antibody levels in serum or intestinal fluids, the following controls were performed.

(a) **Sample control:** Consists only of incubating (6 hours at 25°) serum or intestinal fluid with LPS-coated plates and then washing prior to addition of substrate. Such controls measure the effect of non-specifically bound molecules (e.g., alkaline phosphatase) which contribute to the extinction value of the test sample. The extinction value of the sample control, carried out at every dilution of sample used in the assay, was deducted from the extinction value obtained for each dilution of the test sample prior to plotting (as in Figs. 5.18, 5.19, 5.20).

(b) **Substrate control:** Consists only of substrate added directly to LPS-coated wells; such controls yield negligible extinction values and are used as a blank, being deducted from every extinction value obtained from a given plate.

Figure 5.14

The effect of the sample diluting buffer.

The serial dilutions of mouse anti-F885 intestinal fluids was made in PBS-Tween or PBS-Tween-BSA20 (PBS-Tween containing 2% BSA) were tested with F885 LPS coated plates. The anti-mouse α-chain-alkaline phosphatase conjugate 1:1000 was added. After 18 hours incubation at room temperature the substrate was added. The enzyme activity was measured after 180 minutes incubation.

- ● Diluted in PBS-Tween-BSA20.
- O Diluted in PBS-Tween.

FIGURE 5.14

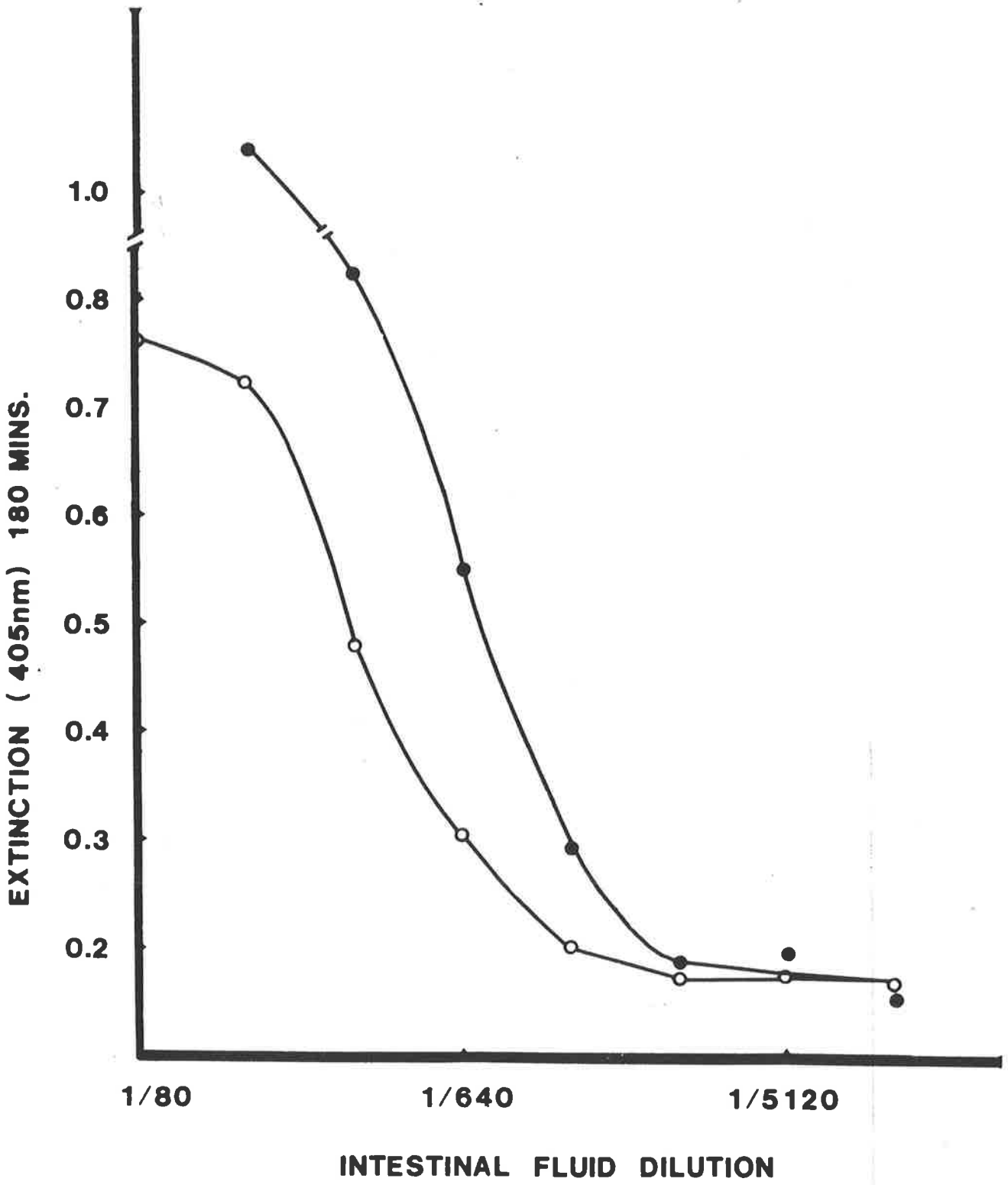

EXTINCTION (405nm) 180 MINS.
1.0
0.8
0.7
0.6
0.5
0.4
0.3
0.2
1/80
1/640
1/5120
INTESTINAL FLUID DILUTION

Figure 5.15

Attempts to decrease non-specific binding.

F885 LPS coated plates were incubated with 2% Bovine serum albumin (BSA) or 4% BSA or 5% Human serum albumin (HSA) for 1 hour at 37°. After the plates were washed, serial dilutions of mouse anti-F885 serum was added to each plate. The enzyme anti-mouse Fab conjugate 1:500 was added and incubated for 18 hours at 25°. The substrate was then added and incubated at 37° for 180 minutes before measuring the enzyme activity.

- ○ Without BSA.
- ● With 2% BSA.
- ▲ With 4% BSA.
- △ With 5% HSA.

FIGURE 5.15

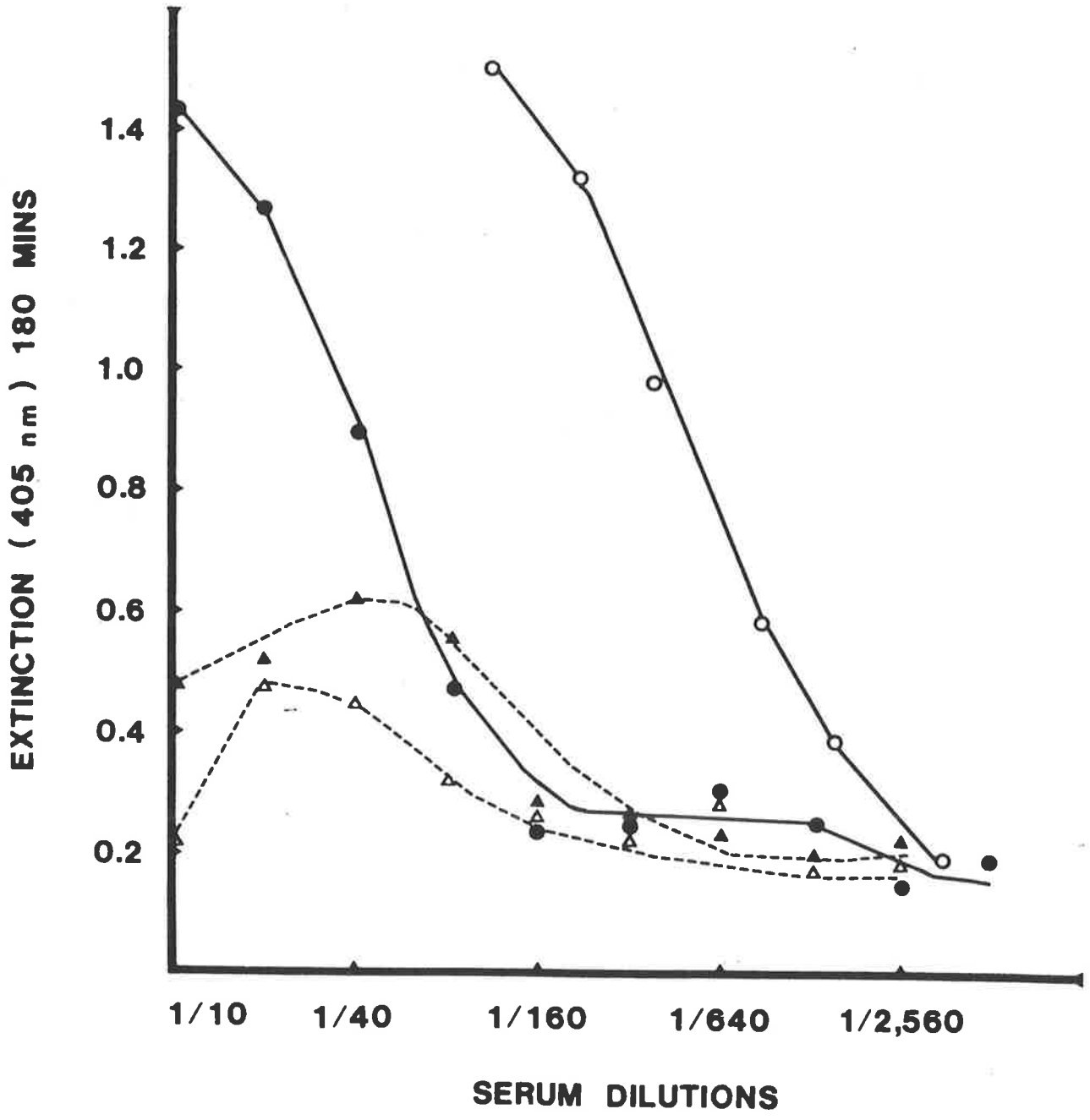

EXTINCTION (405 nm) 180 MINS
1.4
1.2
1.0
0.8
0.6
0.4
0.2
1/10
1/40
1/160
1/640
1/2,560
SERUM DILUTIONS

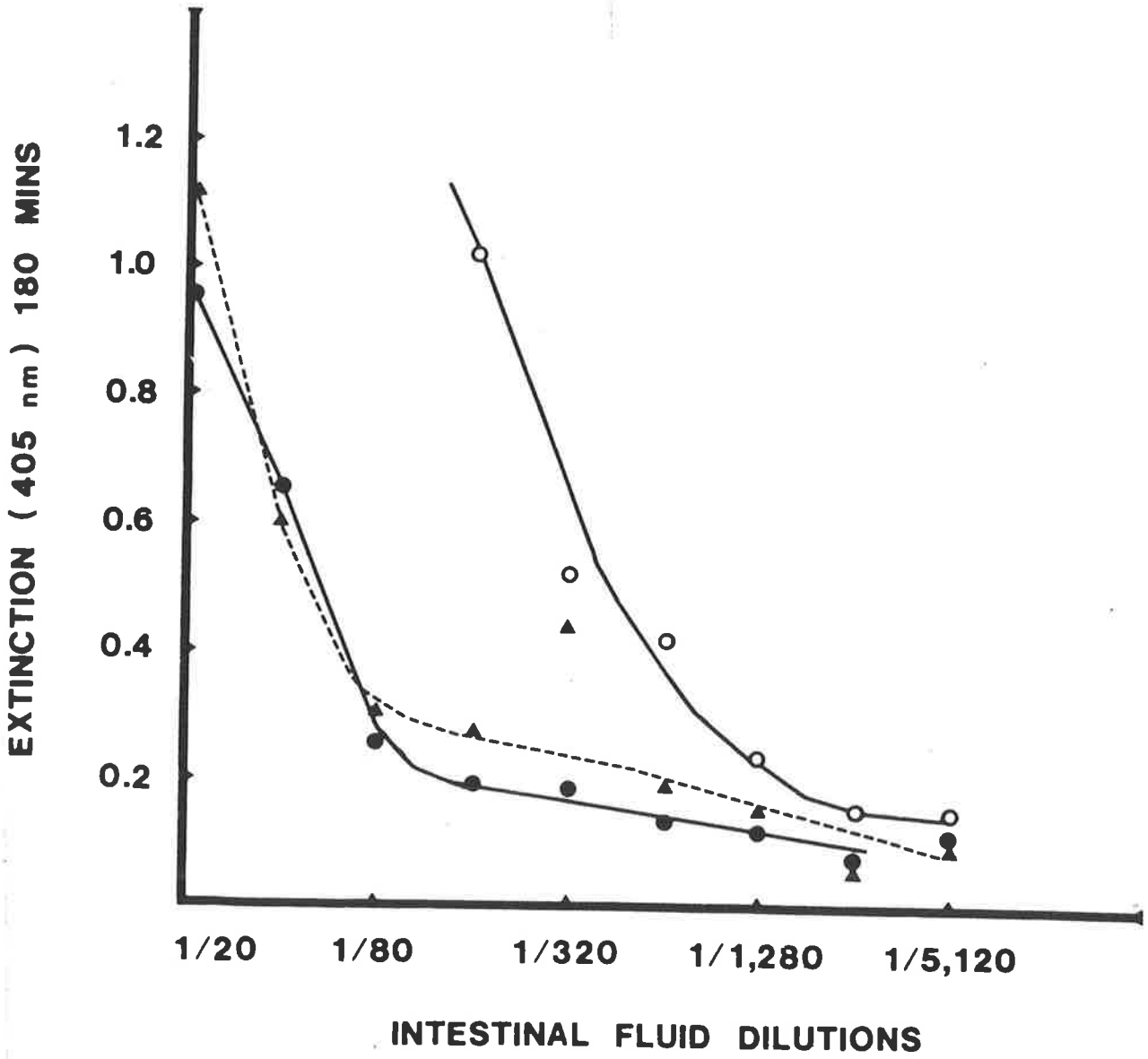
Figure 5.16

Attempts to decrease non-specific binding.

F885 LPS coated plates were incubated with 2% BSA or 4% BSA for 1 hour at 37°. After the plates were washed, serial dilutions of mouse anti-F885 intestinal fluid were added to each plate. The enzyme anti-mouse α-chain 1:1000 was added and incubated for 18 hours at 25°. The substrate was added and incubated for 180 minutes before measuring the enzyme activity.

- ○ Without BSA.
- ● With 2% BSA.
- ▲ With 4% BSA.

FIGURE 5.16


EXTINCTION (405 nm) 180 MINS
1.2
1.0
0.8
0.6
0.4
0.2
1/20
1/80
1/320
1/1,280
1/5,120
INTESTINAL FLUID DILUTIONS

(c) **Conjugate controls:** These consist of LPS-coated wells plus conjugate, and after incubation and washing substrate was added.

(d) **Negative controls:** Dilutions of conventionalized normal mouse serum and normal intestinal fluid samples were used.

5.5 Summary and Conclusions

The dedicated work of Pesce et al. (1977, 1978) has shown that antibody affinity for its antigen is the main factor which limits the sensitivity of immunoassays whether enzymic or isotopic. However, other limiting factors include the possible denaturing effect of the solid phase support on antibody or antigen as well as the volume of the assay solution.

Variations in the physico-chemical properties of plastic plates occur which markedly affect the binding of antigens or antibodies. Such variations may occur even between batches of the same product. Consequently, each batch of plates used must be tested with reference positive and negative sera prior to use. In this work, round-bottom polystyrene microtitre plates were used as described earlier. In the indirect ELISA to determine antibody, antigens were bound to the wells either by direct adsorption in the case of protein antigens or after complexing with methylated bovine serum albumin in the case of LPS. With both antigen types the optimal concentration for coating the plates was 5 μg/ml.

The glutaraldehyde coupled antibody-enzyme conjugates, prepared from purified antibodies, were highly specific and gave great sensitivity. The optimal working dilution of the goat anti-mouse α-chain enzyme conjugate was 1 in 1000 and for the rabbit anti-mouse Fab-enzyme conjugate 1 in 500. At these levels, extinction values

for conjugate controls were low and sensitivity was high (Figs. 5.5 and 5.6). The relative lack of sensitivity when determining IgM is thought to be due to the fact that the Fab fragments used to immunize the rabbits were obtained from mouse IgG1. Absolute quantitation of serum and intestinal antibodies is discussed in Chapter 6.

Incubation for 6 hours at room temperature was routinely used to complete the binding of serum or intestinal antibody to its antigen; this allowed low concentrations of antibody to become efficiently bound. The degree of binding over this period was similar at 4^{o} and at room temperature, but a significant decrease was observed at 37^{o}; this was thought to be due to detachment of antibody or antigen from the plates. Further, at 37^{o}, the effect of intestinal enzymes on the system would be most apparent; at 4^{o} and at room temperature, the binding system appeared to be totally unaffected by intestinal fluid enzymes or contents such as bile salts.

On the other hand, it was found that, at 37^{o}, maximum binding of enzyme conjugate to antibody occurred between 2 and 4 hours. At 25^{o} (or room temperature), maximum binding was attained over this same time period with relatively high concentrations of antibody, but 6 to 8 hours was needed with low concentrations. For practical purposes, therefore, overnight incubation at room temperature was routinely used for conjugate binding.

The degree of hydrolysis of the chromogenic substrate was directly proportional to the incubation time. It was found that incubation at 37^{o} for 180 minutes gave low backgrounds and high sensitivity and this was generally used. We have also compared the sensitivities of immunosorbent assay systems employing enzymic markers and isotopic markers. Here, we measured rabbit serum antibody against

Salmonella derby. The results of such comparison, using polystyrene plates coated with homologous LPS using I^{125} labelled and alkaline phosphatase conjugated goat anti-mouse light chain, respectively, are shown in Fig. 5.17. If end-points are compared equivalent to 10% of the maximum binding for the solid phase RIA and an extinction value of 0.2 for the ELISA, then these two assays give comparable serum antibody titres.

Figure 5.17

Comparison of antibody quantitation using the ELISA and the solid phase RIA (SRIA) techniques.

The polystyrene plates were coated with S. derby LPS*, 5 µg/ml for 3 hours at 37°, followed by 18 hours at 4°. To these was added, in 2-fold serial dilution, mouse anti-S. derby serum**. After incubation and washing, goat anti-mouse immunoglobulin I^{125} conjugate was used for the SRIA≠. The bound I^{125} was measured in a gamma-counter; the bound alkaline phosphatase was measured by incubating with the enzyme substrate.

ELISA controls:

Conjugate control = 0.03 O.D.

Sample control = 0.1 O.D.

SRIA control:

Antigen control = 51 cpm (LPS coated plate + I^{125} conjugate).

* The LPS was complexed with methylated BSA.

** Four oral doses of $1x10^9$ organisms twice weekly, followed by $1x10^6$ i.p. boosting dose.

Insert:
≠ The goat anti-mouse immunoglobulin conjugate with alkaline phosphatase was used in the ELISA.

FIGURE 5.17

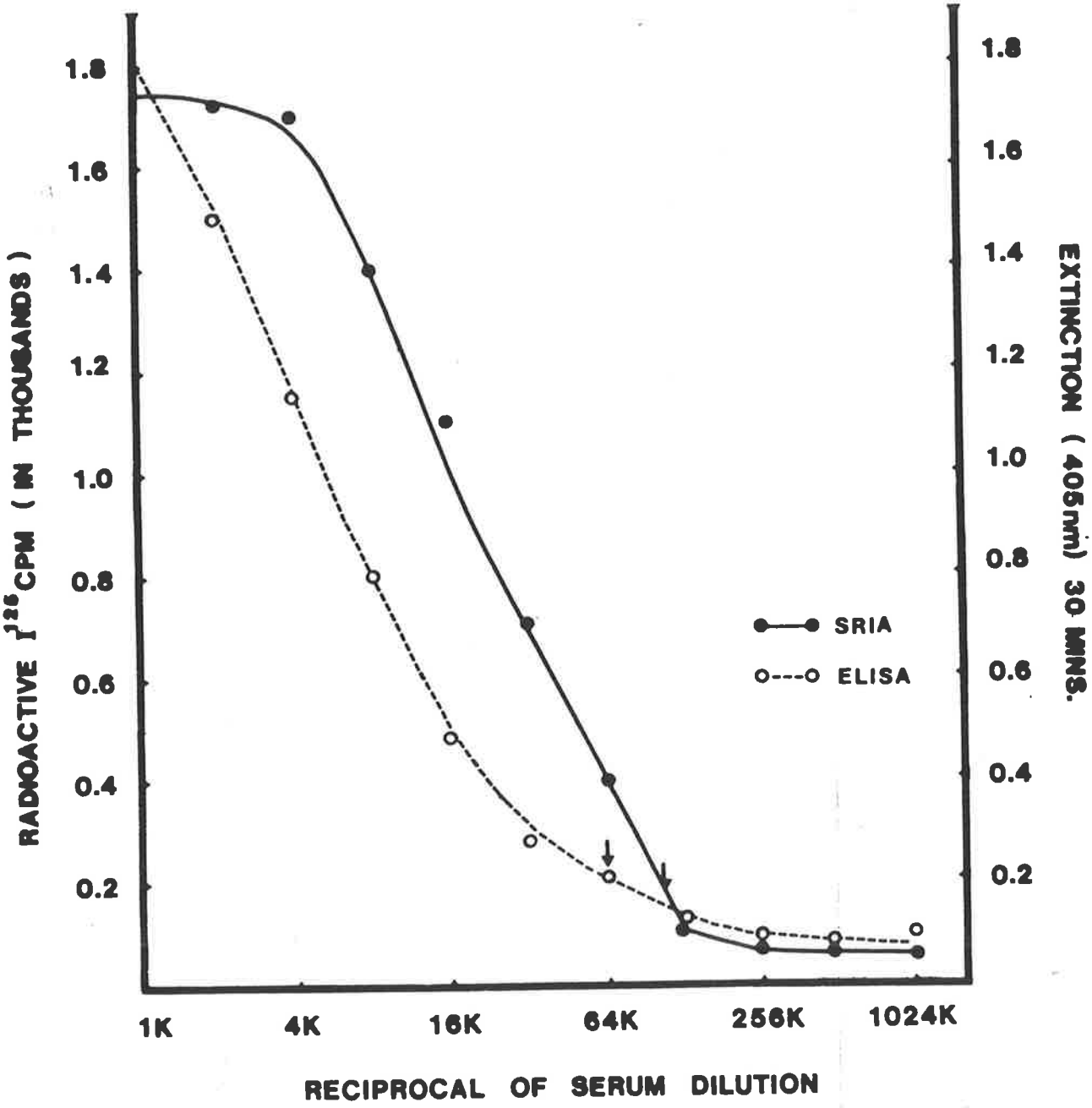

RADIOACTIVE I^{125} CPM (IN THOUSANDS)
EXTINCTION (405nm) 30 MINS.
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
SRIA
ELISA
1K
4K
16K
64K
256K
1024K
RECIPROCAL OF SERUM DILUTION

Figure 5.18

Typical titration curve for the assay of serum antibody by the indirect ELISA (using anti-mouse Fab-alkaline phosphatase conjugate).

The polystyrene plate was coated with F885 LPS, 5 µg/ml for 3 hours at 37°. Two-fold serial dilutions of hyperimmune mouse anti-F885 serum* were tested with the F885 LPS coated plate. After incubation the enzyme anti-mouse Fab 1:500 was added. The bound conjugate was measured after 18 hours incubation at room temperature by adding the substrate.

Hyperimmune serum. ○

Normal mouse serum. ●

Conjugate control. ▲

Every extinction value plotted for each dilution of both serum was the difference between the extinction value of a given dilution of test sample and its corresponding sample control.

* Three oral doses of 1×10^9 organisms at weekly intervals.

FIGURE 5.18

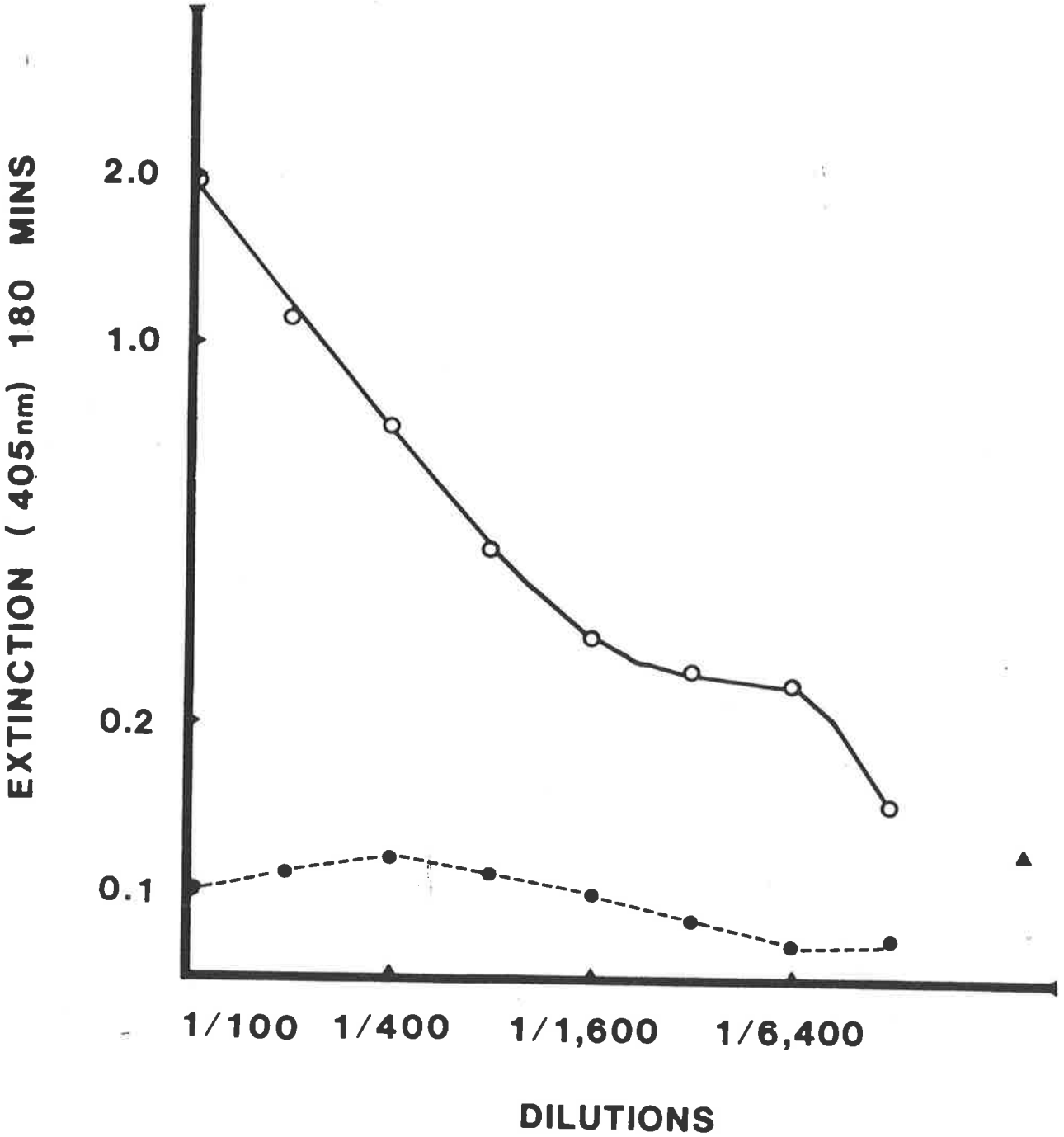

EXTINCTION (405nm) 180 MINS
2.0
1.0
0.2
0.1
1/100
1/400
1/1,600
1/6,400
DILUTIONS

Figure 5.19

Typical titration curve for the assay of serum antibody by the indirect ELISA (using anti-mouse α-chain alkaline phosphatase conjugate).

The same procedure was adopted as described in Figure 5.18.

Mouse anti-F885 serum. O

Normal mouse serum. ●

Conjugate control. ▲

Every extinction value plotted for each dilution of both serum was the difference between the extinction value of a given dilution of test sample and its corresponding sample control.

FIGURE 5.19

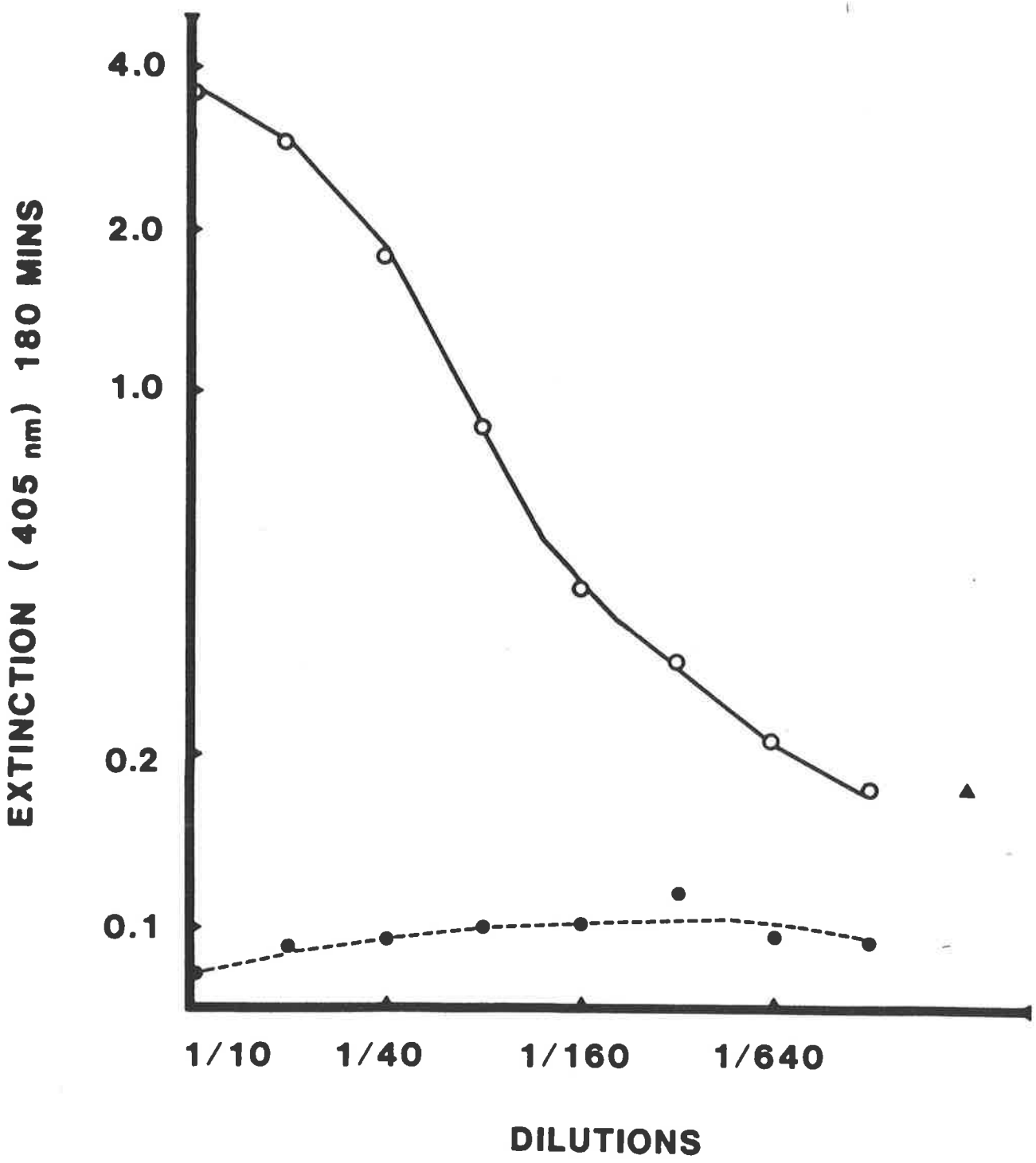

4.0
2.0
1.0
0.2
0.1
EXTINCTION (405 nm) 180 MINS
1/10
1/40
1/160
1/640
DILUTIONS

Figure 5.20

Typical titration curve for the assay of intestinal antibody tested by the indirect ELISA (using anti-mouse α-chain alkaline phosphatase conjugate).

The polystyrene plate was coated with F885 LPS, 5 μg/ml for 3 hours at 37°. Two-fold serial dilutions of mouse anti-F885 intestinal fluid* washings were incubated with the antigen coated plate. Enzyme anti-mouse α-chain 1:1000 was then added. The bound enzyme activity was determined after 18 hours incubation at room temperature by adding the substrate.

Mouse anti-F885 intestinal fluid. O

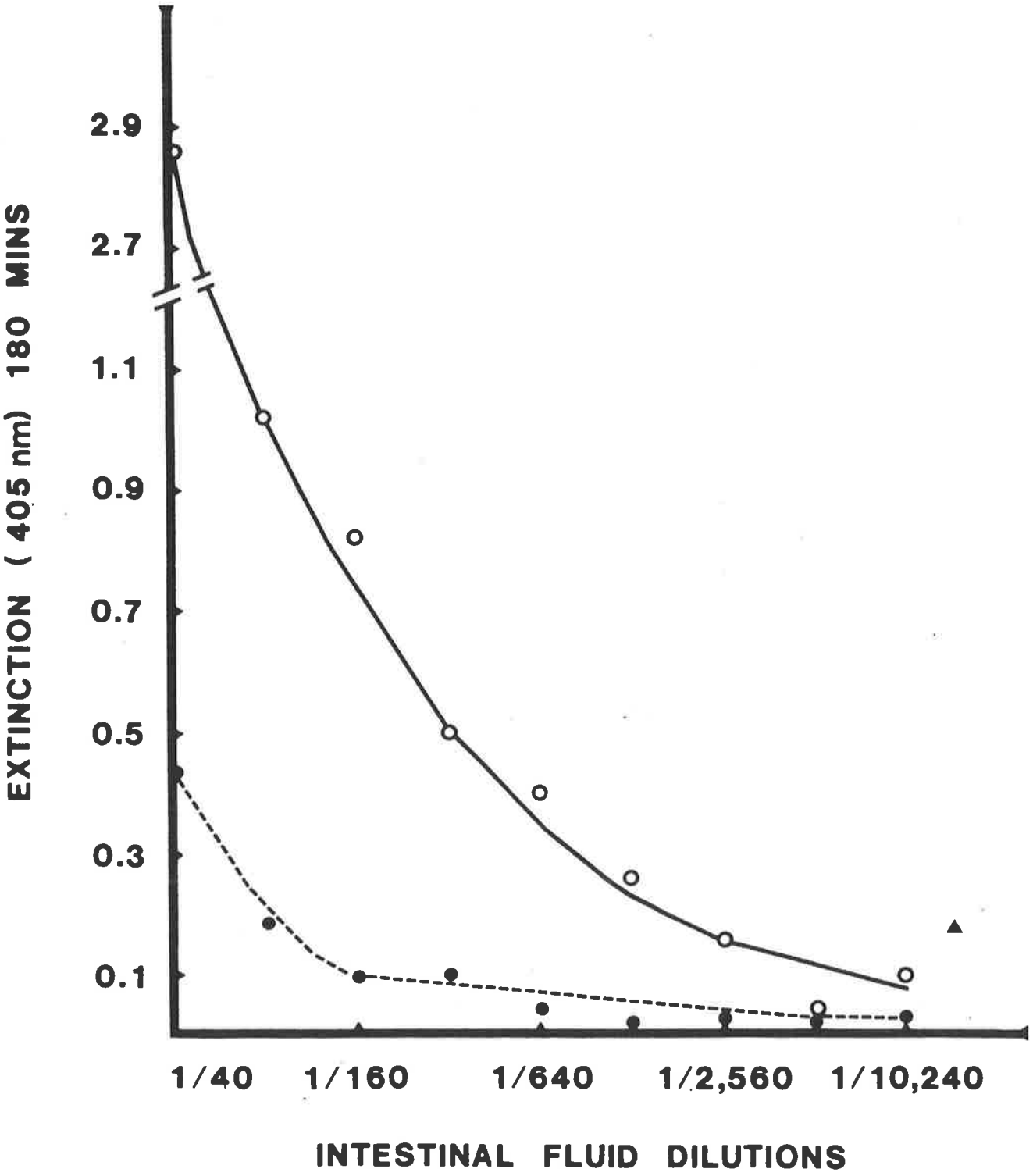
Normal mouse intestinal fluid. ●

Conjugate control. ▲

Every extinction value plotted for each dilution of both intestinal fluid washing was the difference between the extinction value of a given dilution of test sample and its corresponding sample control.

* Three oral doses of 1×10^9 organisms at weekly intervals.

FIGURE 5.20


EXTINCTION (405 nm) 180 MINS
2.9
2.7
1.1
0.9
0.7
0.5
0.3
0.1
1/40
1/160
1/640
1/2,560
1/10,240
INTESTINAL FLUID DILUTIONS

CHAPTER 6

The Correlation Between IgA Levels and Resistance to Mouse Typhoid After Oral Immunization with Various *Salmonellae*

Introduction

As discussed in Chapter 2, the antibody levels in the serum do not necessarily bear any relationship to those levels found at secretory surfaces such as the intestine. The independence of the mucosal (local) immune response has been firmly established with the concept of the secretory immune system containing predominantly secretory IgA (sIgA) that is synthesized in local plasma cells (Tomasi et al., 1965; Tomasi and Bienenstock, 1968). A specialized sIgA molecule in secretions provided an explanation for the long-standing observation that specific immunological resistance to mucosal infection could exist in the absence of demonstrable serum antibody, and there is much evidence for the local synthesis of secretory IgA in various mucous membranes and glands. In such tissues there are an abundance of plasma cells synthesizing IgA and these predominate over plasma cells producing other classes of immunoglobulin (Crabbé et al., 1965, 1970; Tomasi et al., 1965; Crabbé and Heremans, 1966; Brandtzaeg et al., 1967; Tourville et al., 1969; Nash et al., 1969; Franklin et al., 1973; Bienenstock et al., 1973).

Besredka (1927) originally postulated the primary role of mucosal (local) immune mechanisms in protection against enteric bacterial infections over 50 years ago. Since then, however, in spite of many studies, the mechanisms of mucosal immunity in the intestine following

oral challenge have not been fully elucidated. Burrows et al. (1947, 1950) and Burrows and Havens (1948), studying experimental cholera, clearly demonstrated that intestinal antibody, after local immunization, is not merely a transudate of serum antibody, as it appeared sooner, declined earlier and correlated better with resistance to intestinal infection. Studies in humans have also suggested that local antibodies are more important for protective immunity in cholera than are serum antibodies (Freter et al., 1965). A correlation was also found between the synthesis of specific intestinal IgA and the protection of mice against experimental cholera (Svennerholm et al., 1978), and Du Pont et al. (1972) showed that oral immunization with live attenuated Shigellae gave man protection against bacillary dysentery. Nevertheless, the protective role of sIgA antibody is best documented in the case of viral disease. Local immunity against enteric and respiratory viruses has been reviewed respectively by Ogra and Karzon (1971) and Rossen et al. (1971).

6.1 Protection Against Salmonellosis

There have been many demonstrations that animals and man can be effectively protected against some Salmonella infections by oral immunization with live Salmonellae.

A recent example is provided by the trials in Egyptian children vaccinated orally with the epimeraseless mutant, Ty 21a strain, of S. typhi (Germanier and Fürer, 1975) which afforded excellent protection against typhoid (Wahdan et al., 1982). Similarly, mice can be protected against S. typhimurium challenge by previous feeding of a variety of Salmonella strains, none of which need to be antigenically related in terms of their O-antigens (Moser et al., 1980).

The role of cellular and/or humoral factors in immunity to Salmonellosis has long been debated. Since Salmonellae are ubiquitous human pathogens, however, an understanding of events during the immune response is of great importance in the development of truly effective vaccines. Recent publications (Moser et al., 1980; Maneerashapisal and Rowley, 1981) suggest that intestinal immunity is essentially cellular in character, and in this kind of immunity the T cells of the recipients must be sensitized to some unidentified bacterial antigens so that a later contact with these antigens results in a rapid liberation from these T cells of effective mediator substances (e.g., lymphokines), which activate the local macrophages in ways which enable them to kill the invading pathogens (Mackaness, 1969). The Salmonella strains which are best at inducing resistance are those which colonize the animal for a prolonged period without inducing disease. The early events which lead to such resistance appear to take place in the Peyer's patches of the small intestine, and within a few days of oral vaccination this lymphoid tissue acquires a much larger population of macrophages which have the anti-bacterial properties of activated cells (Maneerashapisal and Rowley, 1981, and in the press). This local cellular immunity can be rapidly recalled many months later by oral challenge with live Salmonella vaccines.

Whilst accepting that induced resistance to enteric infection is mainly due to local cellular factors, it is interesting that the factors required to initiate this immunity and those required to initiate IgA antibody formation both reside in the Peyer's patches of the small intestine. Both require penetration of the organism or antigen into this local lymphoid tissue. For example, delayed-type

hypersensitivity (DTH) develops generally following oral administration of Salmonella vaccines, and IgA antibody can be detected in most secretory fluids and in serum following local intestinal stimulation (Collins and Carter, 1974; Moser et al., 1980). The question then arises as to whether these two immuno-competent pathways are usually or always activated together and whether a rise in serum IgA can be used as an indication that local cellular immunity has also been activated. At the moment, it is not possible to measure cellular immunity in the intestine of man under normal conditions, whereas it is possible to obtain secretory fluids or serum for IgA antibody measurement. The purpose of this study, therefore, is to determine whether Salmonella strains which induce protection against S. typhimurium infection in mice following oral immunization also induce IgA antibodies against the lipopolysaccharide of the various strains and whether the levels of serum IgA or secretory IgA are positively correlated with such protection.

6.2 Colonization of Various Strains of Salmonellae in the Peyer's Patches of Mice After Oral Immunization

Following oral infection with S. enteritidis, Collins and Carter (1974) observed the primary focus of infection was in the Peyer's patches of the ileum. The infection then spread quickly to the liver and spleen. Hohmann et al. (1978) found that only virulent strains of Salmonellae can persist and colonize the Peyer's patches of the small intestine after oral feeding. It would seem that a most essential step in the pathogenesis of, or immunity to, typhoid fever in mice or humans is the establishment of a systemic infection after oral challenge (Hohmann et al., 1978). This systemic infection

usually develops from foci established in the small intestinal Peyer's patches. Thus, in order to study immunity to Salmonella infection, one needs to compare the effects produced by strains which do persist in the Peyer's patches of mice with those that do not. We have, therefore, tested 14 strains of Salmonellae, each belonging to different O-antigenic groups, for their ability to persist in and to colonize the Peyer's patches of the small intestine.

These strains were administered once orally in a dose of $1x10^9$ organisms to each member of groups of the LAC strain of mice. At days 1, 2, 3, 4, and 7 or 8 or 9 after feeding, the Peyer's patches were removed from a group of 6 mice. The organisms that could be recovered from the patches were determined by plating out on solid media. Figs. 6.1a, b and c show the results of such experimentation.

Table 6.1 summarizes the persistence of the immunizing strains in the Peyer's patches. The results confirm that there appears to be no correlation between O-antigen components and ability to colonize the patches; this was previously found by Hohmann et al. (1978). For example, strains of the same serotype (1, 4, 5 and 12), namely, S. stanley, S. derby, S. typhimurium M206 and E. coli F1142 show different abilities in colonizing and persisting in the Peyer's patches.

6.3 Serum and Intestinal Antibody Levels After One Oral Feeding with Various Peyer's Patch Persistent and Non-persistent Salmonella Strains

As seen in section 6.2, the strains of Salmonellae used in this study fall basically into two groups: those which persist in Peyer's

Figure 6.1(a)

Persistence in the small intestinal Peyer's patches.

The number of Salmonella organisms and hybrid strains recovered from the Peyer's patches of the small intestine at specified times following feeding of 10^9 organisms to LAC mice. Each point represents the mean number of organisms recovered from 5 to 6 mice.

- ● *S. typhimurium* F885.
- ▲ *S. salford*.
- ■ *S. bonariensis*.
- □ *S. budapest*.
- ○ *E. coli* F1142.
- △ *S. humber*.

FIGURE 6.1a

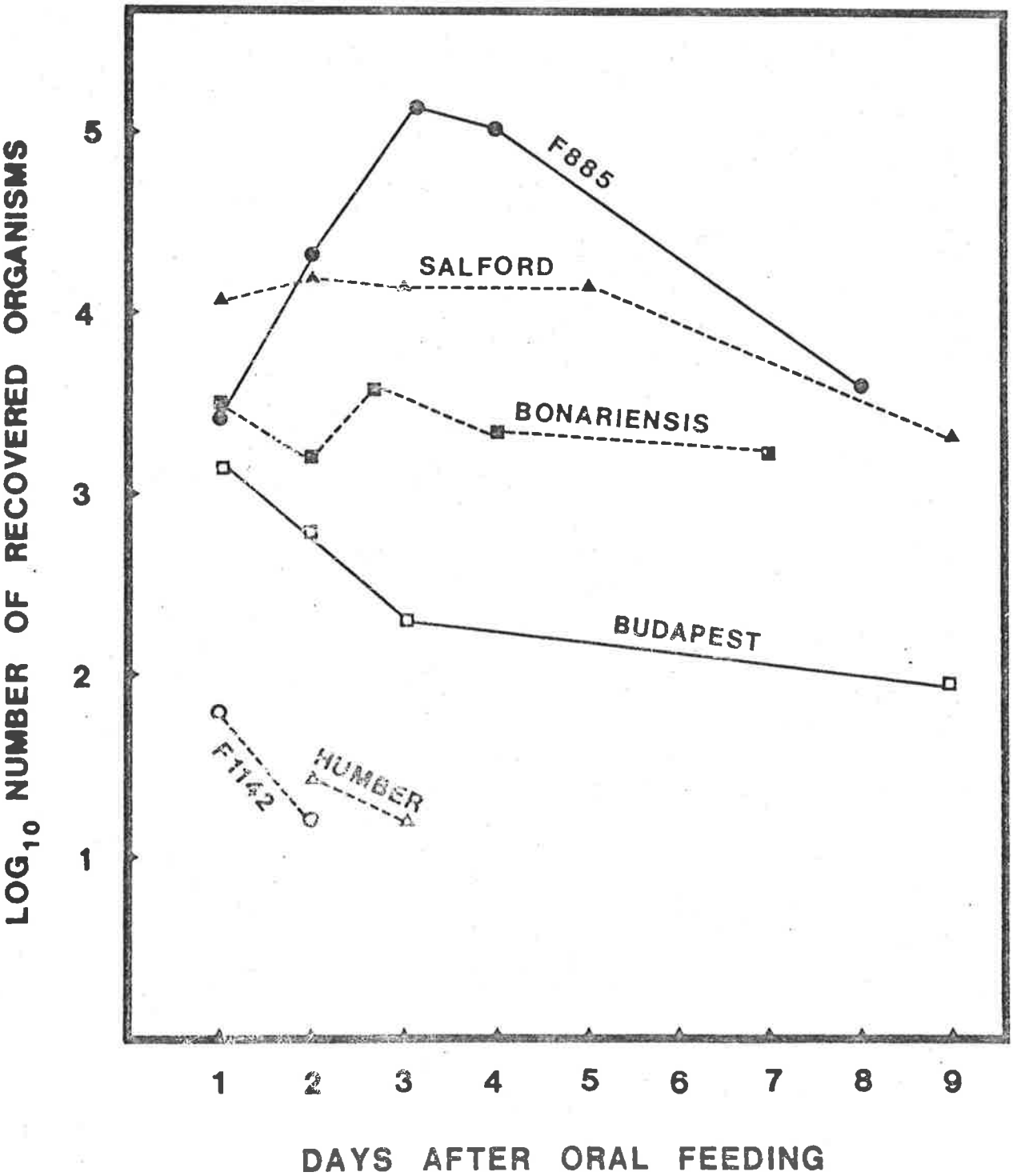

LOG10 NUMBER OF RECOVERED ORGANISMS
5
4
3
2
1
F885
SALFORD
BONARIENSIS
BUDAPEST
F1142
HUMBER
1
2
3
4
5
6
7
8
9
DAYS AFTER ORAL FEEDING

Figure 6.1(b)

Persistence in the small intestinal Peyer's patches.

The number of Salmonella organisms recovered from the Peyer's patches of the small intestine at specified times following feeding of 10^9 organisms to LAC mice. Each point represents the mean number of organisms recovered from 5 to 6 mice.

- ● S. derby.
- ▲ S. fridenau.
- ■ S. chester.
- O S. enteritidis 11RX.

FIGURE 6.1b

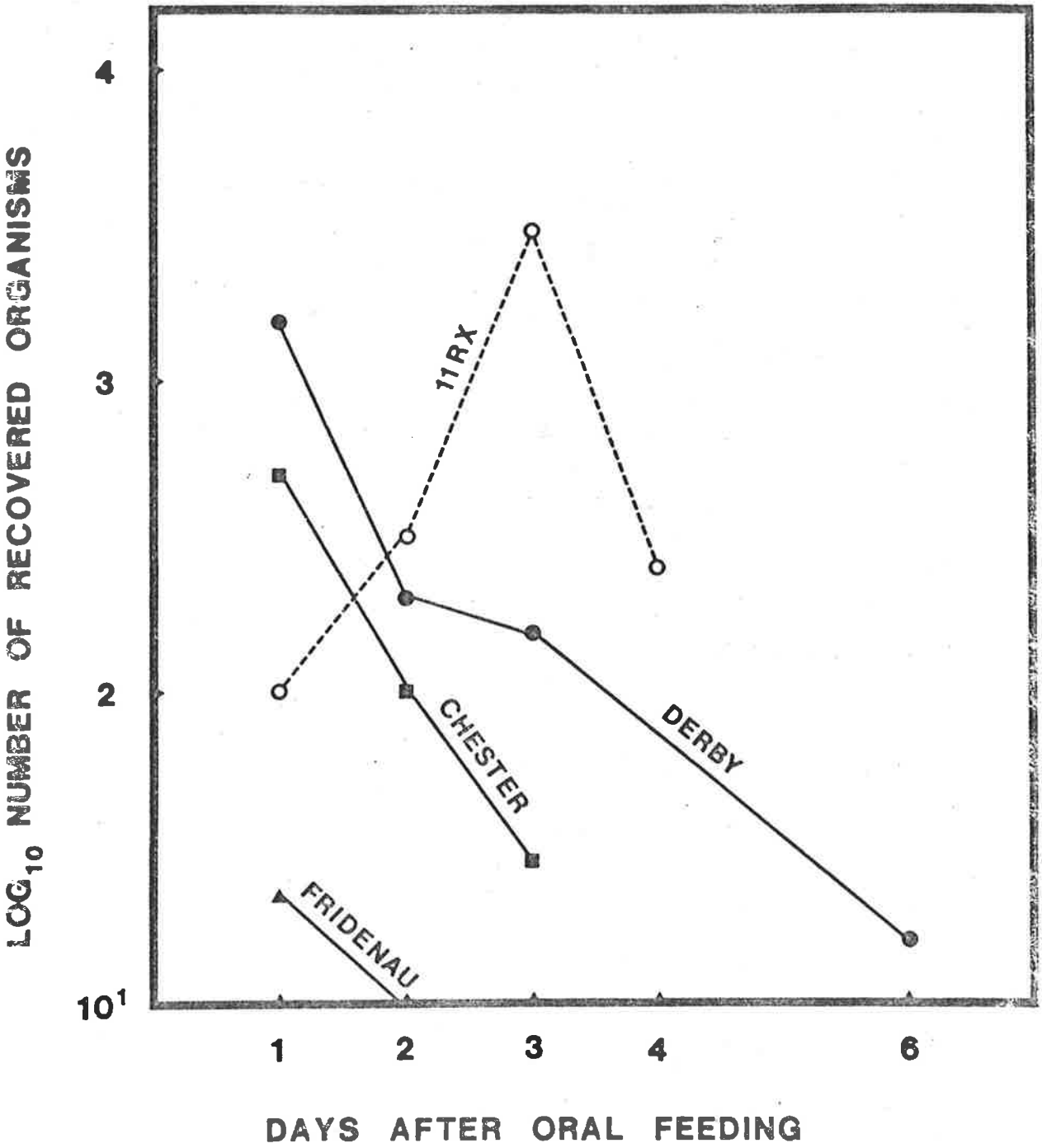

LOG10 NUMBER OF RECOVERED ORGANISMS
4
3
2
10¹
11RX
CHESTER
DERBY
FRIDENAU
1
2
3
4
6
DAYS AFTER ORAL FEEDING

Figure 6.1(c)

Persistence in the small intestinal Peyer's patches.

The number of Salmonella organisms recovered from the Peyer's patches of the small intestine at specified times following feeding of 10^9 organisms to LAC mice. Each point represents the mean number of organisms recovered from 5 to 6 mice.

- ● S. strasbourg.
- ▲ S. stanley.
- △ S. kirkee.
- O S. typhimurium M206.

FIGURE 6.1C

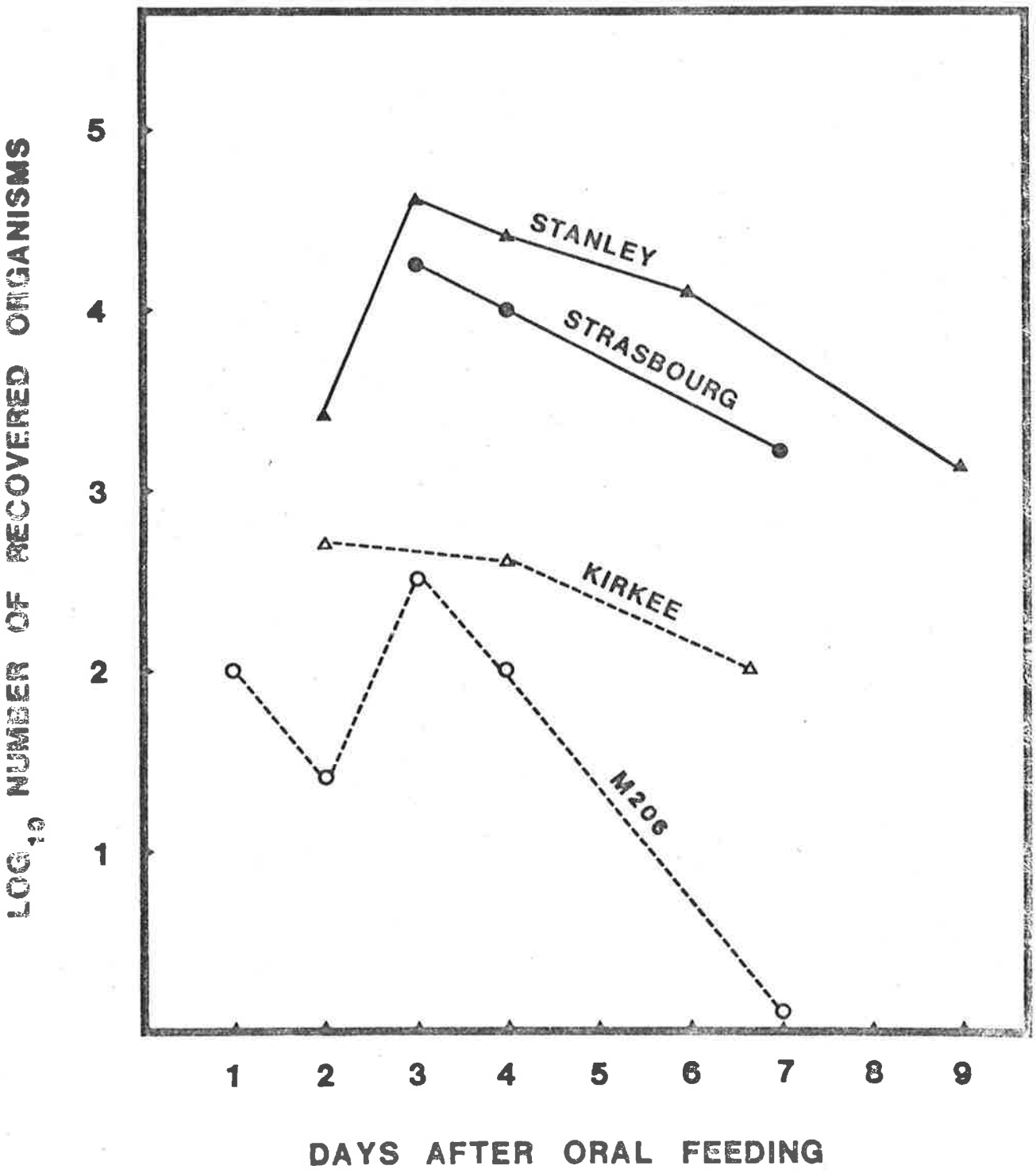

LOG10 NUMBER OF RECOVERED ORGANISMS
5
4
3
2
1
STANLEY
STRASBOURG
KIRKEE
M206
1
2
3
4
5
6
7
8
9
DAYS AFTER ORAL FEEDING

Table 6.1

Persistence of Salmonellae in the

Peyer's patches of the small intestine.

Immunizing strains	'O' somatic antigens	Persistence in the Peyer's patches
S. typhimurium F885	E. coli 'O' 8	+ [a]
S. bonariensis	6, 8	+
S. salford	16	+
S. stanley	1, 4, 5, 12	+
S. strasbourg	(9), 46	+
S. kirkee	17	± [b]
S. enteritidis 118X	1, 9, 12	±
S. budapest	1, 4, 12	±
S. typhimurium M206	1, 4, 5, 12	- [c]
S. fridenau	13, 22	-
S. derby	1, 4, 5, 12	-
S. chester	4, 5, 12	-
S. humber	53	-
E. coli F1142	Salmonella 'O' 1, 4, 5, 12	-

a = More than 10^3 organisms recovered at day 7 after oral administration.

b = Approximately 10^2 organisms or less recovered at day 7.

c = Less than 10^2 organisms recovered at day 1, and the number declined rapidly.

patches and those which do not. We became interested to see whether a local immune response, such as an increase in intestinal IgA levels, was concomitant with persistence in Peyer's patches.

Thus, groups of 50 mice were immunized orally, each mouse receiving $1x10^9$ organisms, one of the 14 Salmonella strains being used per group. Twenty-one days after immunization, 20 to 25 mice were removed from each group, killed, and their serum and intestinal juice were collected. The serum antibodies and the intestinal antibodies directed against their homologous antigens were assayed by the indirect ELISA technique. Homologous lipopolysaccharide (LPS) was used as the antigen in each case. The results (Fig. 6.2) show that S. salford (Fig. 6.2a), a persistent strain, was able to induce high serum levels of all immunoglobulin classes, including IgA, and also of intestinal sIgA. On the other hand, the non-persistent strains, such as S. humber (Fig. 6.2b), developed only low serum antibody of any immunoglobulin class and low intestinal sIgA. The results from other strains are shown in Table 6.2. These results were obtained as specific antibody titres directed against homologous LPS. There was no cross-reaction between the antibodies induced by the various strains, as shown by the data of Fig. 6.3, where the serum and intestinal fluids collected from S. bonariensis immunized mice (Fig. 6.3a) and S. salford immunized mice (Fig. 6.3b) showed no significant extinction value when tested against S. typhimurium F885 LPS, with either anti-mouse Fab conjugate or anti-mouse α-chain conjugate. Further, when an outer-membrane protein preparation obtained from S. typhimurium C5 was used as the coating antigen in this ELISA technique, negligible amounts of serum IgA or intestinal IgA were found in the samples collected from mice immunized by any

Figure 6.2(a)

Anti-S. salford antibodies after one oral immunization.

1. ELISA Ab titration curves of total anti-S. salford antibodies (●—●) and IgA anti-S. salford antibodies (▲--▲) directed against S. salford LPS recovered from serum of the mice immunized with live S. salford. Anti-mouse Fab-alkaline phosphatase conjugate was used for detecting total Ab in serum, while anti-mouse α-chain-alkaline phosphatase conjugate was used for detecting IgA Ab.

■—■ Total anti-S. salford Ab recovered from normal serum.

□--□ IgA anti-S. salford Ab recovered from normal serum.

△ Anti-mouse α-chain conjugate control.

○ Anti-mouse Fab conjugate control.

2. ELISA Ab titration curves of sIgA anti-S. salford antibodies (●—●) directed against S. salford LPS recovered from the intestinal fluid washings of the mice immunized with live S. salford.

○--○ Normal mouse intestinal fluid washing.

△ Conjugate control.

FIGURE 6.2a

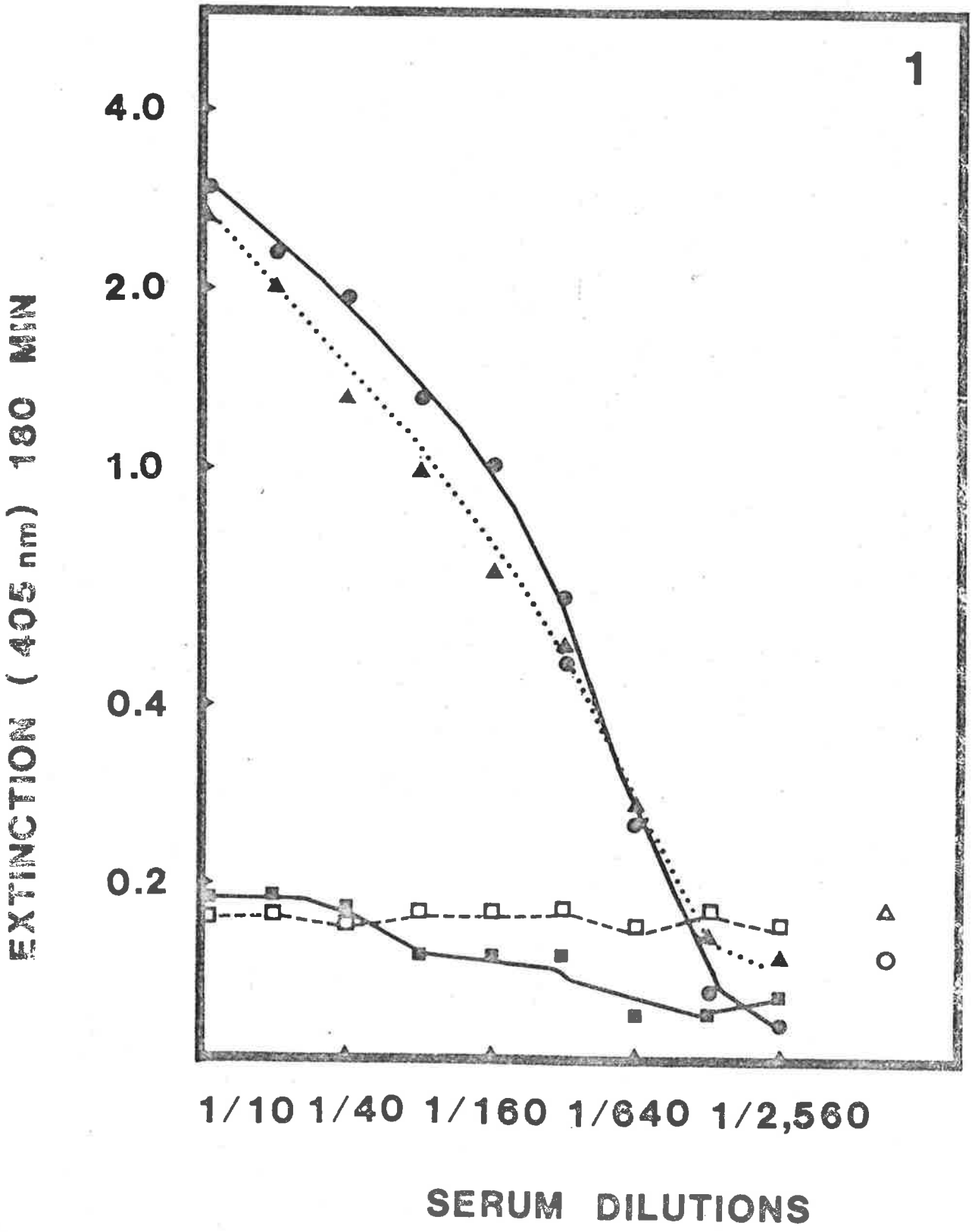

1
4.0
2.0
1.0
0.4
0.2
EXTINCTION (405 nm) 180 MIN
1/10 1/40 1/160 1/640 1/2,560
SERUM DILUTIONS

FIGURE 6.2a

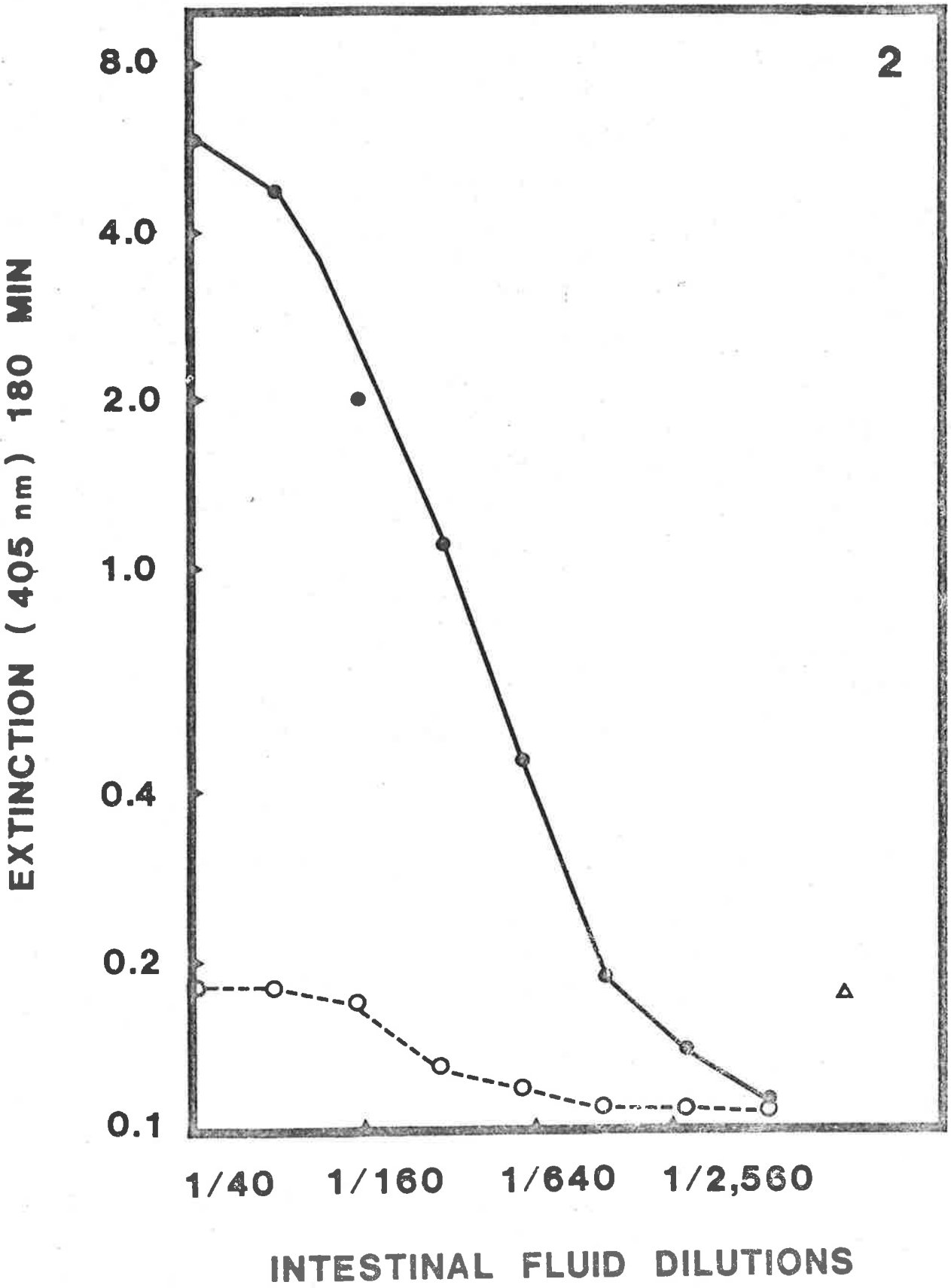

2
8.0
4.0
2.0
1.0
0.4
0.2
0.1
EXTINCTION (405 nm) 180 MIN
1/40
1/160
1/640
1/2,560
INTESTINAL FLUID DILUTIONS

Figure 6.2(b)

Anti-*S. humber* antibody after one oral immunization.

1. ELISA Ab titration curves of total anti-*S. humber* antibodies (●—●) and IgA anti-*S. humber* antibodies (▲- - -▲) directed against *S. humber* LPS recovered from serum of the mice immunized with live *S. humber*.

■—■ Total anti-*S. humber* Ab from normal serum.

□- -□ IgA anti-*S. humber* Ab from normal serum.

△ Anti-mouse α-chain conjugate control.

○ Anti-mouse Fab conjugate control.

2. ELISA Ab titration curves of sIgA anti-*S. humber* antibodies (●—●) directed against *S. humber* LPS recovered from the intestinal fluid washings of the mice immunized with live *S. humber*.

○- -○ Normal mouse intestinal fluid washings.

△ Anti-mouse α-chain conjugate control.

FIGURE 6.2b

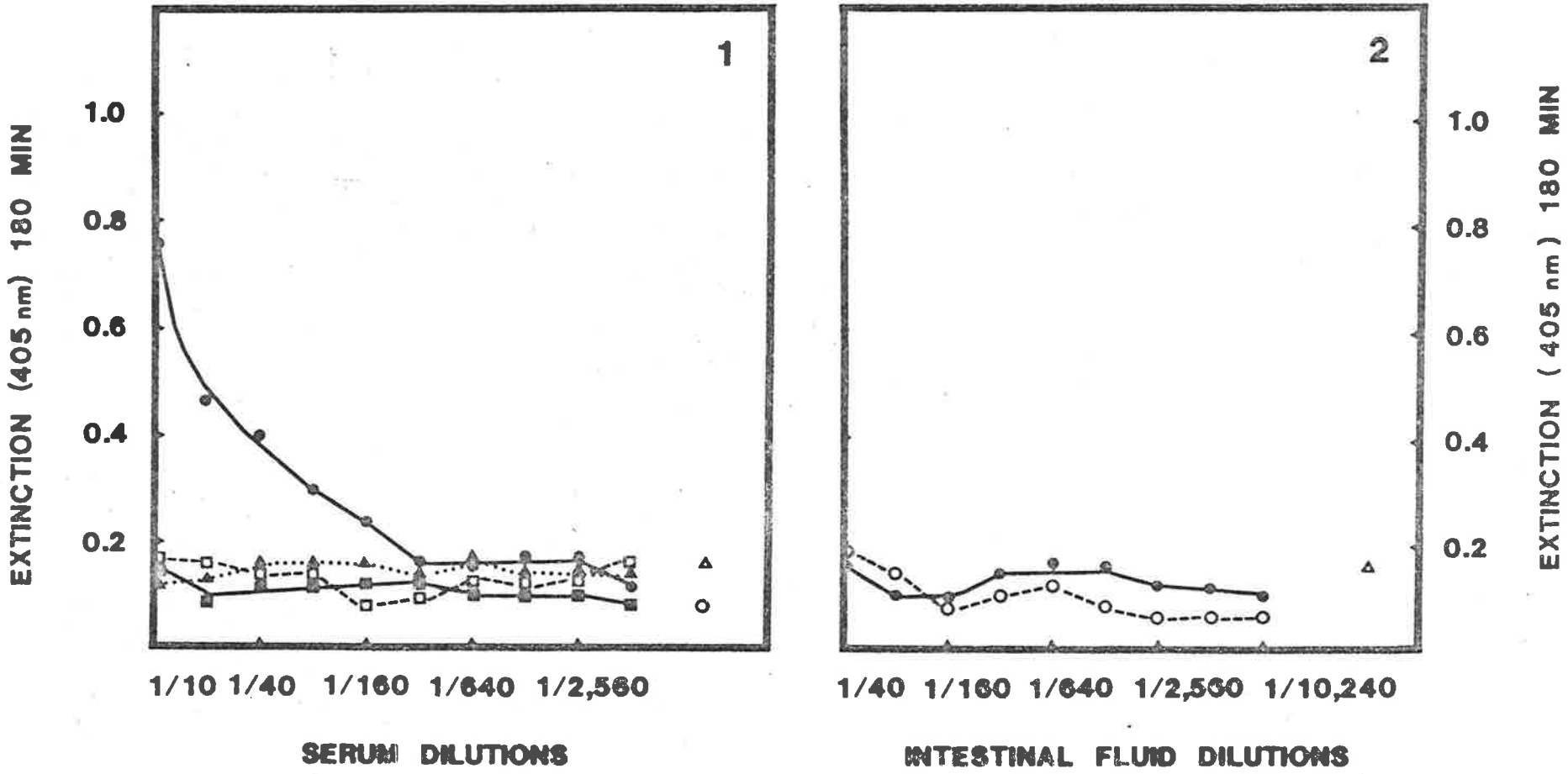

1
2
EXTINCTION (405 nm) 180 MIN
1.0
0.8
0.6
0.4
0.2
1/10 1/40 1/160 1/640 1/2,560
SERUM DILUTIONS
1/40 1/160 1/640 1/2,560 1/10,240
INTESTINAL FLUID DILUTIONS

Figure 6.3(a)

Cross-reactions among the anti-Salmonella antibodies.

Anti-S. bonariensis antibody serum was tested for antibody activities directed against S. typhimurium F885 LPS, using anti-mouse Fab-alkaline phosphatase conjugate (O--O) and anti-mouse α-chain-alkaline phosphatase conjugate (Δ###Δ), and against its homologous (S. bonariensis) LPS, using anti-mouse Fab-alkaline phosphatase conjugate (●—●) and anti-mouse α-chain-alkaline phosphatase conjugate (▲--▲).

Figure 6.3(b)

Anti-S. salford antibody serum was tested for antibody activities directed against S. typhimurium F885 LPS, using anti-mouse Fab-alkaline phosphatase conjugate (O--O) and anti-mouse α-chain-alkaline phosphatase conjugate (Δ###Δ), and against its homologous (S. salford) LPS, using anti-mouse Fab-alkaline phosphatase conjugate (●—●) and anti-mouse α-chain-alkaline phosphatase conjugate (▲--▲).

FIGURE 6.3

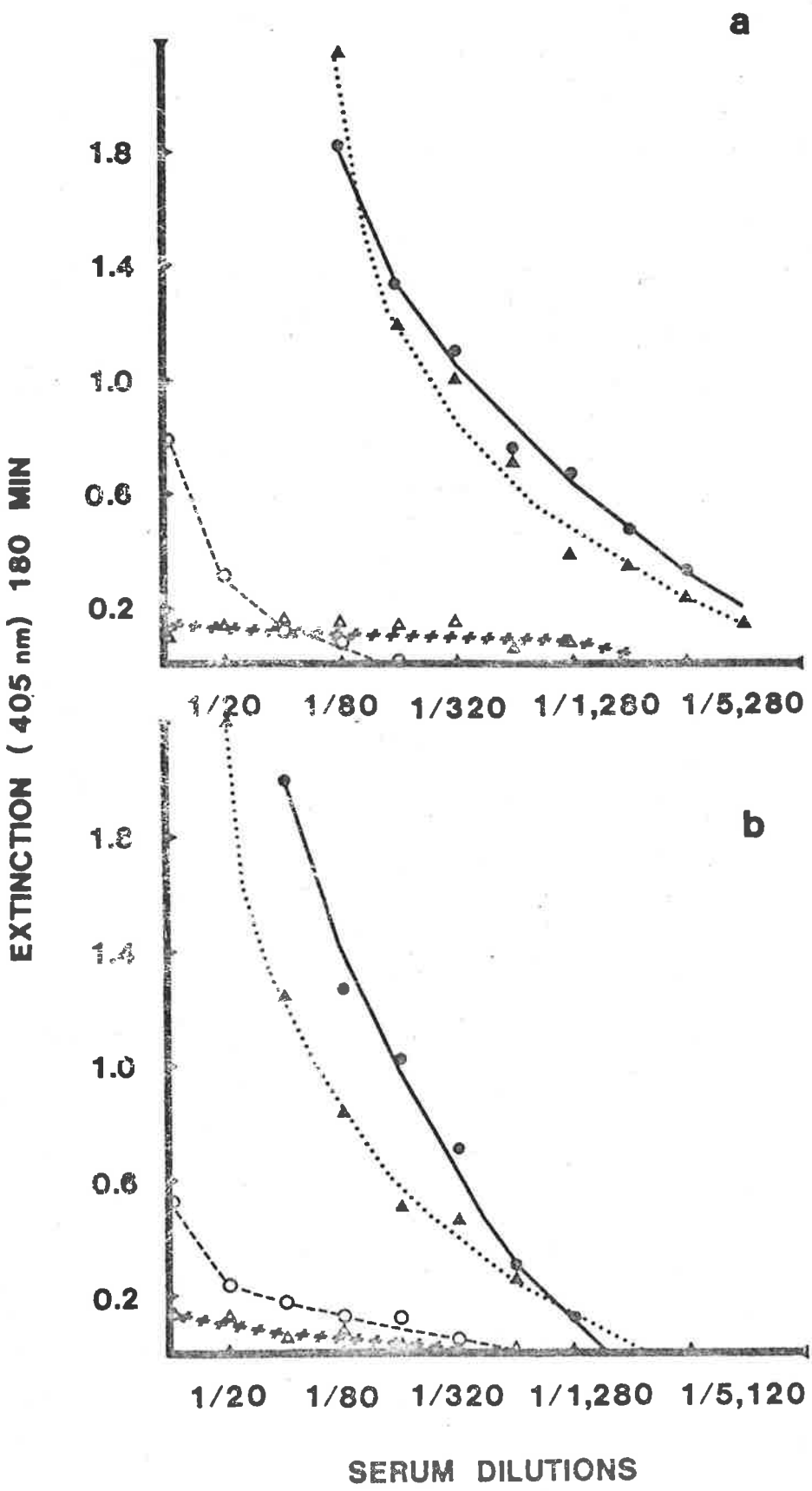

a
b
1.8
1.4
1.0
0.6
0.2
EXTINCTION (405 nm) 180 MIN
1/20
1/80
1/320
1/1,280
1/5,280
1/5,120
SERUM DILUTIONS

Table 6.2

Anti-Salmonella antibodies in serum and intestinal fluids after one oral immunization using indirect ELISA[a] technique.

Immunizing strains	Anti-Salmonella ELISA Ab Titre		
	Serum		Intestinal fluid
	Total Ab [b]	IgA [b]	sIgA [b]
S. typhimurium F885	1280	640	1280
S. bonariensis	640	1280	1280
S. salford	640	640	640
S. stanley	320	320	160
S. strasbourg	640	320	160
S. kirkee	160	160	160
S. enteritidis 11RX	320	40	40
S. budapest	320	<10	80
S. typhimurium M206	20	10	<40
S. fridenau	10	20	<40
S. derby	320	20	<40
S. chester	160	<10	<40
S. humber	320	10	<40
E. coli F1142	320	10	<40
Normal sample	<10	<10	40

a = Using homologous LPS as the antigen.

b = Greatest dilution of serum or intestinal fluid giving an extinction of 0.2 after 180 minutes incubation with substrate.

Table 6.3

Anti-Salmonella antibodies directed against

S. typhimurium C5 outer-membrane proteins after one

oral immunization[a] with various strains of *Salmonellae*.

Immunizing strains	ELISA Antibody Titres Against C5 OMP		
	Total serum Abs	Serum IgA	Intestinal IgA
S. typhimurium F885	160	<10	<40
S. bonariensis	160	<10	<40
S. salford	160	<10	<40
S. stanley	80	10	<40
S. strasbourg	80	<10	<40
S. kirkee	80	<10	<40
S. enteritidis 11RX	80	<10	<40
S. budapest	40	<10	<40
S. typhimurium M206	80	<10	<40
S. derby	40	10	<40
Normal mouse	40	<10	<40

a = 1×10^9 of live organisms.

of the Salmonella strains (Table 6.3).

6.3 (a) Standard Curves for the Determination of Immunoglobulin Class Detected in the ELISA by Using the Anti-mouse α-Chain Enzyme Conjugate

In order to measure the actual amount of antibody present in samples, standard curves were obtained as follows using the sandwich ELISA technique.

1. Standard curve for quantitating IgA antibody in serum and intestinal fluids: The polystyrene plate was coated with goat anti-mouse α-chain, 5 μg/ml, 37° for 3 hours. After washing various amounts of standard mouse IgA (kindly given by Dr P. L. Ey of this department) were added to each well and the antigen-antibody reaction was allowed to proceed for 6 hours at 25°. After washing 1:1000 goat anti-mouse α-chain alkaline phosphatase conjugate was added to every well and the plate was incubated at 25° overnight prior to washing. The substrate, p-nitrophenyl phosphate, was then added to each well and after 90 minutes at 25° the absorbance was measured at 405 nm.

The resultant curve was linear between 0.045 μg/ml and 0.75 μg/ml (Fig. 6.4a). The standard curve was determined by two separate experiments; each gave similar values.

2. Standard curves for quantitating IgG1, IgG2a, IgG2b, IgM and IgA in serum: Doubling dilutions of the purified mouse IgG1, IgG2a, IgG2b, IgM and IgA (kindly given by Dr P. L. Ey of this department) were employed in the "sandwich" ELISA to produce standard curves, using rabbit anti-mouse Fab (5 μg/ml) to coat the plates and 1 in 500 rabbit anti-mouse Fab alkaline phosphatase. The steps in these assays were described above. The resultant curves are shown in Figs.

6.4b, 6.4c and 6.4d.

6.3 (b) Quantitation of Salmonella Antibodies in Mouse Serum and Intestinal Fluid After One Oral Immunization

It is of interest to follow the development of both serum and intestinal antibodies after giving one oral immunizing dose of Salmonellae. Although it is generally accepted that serum antibodies alone play a minor role in the defense against Salmonella infection (Hornick et al., 1970), it is known that antibodies against somatic antigens enhance the antibacterial efficiency of cell-mediated mechanisms (Davies and Kotlarski, 1976) and may be important in defense for this reason. As mentioned earlier in this section, the serum and intestinal fluid samples from each immunized group of mice were examined by the indirect ELISA for anti-Salmonella antibodies. The goat anti-mouse α-chain alkaline phosphatase conjugate was used when IgA class antibody was examined in serum or intestinal fluids and the rabbit anti-mouse Fab-alkaline phosphatase conjugate was used when total antibody in serum was examined. Fig. 6.2 (a, b) shows the types of positive and negative results obtained by plotting the ELISA results (extinction value at 405 nm) against serum or intestinal fluid dilution. The concentration of anti-Salmonella IgA or other classes in each sample was quantitated by determining the extinction coefficient at 405 nm at the end-point titre and by reference to the standard curves. The end-point titre is the highest dilution of serum or intestinal fluids which give an extinction value of 0.2. Table 6.4 gives a summary of the amount of anti-Salmonella antibodies found in serum and intestinal fluid washings after one oral feeding. The data were obtained from the pooled serum or intestinal

Figure 6.4(a)

Standard curve for quantitating IgA using the sandwich ELISA technique.

Serial dilutions of standard IgA were incubated with anti-mouse α-chain adsorbed to the plates. Anti-mouse α-chain-alkaline phosphatase conjugate was then added, and after subsequent incubation the enzyme activity was determined using the substrate. At the limiting O.D. 405 of 0.2, this detected 45 ng/ml of IgA[a]. Since we had no purified sIgA to use as a standard, we converted IgA values to sIgA values in proportion to their molecular weights.

a = Incubation time with substrate was 90 minutes.

FIGURE 6.4a

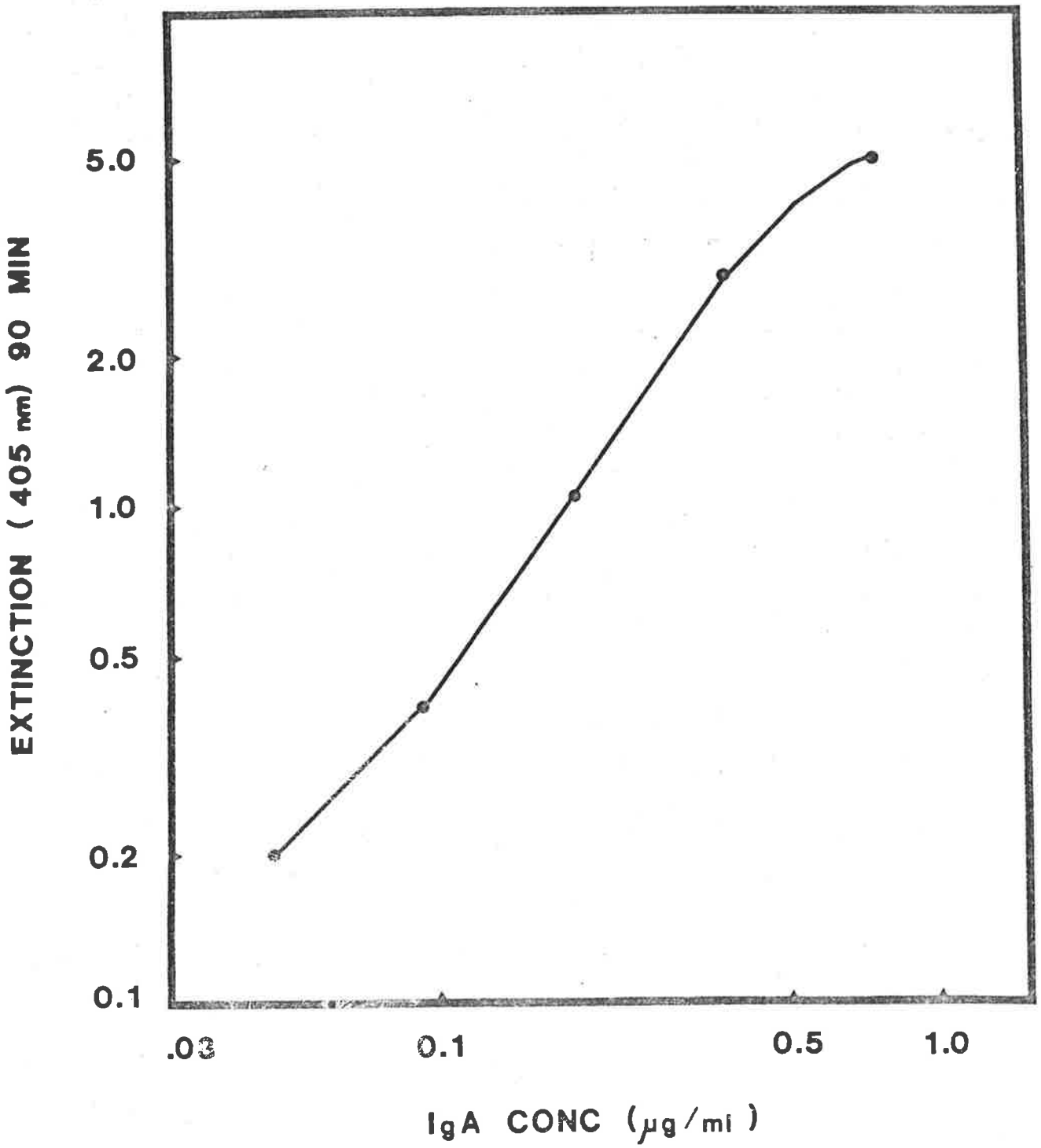

EXTINCTION (405 nm) 90 MIN
5.0
2.0
1.0
0.5
0.2
0.1
.03
0.1
0.5
1.0
IgA CONC (µg/ml)

Figure 6.4(b)

Standard curves for quantitating IgG using the sandwich ELISA technique.

Serial dilutions of standard IgG1, IgG2a, IgG2b were incubated with anti-mouse Fab adsorbed to the plate. Anti-mouse Fab-alkaline phosphatase conjugate was then added, and after subsequent incubation the enzyme activity was determined using its substrate. At the limiting O.D. 405 of 0.2, this detected approximately 30 ng/ml of each of the IgG sub-classes[a].

a = Incubation time with substrate was 90 minutes.

FIGURE 6.4b

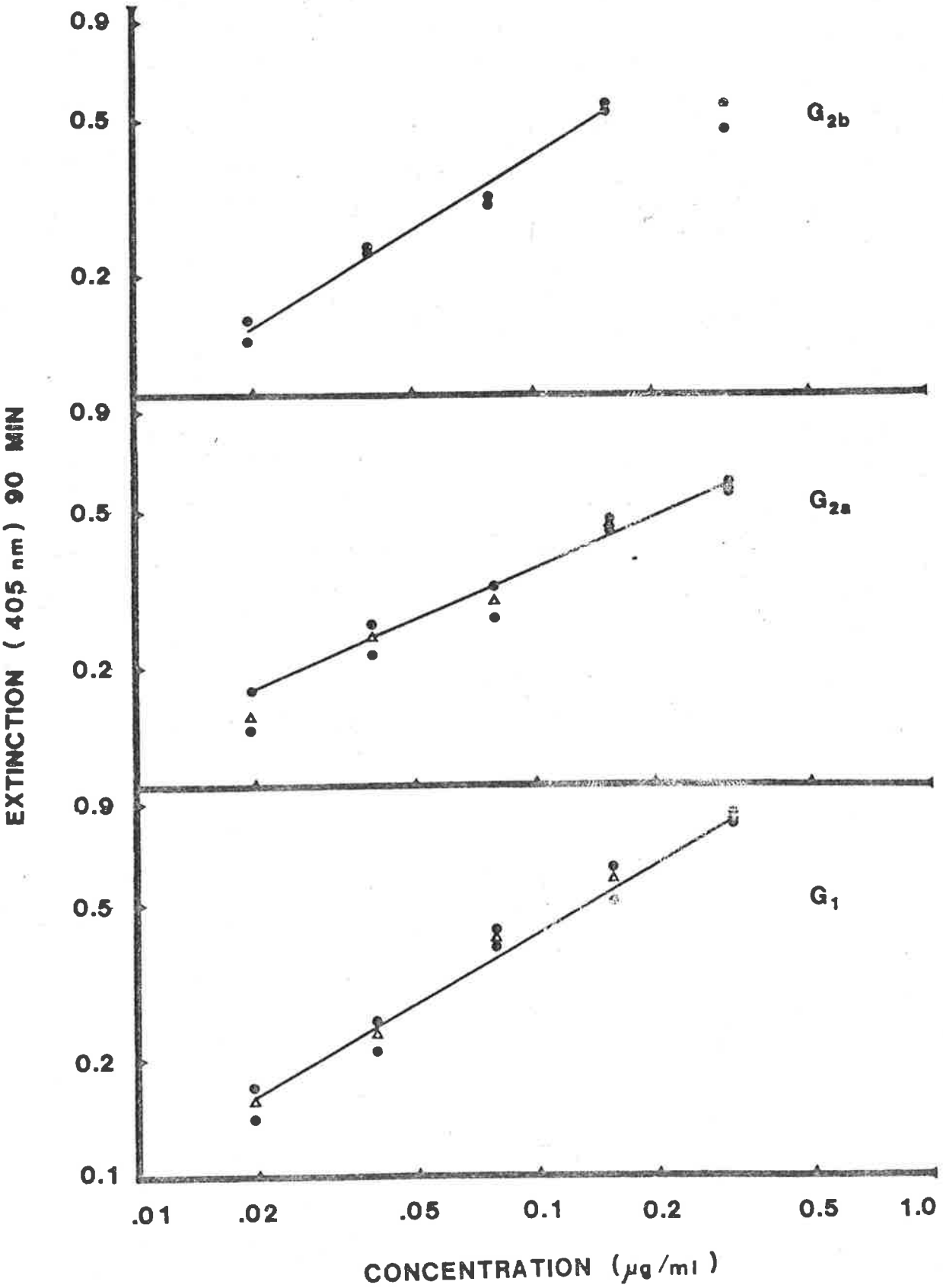

0.9
0.5
0.2
G_{2b}
0.9
0.5
0.2
G_{2a}
0.9
0.5
0.2
0.1
G_1
EXTINCTION (405 nm) 90 MIN
.01
.02
.05
0.1
0.2
0.5
1.0
CONCENTRATION (µg/ml)

Figure 6.4(c)

Standard curves for quantitating IgM using the sandwich ELISA technique.

Serial dilutions of standard IgM were incubated with anti-mouse Fab adsorbed to the plate. Anti-mouse Fab-alkaline phosphatase conjugate was then added, and after subsequent incubation the enzyme activity was determined using its substrate. At the limiting O.D. 405 of 0.2, this detected approximately 50 ng/ml of IgM[a].

a = Incubation time with substrate was 90 minutes.

FIGURE 6.4C

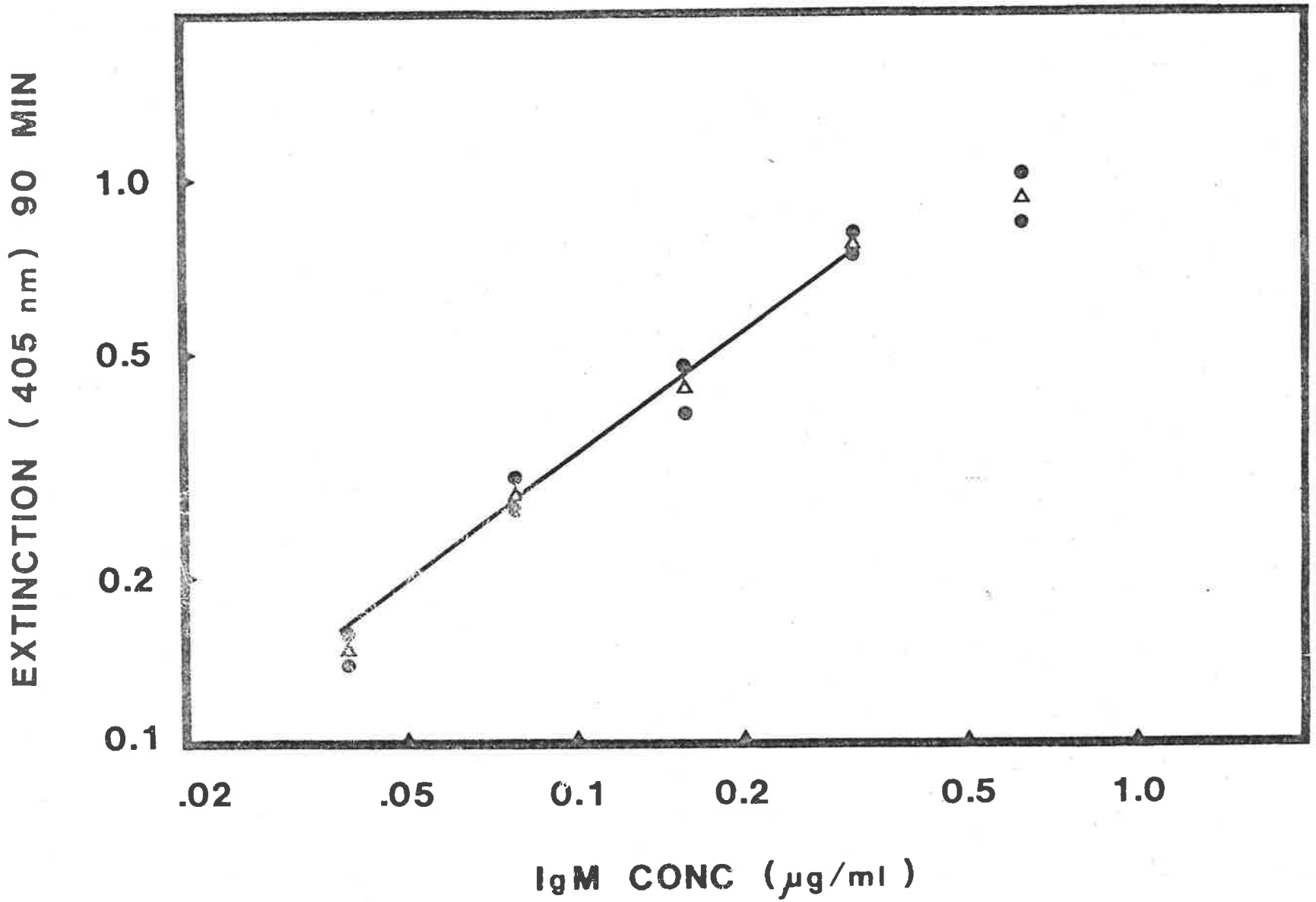

EXTINCTION (405 nm) 90 MIN
1.0
0.5
0.2
0.1
.02
.05
0.1
0.2
0.5
1.0
IgM CONC (µg/ml)

Figure 6.4(d)

Standard curves for quantitating IgA using anti-mouse Fab-alkaline phosphatase conjugate in the sandwich technique.

Serial dilutions of standard IgA were incubated with anti-mouse Fab adsorbed to the plate. Anti-mouse Fab-alkaline phosphatase conjugate was then added, and after subsequent incubation the enzyme activity was determined using its substrate. At the limiting O.D. 405 of 0.2, this detected approximately 250 ng/ml of IgA[a]. (Clearly, this system is much less sensitive for IgA than the anti-mouse α-chain-alkaline phosphatase conjugate which detects 45 ng/ml at the limiting O.D.)

a = Incubation time with substrate was 90 minutes.

FIGURE 6.4d

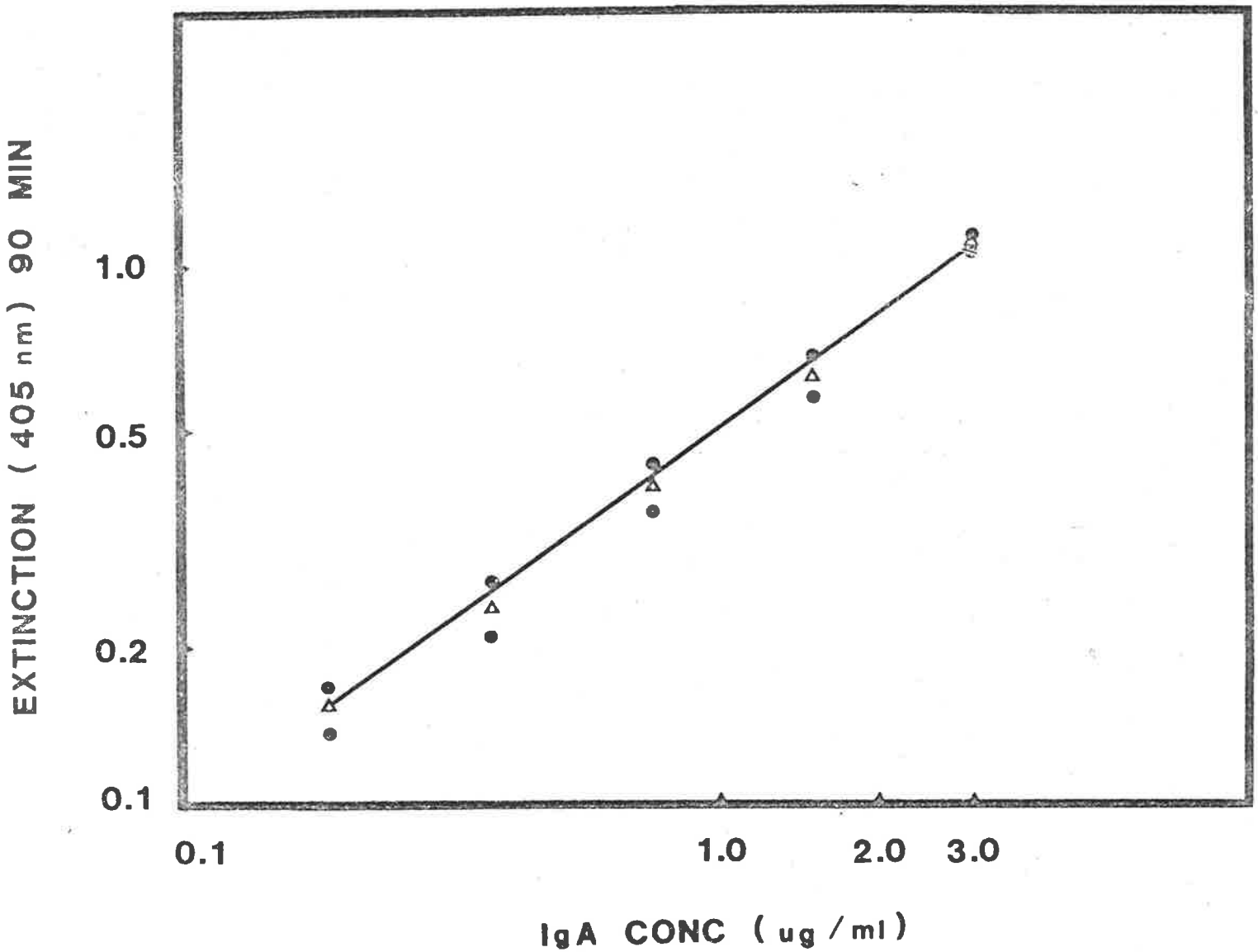


Table 6.4

Anti-Salmonella antibodies in serum and intestinal fluids after one oral immunization[a]. Concentration in µg/ml.

Immunizing strains	Anti-Salmonella Ab Concentration: µg/ml		
	Serum		Intestinal fluid
	IgG	IgA	sIgA
S. typhimurium F885	16.5	15	75
S. bonariensis	4.5	30	75
S. salford	7.0	15	37.5
S. stanley	3.5	7.5	9.5
S. strasbourg	8.5	7.5	9.5
S. kirkee	2.0	3.5	9.5
S. enteritidis 11RX	5.0	1.0	2.5
S. budapest	5.0	<0.25	5.0
S. typhimurium M206	<1.0	<0.25	<2.5
S. fridenau	<1.0	<0.25	<2.5
S. derby	5.0	<0.25	<2.5
S. chester	2.5	<0.25	<2.5
S. humber	5.0	<0.25	<2.5
E. coli F1142	5.0	<0.25	<2.5
Normal sample	1.0	<0.25	<2.5

a = 1×10^9 organisms fed orally.

fluids obtained from groups of 20 to 25 mice 21 days after oral feeding with $1x10^9$ organisms. Serum antibodies were determined either totally or specifically for IgA. Since, at 21 days, it may be assumed that serum antibody is principally of IgG or IgA class, the serum antibody is shown as either IgG or IgA calculated in terms of the data shown in Figs. 6.4a, b, c and d.

All the persistent strains, such as S. typhimurium F885, S. salford and S. bonariensis, give relatively high amounts of IgA anti-Salmonella antibodies in both serum and intestinal fluid washings and high total serum antibodies. On the other hand, the non-persistent strains, such as S. derby, S. humber and E. coli F1142, produce the Salmonella antibody levels in either serum or intestinal fluid washings similar to those for normal mice. However, among the non-persistent strains, some do give relatively high total serum antibodies. These antibodies probably reflect a "spillover" from the intestinal lumen of bacterial antigen.

6.4 Resistance to Salmonella typhimurium Infection After One Oral Immunization with Various Strains of Salmonellae

(a) Immunization

Each 14 groups of mice were given one dose orally with $1x10^9$ live organisms of each 14 Salmonella strains (see Materials and Methods). Each group contained 40-50 mice, and 21 days after the immunization 20-25 mice were killed and serum and intestinal fluids collected; the other 20-25 mice were used in the protection test. The sera and intestinal fluids were tested for antibodies directed against their homologous antigens using the ELISA indirect technique.

(b) Protection test

Protection against S. typhimurium C5 infection (mouse typhoid) was studied by challenging those groups of immunized mice with lethal doses of S. typhimurium C5 organisms. On day 21 after oral immunization, 20-25 mice from each group were fed orally with $1x10^9$ S. typhimurium C5. The number of deaths were recorded 30 days after the challenge. The control mice received in the department on the same day as the test mice were kept in the animal house and given food and drinking water for 21 days before challenge. Table 6.5 shows the protective capabilities of orally administered live vaccines. Using the per cent. survivors of the immunizing groups and their controls (non-immunizing mice), probability values were obtained for significant protection using "Table for Testing Significance in a 2x2 Contingency Table", compiled by D. J. Finney, R. Latscha, B. M. Bennett and P. Hsu, published for the Biometrika Trustees by the Cambridge University Press, 1963.

Values of <0.005 are considered to indicate highly significant protection.

6.5 The Correlation Between the Serum IgA and Intestinal IgA and the Protection After One Oral Immunization

From the data shown in Table 6.6 it can be seen that there is a good correlation between both serum IgA and intestinal sIgA levels and protection against the secondary oral challenge with S. typhimurium C5. Those strains which are able to induce large amounts of both serum and intestinal IgA after oral administration are able to afford protection in mice against challenge with a virulent Salmonella strain (S. typhimurium C5). Consequently, S. typhimurium F885,

Table 6.5

Protection[a] from S. typhimurium C5 infection afforded by one oral live Salmonella feeding.

Immunization orally with $1x10^9$ living cells of	Protection: Survivors/Total after oral challenge with S. typhimurium C5	% Survival	Significance[c] of test
S. typhimurium F885	18/20 (0/10)[b]	90 (0)[b]	<0.005
S. bonariensis	12/13 (1/10)	93 (10)	<0.005
S. salford	18/20 (0/10)	90 (0)	<0.005
S. stanley	17/20 (0/10)	85 (0)	<0.005
S. strasbourg	18/20 (0/10)	90 (0)	<0.005
S. kirkee	6/12 (2/10)	50 (20)	>0.05
S. enteritidis 11RX	12/20 (3/20)	60 (15)	0.01
S. budapest	5/12 (2/13)	42 (15)	>0.05
S. typhymurium M206	3/11 (1/10)	27 (10)	>0.05
S. fridenau	1/10 (1/10)	10 (10)	>0.05
S. derby	1/11 (1/10)	9 (10)	>0.05
S. chester	4/20 (0/13)	20 (0)	>0.05
S. humber	0/20 (0/10)	0 (0)	>0.05
E. coli F1142	2/20 (1/10)	10 (10)	>0.05

a = Oral challenge with 10^9 living S. typhimurium C5 after orally immunizing with various living cells of Salmonella strains. The survivors or the death were observed within 30 days after challenge.

b = Control, non-immunized mice.

c = Values of <0.005 indicate highly significant protection.

S. bonariensis, S. salford, S. stanley and S. strasbourg that are able to induce the high level of both serum and intestinal IgA after one oral administration are able to protect mice from S. typhimurium C5 infection, while those strains that are not able to induce the high level of serum and intestinal IgA, such as S. fridenau, S. derby, S. humber, E. coli F1142, S. typhimurium M206, are not able to afford the protection. S. kirkee, S. budapest and S. enteritidis 11RX, which induce generally lower levels of these IgAs, are seen to afford some slight protection (Table 6.5).

6.6 Conclusion and Discussion

Conclusion

The results reported in this chapter suggest that (1) there is a significant correlation between both serum IgA and intestinal sIgA directed against the immunizing strains and the resistance to mouse typhoid. Conversely, the serum IgG appears to show only weak correlation, if any; (2) the persistence of some immunizing Salmonella strains in the Peyer's patches (which is claimed to be responsible for local cellular immunity; Maneerashapisal and Rowley, 1981; in press) correlates with a high level of both serum and intestinal sIgA, while non-persistent strains do not produce high IgA levels.

Discussion

It is evident from these experiments that oral immunization with various persistent strains of Salmonellae confers significant protection against death from subsequent challenge with S. typhimurium C5, the causative agent of mouse typhoid. IgA antibodies in both serum and in the intestine correlate very well with this protection.

Table 6.6

Correlation between protection and serum IgA and intestinal IgA anti-Salmonella antibody.

Immunizing strains	Serum IgG µg/ml	Serum IgA µg/ml	Intestinal sIgA µg/ml	Significance[a] of protection test
S. typhimurium F885	16.5	15	75	<0.005
S. bonariensis	4.5	30	75	<0.005
S. salford	7.0	15	37.5	<0.005
S. stanley	3.5	7.5	9.5	<0.005
S. strasbourg	8.5	7.5	9.5	<0.005
S. kirkee	2.0	3.5	9.5	>0.05
S. enteritidis 11RX	5.0	1.0	2.5	0.01
S. budapest	5.0	<0.25	5.0	>0.05
S. typhimurium M206	<1.0	<0.25	<2.5	>0.05
S. fridenau	<1.0	<0.25	<2.5	>0.05
S. derby	5.0	<0.25	<2.5	>0.05
S. chester	2.5	<0.25	<2.5	>0.05
S. humber	5.0	<0.25	<2.5	>0.05
E. coli F1142	5.0	<0.25	<2.5	>0.05
Normal mouse	1.0	<0.25	<2.5	

a = Values of <0.005 are considered to indicate highly significant protection.

Although the protective action of IgA, the predominant immunoglobulin in external secretions, against viral infection in the intestinal tract (Ogra et al., 1968) and elsewhere (Smith et al., 1966) has been well established, its action against bacteria is less evident and is indeed most unlikely to have any direct bearing on the protection observed in this work, since the induced IgA is generally non-specific for the challenge strains. Further, a negligible amount of intestinal IgA was found able to interact with the outer membrane protein of the challenge strain.

CHAPTER 7

Discussion

7.1 Reiteration of Aims

The aim of this work has been to determine whether a simple technique which measures the levels of either serum IgA or sIgA can be used as an index of protection against typhoid fever in mice.

As previously discussed in Chapters 3 and 5, the ELISA proves to be at least as exquisitely sensitive and specific as radioimmunoassay, and because it can be readily adapted to field use as well as in the routine laboratory, it affords an ideal screening technique. Consequently, we have used the ELISA for such IgA determinations.

7.2 ELISA Technique

As has been discussed in 3.2.1 and 5.4.1, we found that the nature of the carrier surface (i.e., polystyrene microtitre plates) had a very marked influence on the binding of lipopolysaccharides and proteins. Consistently adequate binding was obtained by coating plates with LPS which had been previously coupled to methylated bovine serum albumin. Using a sandwich technique, we were able to detect as little as 45 ng/ml of IgA, 30 ng/ml of IgG and 50 ng/ml of IgM at the limiting O.D. 405 of 0.2.

7.3 Local Immunological Defense Mechanisms

The defense mechanisms of the gastrointestinal tract have already been discussed in some detail. The non-immunologic mechanisms in Chapter 1 and the immunologic, such as the production of sIgA and

cell-mediated immunity, in Chapter 2. This work has thrown some new light on these local immune mechanisms. For example, it is clear that those Salmonellae that afford protection against mouse typhoid after one oral feeding of live organisms are able: (1) to persist in the Peyer's patches of the small intestine and thus presumably cause the committal of T-lymphocytes to a cell-mediated immunity so that they respond to antigen present within the intestinal lumen, as suggested by Muller-Schoop and Good (1975) and by the work of Maneerushapisal and Rowley (1981); (2) to cause a marked increase in the titres of both serum IgA and, more particularly, in the sIgA of the intestinal juice.

The data presented in Table 6.5 clearly demonstrated a correlation between the levels of both serum IgA and intestinal sIgA and protection to a subsequent challenge with S. typhimurium C5. (In general, the levels of serum IgG remain relatively unchanged after administration of the oral immunizing dose, presumably as a result of suppressor T cells, as indicated by the work of Richman et al. (1981) and Kawanishi et al. (1982). Also, negligible quantities of IgG and IgM by ELISA were found in the gastric juice.) Of those strains which afford protection against mouse typhoid, only S. stanley has O-somatic antigens (4, 5, 12), in common with the challenge strain. Indeed, when S. typhimurium C5 LPS was used to coat plates, negligible quantities of serum IgA or sIgA were found in serum or intestinal fluid samples of mice immunized with the various strains of Salmonella, except from the mice orally immunized with S. stanley. Therefore, it seems most unlikely that the high levels of sIgA obtained by immunization with some organisms are, as such, responsible for the protection observed, unless the sIgA is directed against some

common antigen such as ECA. (Our results show that such a common antigen is not in the outer membrane proteins of the challenge strain, S. typhimurium C5.)

7.4 Concentration of Serum IgA or sIgA as an Indicator of Protection

Clearly, a single oral feeding of antigen can elicit a local humoral response in the gut which results in the production of sIgA locally. The concomitant increase in serum IgA is due presumably to the presence of IgA secreting plasma cells in the peripheral blood as they cycle back to the gut (see 2., 'IgA cell cycle') where the SC is then added to produce sIgA. This increase in the levels of serum IgA and intestinal sIgA correlates very well with protection against mouse typhoid and can, therefore, be used as an easily measured indicator of immune status.

However, it also seems clear that these induced immunoglobulins play little, if any, part in the protection process as such, which is almost certainly due to cellular immunity. Maneerushapisal and Rowley (in press) consider that the T-cells of recipients must be sensitized to some unidentified bacterial antigens which, on secondary challenge, liberate lymphokines which activate the local macrophages in ways which enable them to kill the invading pathogens. Those Salmonellae which are best at inducing the resistance are those which are able to colonize the Peyer's patches of the small intestine for a relatively long period of time. This lymphoid tissue then acquires a much larger population of macrophages which have the antibacterial properties of activated cells (Maneerushapisal and Rowley, 1981). Such mechanisms are almost certainly responsible for the successful protection obtained by Wahdan et al. (1982) in the Egyptian trials

involving oral administration of live typhoid vaccine.

Since, at present, it is not easily possible to measure cellular immunity and use it as an index of immunological defense, the correlation between protection and IgA levels which follow the persistence of living Salmonellae in the Peyer's patches of the small intestine seems a simply measured and an appropriate indicator of the immune status of an animal. In consequence, such simple measurements of IgA levels offer a very attractive alternative to the mounting of expensive and extensive field trials of vaccines. Clearly, aberrations due to the incorrect or inadequate feed-back of information in such trials are overcome.

BIBLIOGRAPHY

Abe, K., and Ito, T. (1977). A qualitative and quantitative morphologic study of Peyer's patches of the mouse. Arch. Histol. Jpn, 40, 407.

Abraham, R., Fabian, R. J., Goldberg, L., and Coulston, F. (1974). Role of lysosomes in carragenan-induced cecal ulceration. Gastroenterology, 67, 1169.

Abrams, G. D., and Bishop, J. E. (1966). Effect of the normal microbial flora on the resistance of the small intestine to infection. J. Bact., 92, 1604.

Ackert, J. E., Edgar, S. A., and Frick, L. P. (1939). Goblet cells and age resistance of animals to parasitism. Trans. Am. Micro. Soc., 58, 81.

Adinolfi, M., Glynn, A. A., Lindsay, M., and Milne, C. M. (1966). Serological properties of γA antibodies to E. coli present in human colostrum. Immunology, 10, 517.

Allen, W. D., Smith, C. G., and Porter, P. (1973). Localization of intracellular immunoglobulin A in porcine intestinal mucosae using enzyme-labelled antibody. Immunology, 25, 55.

André, C., Lambert, R., Bazin, H., and Heremans, J. F. (1974). Interference of oral immunization with the intestinal absorption of heterologous albumin. Eur. J. Immunol., 4, 701.

Arnold, L., and Brody, L. (1926). The gastro-duodenal bactericidal mechanism. Am. J. Hyg., 6, 672.

Ashcroft, M. T., Singh, B., Nicholson, C. C., Ritchie, J. M., Sobryan, E., and Williams, F. (1967). A seven-year field trial of two typhoid vaccines in Guyana. Lancet, 2, 1056.

Avrameas, S. (1969). Coupling of enzymes to proteins with glutaraldehyde. Use of the conjugate for the detection of antigens and antibodies. Immunochem., 6, 43.

Avrameas, S., and Ternynck, T. (1971). Peroxidase-labelled antibody and Fab conjugates with enhanced intracellular penetration. Immunochem., 8, 1175.

Avrameas, S., Ternynck, T., and Guesdon, J. L. (1978). Coupling of enzymes to antibodies and antigens. Scand. J. Immunol., 8, Supp. 7, 7.

Auzins, I., and Rowley, D. (1969). The response to S. typhimurium antigens by two different strains of mice. Immunol., 17, 579.

Barclay, W. R., Busey, W. M., Dalgard, D. W., Good, R. C., Janicki, B. W., Kazik, J. E., Ribi, E., Ulrich, C. E., and Wolinsky, S. (1973). Protection of monkeys against airborne tuberculosis by aerosol vaccination with Bacillus Calmette-Guerin. Am. Rev. Resp. Dis., 107, 351.

Beale, D., and Feinstein, A. (1976). Structure and function of the constant regions of immunoglobulins. Quart. Rev. Biophys., 9, 135.

Bellamy, J. E. C., and Nielsen, N. O. (1974). Immune mediated migration of neutrophils into the lumen of the small intestine. Infect. Immun., 9, 615.

Bennich, H., and von Bahr-Lindström, H. (1974). Structure of immunoglobulin E (IgE). In "Progress in Immunology II," Vol. 1 (eds. Brent L., and Holbrow, J.), pp 49-58, Amsterdam, North Holland.

Bernard, R. P. (1965). The Zermatt typhoid outbreak in 1963. J. Hyg., Camb., 63, 537.

Besredka, A. (1927). "Local Immunization." Williams and Wilkins,

Baltimore, Md.

Bienenstock, J., and Dolezel, J. (1971). Peyer's patches: lack of specific antibody containing cells after oral and parenteral immunization. J. Immunol., 106, 938.

Bienenstock, J., Gauldie, J., and Perey, D. Y. E. (1973). Synthesis of IgG, IgA, IgM by chicken tissues: Immunofluorescent and ^{14}C amino acid incorporation studies. J. Immunol., 111, 1112.

Birbeck, M. S. C., Cartwright, P., and Hall, J. G. (1979). The transport by hepatocytes of IgA from blood to bile visualized by autoradiography and electron microscope. Immunology, 37, 477.

Bloom, L. D., and Rowley, D. (1979a). Local immune response in mice to Vibrio cholerae. Aust. J. Exp. Biol. Med. Sci., 57, 313.

Bloom, L., and Rowley, D. (1979b). Persistence in the mouse gut as an important factor in oral immunogenicity of strains of V. cholerae. Aust. J. Exp. Biol. Med. Sci., 57, 325.

Bohnhoff, M., Miller, C. D., and Martin, W. R. (1964). Resistance of the mouse intestinal tract to experimental Salmonella infection. I. Factors which interfere with the initiation of infection by oral inoculation. J. Exp. Med., 120, 805.

Boorsma, D. M., and Streefkerk, J. G. (1976). Peroxidase-conjugate chromatography. Isolation of conjugates prepared with glutaraldehyde or periodate using polyacrylamide-agarose gel. J. Histochem. Cytochem., 24, 481.

Brandtzaeg, P. (1974). Mucosal and glandular distribution of Ig components: differential localization of free and bound SC in secretory epithelial cells. J. Immunol., 112, 1553.

Brandtzaeg, P. (1978). Polymeric IgA is complexed with secretory component (SC) on the surface of human intestinal epithelial

cells. Scand. J. Immunol., 8, 39.

Brandtzaeg, P., Fjellanger, I., and Gjeruldsen, S. T. (1967). Localization of immunoglobulins in human nasal mucosa. Immunochemistry, 4, 57.

Branefors-Helander, P., and Dahlberg, T. (1975). Abstract. Acta. Soc. Med. Suecanae, 84, 270.

Brown, W. R., Nagura, H., Smith, P. D., and Nakane, P. K. (1980). The human liver and the secretory immune system in the mucosal immune system in health and disease. The 81st Ross Conference on Pediatric Research (eds. Ogra and Bienenstock), pp 16-22.

Budd, W. (1856). Lancet 2, 618, 694.

Budd, W. (1873). "Typhoid Fever" London.

Burrows, W., Deupree, N. G., and Moore, D. E. (1950). Effect of X-irradiation on experimental enteric cholera in guinea pig. J. Infect. Dis., 87, 158.

Burrows, W., Elliott, M. E., and Havens, I. (1947). Studies on immunity to Asiatic cholera. IV. The excretion of copro-antibody in experimental enteric cholera in the guinea pig. J. Infect. Dis., 81, 261.

Burrows, W., and Havens, I. (1948). Studies on immunity to Asiatic cholera. V. The absorption of immune globulin from the bowel and its excretion in the urine and faeces of experimental animals and human volunteers. J. Infect. Dis., 82, 231.

Calderon, E. (1974). Amoxicillin in the treatment of typhoid fever due to chloramphenicol-resistant Salmonella typhi. J. Infect. Dis., 129, Suppl. S219-2.

Carlsson, H. E., and Lindberg, A. A. (1978). Application of enzyme immunoassay for diagnosis of bacterial and mycotic infections.

Scand. J. Immunol., 8, Suppl. 7, 97.

Carlsson, H. E., Hurvell, B., and Lindberg, A. A. (1976). Enzyme-linked immunosorbent assay (ELISA) for titration of antibodies against Brucella abortus and Yersinia enterocolitica. Acta. Pathol. Microbiol. Scand., 84, 168.

Carlsson, H. E., Lindberg, A. A., and Hammarström, S. (1972). Titration of antibodies to Salmonella O antigen by enzyme-linked immunosorbent assay. Infect. Immun., 6, 703.

Carlsson, H. E., Lindberg, A. A., Hammarström, S., and Ljunggren, A. (1975). Quantitation of Salmonella O-antibodies in human sera by enzyme-lined immunosorbent assay (ELISA). Int. Arch. Allerg. Appl. Immunol., 48, 485.

Carter, P. B. (1975). Spread of enteric fever bacilli from the intestinal lumen. In "Microbiology 1975" (ed. Schlessinger, D.). Am. Soc. Microbiol., Washington, D.C., p 182.

Carter, P. B., and Collins, F. M. (1974). The route of enteric infection in normal mice. J. Exp. Med., 139, 1189.

Carter, P. B., Woolcock, J. B., and Collins, F. M. (1975). Involvement of the upper respiratory tract in orally induced Salmonellosis in mice. J. Infect. Dis., 131, 570.

Casu, A., and Avrameas, S. (1969). Conjugation of lactic dehydrogenase with proteins by the use of glutaraldehyde: detection of antigens in immunocompetent cells by means of conjugates. Ital. J. Biochem., 13, 166.

Catt, K., and Tregear, G. W. (1967). Solid-phase radioimmunoassay in antibody-coated tubes. Science, 158, 1570.

Cebra, J. J., and Robbins, J. B. (1966). γA-Immunoglobulin from rabbit colostrum. J. Immunol., 97, 12.

Chodirker, W. B., and Tomasi, T. B. (1963). Gamma globulin; quantitative relationships in human serum and non-vascular fluids. Science, 142, 1080.

Collins, F. M. (1970). Immunity to enteric infection in mice. Infect. Immun., 1, 243.

Collins, F. M. (1972). Salmonellosis in orally infected specific pathogen-free C57BL mice. Infect. Immun., 5, 191.

Collins, F. M., and Carter, P. B. (1974). Cellular immunity in enteric disease. Am. J. Clin. Nutr., 27, 1424.

Collins, F. M., and Carter, P. B. (1978). Growth of Salmonellae in orally infected germ-free mice. Infect. Immun., 21, 41.

Colten, H. R., and Bienenstock, J. (1974). Lack of C3 activation through the classical or alternate pathways by human secretory IgA antiblood group A antibody. Adv. Exp. Med. Biol., 45, 305.

Cornes, J. S. (1965). Number, size and distribution of Peyer's patches in the human small intestine. Gut, 6, 225.

Crabbé, P. A. (1967). "Signification du Tissue Lymphoide des Muque uses Digestives." Arscia, Brussels.

Crabbé, P. A., Carbonara, A. O., and Heremans, J. E. (1965). The normal human intestinal mucosa as a major source of plasma cells containing γA-immunoglobulin. Lab. Invest., 14, 235.

Crabbé, P. A., and Heremans, J. F. (1966). The distribution of immunoglobulin-containing cells along the human gastrointestinal tract. Gastroenterology, 51, 305.

Crabbé, P. A., Nash, D. R., Bazin, H., Eyssen, H., and Heremans, J. (1969). Antibodies of the IgA type in intestinal plasma cells of germ-free mice after oral stimulation or parenteral immunization with ferritin. J. Exp. Med., 130, 723.

Crabbé, P. A., Nash, D. R., Bazin, H., Eyssen, H., and Heremans, J. F. (1970). Immunohistochemical observations on lymphoid tissues from conventional and germ-free mice. Lab. Invest., 22, 448.

Crago, S. S., Kulhavy, R., Prince, S. J., and, Mestecky, J. (1978). Secretory component on epithelial cells is a surface receptor for polymeric immunoglobulins. J. Exp. Med., 147, 1832.

Craig, S. W., and Cebra, J. J. (1971). Peyer's patches: an enriched source of precursors for IgA producing immunocytes in the rabbit. J. Exp. Med., 134, 188.

Craig, S. W., and Cebra, J. J. (1975). Rabbit Peyer's patches, appendix, and popliteal lymph node B lymphocytes: A comparative analysis of their membrane immunoglobulin components and plasma cell precursor potential. J. Immunol., 114, 492.

Cvjetanovic, B., and Vemura, K. (1965). The present status of field and laboratory studies of typhoid and paratyphoid vaccines with special reference to studies sponsored by the World Health Organisation. Bull. W.H.O., 32, 29.

Davies, A. (1922). An investigation into the serological properties of dysentery stools. Lancet (Lond.), ii, 1009.

Davies, R., and Kotlarski, I. (1976). A role for antibody in the expression of cellular immunity to Salmonella typhimurium C5. Aust. J. Exp. Biol. Med. Sci., 54, 207.

Donaldson, R. M. (1967). Role of enteric microorganisms in malabsorption. Fed. Proc., 26, 1426.

Dupont, H. L., Hornick, R. B., Snyder, M. J., Libonati, J. P., Formal, S. B., and Gangarosa, E. J. (1972). Immunity in Shigellosis II. Protection induced by oral live vaccine or primary infection. J. Infect. Dis., 125, 12.

Eddie, D. S., Schulkind, M. L., and Robbins, J. B. (1971). The isolation and biologic activities of purified sIgA and IgG anti-Salmonella typhimurium 'O' antibodies from rabbit intestinal fluid and colostrum. J. Immunol., 106, 181.

Edsall, G., Cains, S., Landy, M., Tigertt, W. D., Sprinz, H., Trapaini, R. J., Mandel, A. D., and Beneson, A. S. (1960). Studies on infection and immunity in experimental typhoid fever. I. Typhoid fever in chimpanzees orally infected with Salmonella typhosa. J. Exp. Med., 112, 143.

Engvall, E. (1977). Quantitative enzyme immunoassay (ELISA) in microbiology. Med. Biol., 55, 193.

Engvall, E., Jonsson, K., and Perlmann, P. (1971). Enzyme-linked immunosorbent assay, ELISA: II. Quantitative assay of protein antigen, immunoglobulin G, by means of enzyme-labelled antigen in antibody coated tubes. Biochim. Biophys. Acta., 251, 427.

Engvall, E., and Perlmann, P. (1972). Enzyme-linked immunosorbent assay, ELISA: III. Quantitation of specific antibodies by enzyme-labelled anti-immunoglobulin in antigen coated tubes. J. Immunol., 109, 129.

Fazekas de St Groth, S. (1951). Studies in experimental immunology of influenza. IX. The mode of action of pathotopic adjuvants. Aust. J. Exp. Biol. Med. Sci., 29, 339.

Fazekas de St Groth, S., Donnelley, M., and Graham, D. M. (1951). Studies in experimental immunology of influenza. VIII. Pathotopic adjuvants. Aust. J. Exp. Biol. Med. Sci., 29, 323.

Feinstein, A., Munn, E. A., and Richardson, N. E. (1971). The three-dimensional conformation of γM and γA globulin molecules. Annals N.Y. Acad. Sci., 190, 104.

Fisher, M. M., Nagy, B., and Bazin, H. (1979). Biliary transport of IgA: Role of secretory component. Proc. Nat. Acad. Sci., U.S.A., 76, 2008.

Florey, H. W. (1933). Observations on the functions of mucus and the early stages of bacterial invasion of the intestinal mucosa. J. Path. Bact., 37, 283.

Formal, S. B., Abrams, G. D., Schneider, H., and Sprinz, H. (1963). Experimental Shigella infections. VI. The role of the small intestine in an experimental infection in guinea pigs. J. Bact., 85, 119.

Frankel, M. E., and Gerhard, W. (1979). The rapid determination of binding constants for antiviral antibodies by a radioimmunoassay. An analysis of the interaction between hybridoma proteins and influenza virus. Mol. Immunol., 16, 101.

Franklin, E. M., Kenyon, K. R., and Tomasi, T. B. (1973). Immunohistologic studies of human lacrimal gland: Localization of immunoglobulins, secretory component and lactoferrin. J. Immunol., 110, 984.

Frederick, G. T., and Bohl, E. H. (1976). Local and systemic CMI against transmissible gastroenteritis, an intestinal viral infection of swine. J. Immunol., 116, 1000.

Freter, R. (1955). The fatal enteric cholera infection in the guinea pig, achieved by inhibition of normal enteric flora. J. Infect. Dis., 97, 57.

Freter, R. (1956). Experimental enteric Shigella and vibrio infections in mice and guinea pigs. J. Exp. Med., 104, 411.

Freter, R., De, S. P., and Mondal, A. (1965). Coproantibody and serum antibody in cholera patients. J. Infect. Dis., 115, 83.

Fubara, E. S., and Freter, R. (1973). Protection against enteric bacterial infection by secretory IgA antibodies. J. Immunol., 111, 395.

Gaines, S., Sprinz, H., Tully, J. G., and Tigertt, W. D. (1968). Studies on infection and immunity in experimental typhoid fever. VII. The distribution of Salmonella typhi in chimpanzee tissue following oral challenge and the relationship between the numbers of bacilli and morphologic lesions. J. Infect. Dis., 118, 293.

Galindo, B., and Myrvik, Q. N. (1970). Migratory response of granulomatous alveolar cells from BCG-sensitized rabbits. J. Immunol., 105, 227.

Gelzayd, E. A., Kraft, S. C., and Fitch, F. W. (1967). Immunoglobulin A: Localization in rectal mucosal epithelial cells. Science, 157, 930.

Germanier, R., and Fürer, E. (1975). Isolation and characterization of gal E, mutant Ty 21a of Salmonella typhi: a candidate strain for a live, oral typhoid vaccine. J. Infect. Dis., 131, 553.

Gilman, R. H., Hornick, R. B., Woodward, W. E., Dupont, H. L., Snyder, M. J., Levine, M. M., and Libonati, J. P. (1977). Evaluation of a UDP-glucose-4-epimeraseless mutant of Salmonella typhi as a live oral vaccine. J. Infect. Dis., 136, 717.

Goldsworthy, N. E., and Florey, H. W. (1930). Some properties of mucus, with special reference to its antibacterial functions. Brit. J. Exp. Path., 11, 192.

Gorzynski, E. A., Neter, E., and Ambrus, J. L. (1970). Differences in antibody responses of mouse strains to enterobacterial common antigen. Proc. Soc. Exp. Biol. Med., 134, 776.

Gowans, J. L., and Knight, E. J. (1964). The route of recirculation

of lymphocytes in the rat. Proc. R. Soc., B159, 257.

Greenwood, F. C., Hunter, W. M., and Glover, J. S. (1963). The preparation of I^{131}-labelled human growth hormone of high specific radioactivity. Biochem. J., 89, 114.

Griscelli, C., Vassalli, P., and McCluskey, R. T. (1969). The distribution of large dividing lymph node cells in syngeneic recipient rats after intravenous injection. J. Exp. Med., 130, 1427.

Guy-Grand, D., Griscelli, C., and Vassalli, P. (1974). The gut-associated lymphoid system: nature and properties of the large dividing cells. Eur. J. Immunol., 4, 435.

Guy-Grand, D., Griscelli, C., and Vassalli, P. (1978). The mouse gut T-lymphocyte a novel type of T-cell: nature, origin and traffic in mice in normal and graft-versus host conditions. J. Exp. Med., 148, 1661.

Hall, J.G., Orlans, E., and Reynolds, J. (1979). Occurrence of specific antibodies of the IgA class in the bile of rats. Int. Arch. Allergy Appl. Immunol., 59, 75.

Hall, J.G., Parry, D., and Smith, M. E. (1972). The distribution and differentiation of lymph-borne immunoblasts after intravenous injection into syngeneic recipients. Cell Tissue Kin., 5, 269.

Hall, J. G., and Smith, M. F. (1970). Homing of lymph-borne immunoblasts to the gut. Nature (Lond.), 226, 262.

Halpern, M. S., and Koshland, M. E. (1970). Novel subunit in secretory IgA. Nature (Lond.), 228, 1270.

Hammarström, S., Engvall, E., Johansson, B. G., Svenson, S., Sundblad, G., and Goldstein, I. J. (1975). Nature of the tumor associated determinants of carcinoembryonic antigen (CEA). Proc. Nat. Acad. Sci., U.S.A., 72, 1528.

Hanson, L. A. (1961). Comparative immunological studies of the immune-globulin of human milk and of blood serum. Int. Arch. Allergy Appl. Immunol., 18, 241.

Hanson, L. A., Ahlstedt, S., Carlson, B., Fällström, S. P., Kaijser, B., Lindblad, B. S., and Svanborg-Eden, C. (1978). New knowledge in human milk immunology. Acta Paediatr. Scand., 67, 577.

Hanson, L. A., and Brandtzaeg, P. (1980). The mucosal defense system. In "Immunologic Disorders in Infants and Children" (2nd edition), (eds Shiehm, E. T., and Fulginiti, U. A.), W. B. Saunders & Co., Philadelphia, p 137.

Henney, C., and Waldman, R. H. (1970). Cell mediated immunity shown by lymphocytes from the respiratory tract. Science, 169, 696.

Hejfec, L. B., Salmin, L. V., Lejtman, M. Z., Kuz'minova, M. L., Vasil'eva, A. V., Levina, L. A., Bencianova, T. G., Pavlova, E. A., and Antonova, A. A. (1966). A controlled field trial and laboratory study of five typhoid vaccines in the U.S.S.R. Bull. W.H.O., 34, 321.

Heremans, J. F. (1960). "Les Globulines Sériques due Systéme Gamma." Arscia, Brussels.

Heremans, J. F. (1974). Immunoglobulin A. In "The Antigens," Vol. II (ed. Sela, M.), Academic Press, London, N.Y., pp 365-522.

Heremans, J. F., Vaerman, J. P., and Vaerman, C. (1963). Studies on the immunoglobulins of human serum. II. A study of the distribution of anti-Brucella and anti-Diphtheria antibody activities among γss-, γlm- and γ1a-globulin fractions. J. Immunol, 91, 11.

Heremans, J. F., and Vaerman, J. P. (1971). Biological significance of IgA antibodies in serum and secretions. In "Progress in Immunology I" (ed. Amos, B.) (First International Congress of Immunology)

Academic Press, New York, pp 875-890.

Herrmann, J. E., and Collins, M. F. (1976). Quantitative of immunoglobulin adsorption to plastics. J. Immunol. Meth., 10, 363.

Hill, I. R., and Porter, P. (1974). Studies of bactericidal activity to Escherichia coli of porcine serum and colostral immunoglobulins and the role of lysozyme with secretory IgA. Immunol., 26, 1239.

Hohmann, A. W., Schmidt, G., and Rowley, D. (1978). Intestinal colonization and virulence of Salmonella in mice. Infect. Immun., 22, 763.

Holmgren, J., and Svennerholm, A-M. (1973). Enzyme-linked immunosorbent assays for cholera serology. Infect. Immun., 7, 759.

Hornick, R. B., and Woodward, T. E. (1966). Appraisal of typhoid vaccine in experimentally infected human subjects. Trans. Am. Clin. Climatol. Assn., 78, 70.

Hornick, R. B., Woodward, T. E., McCrumb, F. R., Snyder, M. J., Dawkins, A. T., Bulkeley, J. T., Macorra, F. de la, and Corozza, F. A. (1967). Typhoid fever vaccine - yes or no? Med. Clin. North Am., 51, 617.

Hornick, R. B., Greisman, S. E., Woodward, T. E., Dupont, H. L., Dawkins, A. T., and Snyder, M. J. (1970). Typhoid fever: pathogenesis and immunologic control (Parts 1 and 2). New Eng. J. Med., 283, 686, 739.

Horsfall, D. J. (1977). Antibody synthesis to Vibrio cholerae in the mouse intestine. Ph.D. Thesis, The University of Adelaide, South Australia.

Horsfall, D. J., Heddle, R. J., Gormly, A. A., and Rowley, D. (1978). In vitro degradation of mouse, rabbit and dog antibodies to V. cholerae by Succus entericus. Aust. J. Exp. Biol. Med. Sci.,

56, 681.

Horsfall, D. J., and Rowley, D. (1979). A comparison of methods for measuring intestinal antibodies against Vibrio cholerae in mice. Aust. J. Exp. Biol. Med. Sci., 57, 61.

Huntley, J., Newby, T. J., and Bourne, F. J. (1979). The cell-mediated immune response of the pig to orally administered antigen. Immunol., 37, 225.

Husband, A. J., and Gowans, J. L. (1978). The origin and antigen-dependent distribution of IgA containing cells in the intestine. J. Exp. Med., 148, 1146.

Jackson, G. D. F., Lemaitre-Coelho, I., and Vaerman, J. P. (1978). Rapid disappearance from serum of intravenously injected rat myeloma IgA and its secretion into bile. Eur. J. Immunol., 7, 123.

Jeffries, G. H., Sleisenger, M. H., and Margolis, S. (1965). Studies of parietal cell antibody in pernicious anemia. J. Clin. Invest., 44, 2021.

Jensenius, J. C., and Williams, A. F. (1974). Total immunoglobulin of rat thymocytes and thoracic duct lymphocytes. Eur. J. Immunol., 4, 98.

Jones, P. P., and Cebra, J. J. (1974). Restriction of gene expression in B lymphocytes and their progeny. III. Endogenous IgA and IgM on the membranes of different plasma cells precursors. J. Exp. Med., 140, 966.

Jurgensen, P. F., Olsen, G. N., Jonnson, J. E. III, Swenson, E. W., Ayoub, E. M., Henney, C. S., and Waldman, R. H. (1973). Immune response of the human respiratory tract. II. Cell-mediated immunity in the lower respiratory tract to tuberculin and mumps and influenza viruses. J. Infect. Dis., 128, 730.

Kagnoff, M. F. (1978). Effects of antigen-feeding on intestinal and systemic immune response. I. Priming of precursor cytotoxic T cells by antigen feeding. J. Immunol., 120, 395.

Kato, K., Hamaguchi, Y., Fukui, H., and Ishikawa, E. (1975). Enzyme-linked immunoassay. I. Novel method for synthesis of the insulin-β-D-galactosidase conjugate and its applicability for insulin assay. J. Biochem., 78, 235.

Kauffman, F. (1966). "The Bacteriology of Enterobacteriaceae." Munksgaard, Copenhagen.

Kent, T. H., Formal, S. B., La Brec, E. H., and Takenchi, A. (1966). Diffuse enteritis due to Salmonella typhimurium in opium-treated guinea pigs. Fed. Proc., 25, 1464.

Keren, D. F. (1979). Enzyme linked immunoabsorbent assay for immunoglobulin G and immunoglobulin A antibodies to Shigella flexneri antigens. Infect. Immun., 24, 441.

Knop, J., and Rowley, D. (1975). Antibacterial mechanisms in the intestine. Elimination of V. cholerae from the gastrointestinal tract of adult mice. Aust. J. Exp. Biol. Med. Sci., 53 (Pt 2), 137.

Koshland, M. E. (1975). Structure and function of the J-chain. In "Advances in Immunology," Vol. 20 (eds. Dixon, F. J., and Kunkel, H. G.), Academic Press, N.Y., p 41.

LaBroy, J. (1979). In "Intestinal Antibody Response to Bacterial Gastroenteritis in Humans." Ph.D. Thesis, The University of Adelaide, South Australia.

Lake, A. M., Bloch, K. J., Neutra, M. R., and Walker, W. A. (1979). Intestinal goblet cells mucous release. II. In vitro stimulation by antigen in the immunized rat. J. Immunol., 122, 834.

Lake, A. M., Bloch, K. J., Sinclair, K. J., and Walker, W. A. (1980). Anaphylactic release of intestinal goblet cell mucous. Immunol., 39, 173.

Lamb, V. L., Jones, L. M., Schurig, G. G., and Berman, D. T. (1979). Enzyme-linked immunosorbent assay for bovine immunoglobulin subclass-specific response to Brucella abortus lipopolysaccharides. Infect. Immun., 26, 240.

Laver, R. C., and Erlanger, B. F. (1974). An enzyme-immunoassay of antibody specific for adenosine using β-galactosidase. Immunochem., 11, 533.

Lefevre, M. E., Hammer, R., and Joel, D. D. (1979). Macrophages of the mammalian small intestine. J. Reticuloendoth. Soc., 26, 553.

Lehtonen, O. P., and Viljanen, M. K. (1980). Antigen attachment in ELISA. J. Immunol. Meth., 34, 61.

Leininger, K. I., Cooper, C. N., Epstein, M. M., Falb, R. D., and Grode, G. A. (1966). Nonthrombogenic plastic surfaces. Science, 152, 1625.

Lemaitre-Coelho, I., Jackson, G. D. F., and Vaerman, J. P. (1977). Rat bile as a convenient source of secretory IgA and free secretory component. Eur. J. Immunol., 8, 588.

Lemaitre-Coelho, I., Jackson, G. D. F., and Vaerman, J. P. (1978a). High levels of secretory IgA and free secretory component in the serum of rats with bile duct obstruction. J. Exp. Med., 147, 934.

Lemaitre-Coelho, I., Jackson, G. D. F., and Vaerman, J. P. (1978b). Relevance of biliary IgA antibodies in rat intestinal immunity. Scand. J. Immunol., 8, 459.

Leonore A. Herzenberg, and Herzenberg, L. A. (1973). Mouse immunoglobulin allotypes: description and special methodology. In

"Handbook of Experimental Immunology" (2nd edition) (ed. Weir, D. M.). Blackwell Scientific Publications, Oxford, London.

Levin, D. M., Ottesen, E. A., Reynolds, H. Y., and Kirkpatrick, C. H. (1976). Cellular immunity in Peyer's patches of rats infected with Trichinella spiralis. Infect. Immun., 13, 27.

Lindh, E. (1975). Studies on human secretory immunoglobulin. In gross conformation of sIgA and binding of SC to IgA and IgM. Thesis, Acta Universitats Upsaliensis. Abstracts of dissertations from the Uppsala Faculty of Medicine.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193, 265.

Lum, L. G., Muchmore, A. V., Keren, D., Decker, J., Koski, I., Strober, N., and Blease, R. M. (1979). A receptor for IgA on membrane T lymphocyte. J. Immunol., 122, 65.

Mackaness, G. B. (1969). The influence of immunologically committed lymphoid cells on macrophage activity in vivo. J. Exp. Med., 129, 973.

Magnusson, K-E., Stendahl, O., Stjernström, I., and Edebo, L. (1979). Reduction of phagocytosis, surface hydrophobicity and charge of Salmonella typhimurium 395 MR10 by reaction with secretory IgA. Immunol., 36, 439.

Maiolini, R., and Masseyeff, R. A. (1975). A sandwich method of enzymo-immunoassay. I. Application to rat and human alpha-fetoprotein. J. Immunol. Meth., 8, 223.

Marneerushapisal, V., and Rowley, D. (1981). Local cell-associated immunity in the Peyer's patches of mouse intestines. Infect. Immun., 33, 338.

Masseyeff, R., Maiolini, R., and Bouron, Y. (1973). A method of enzyme immunoassay. Biomedicine, 19, 314.

McDermott, M. R., Heatley, R. V., Befus, A. D., and Bienenstock, J. (1980). Lack of influence of Peyer's patches on the intestinal localization of mesenteric lymphoblasts. Cell. Immunol., 55, 240.

McWilliams, M., Phillips-Quagliata, J. M., and Lamm, M. E. (1975). Characteristics of mesenteric lymph node cells homing to gut-associated lymphoid tissue in syngeneic mice. J. Immunol., 115, 54.

Messih, G. A. (1961). Salmonellosis - a serological survey in rural areas by Gulyub Demonstration & Training Centre, Egypt. J. Egypt Pub. Health Assn., 36, 161.

Mestecky, J., Schrohenloher, R. E., Kulhavy, R., Wright, G., and Tomana, M. (1974). Site of J chain attachment to human polymeric IgA. Proc. Nat. Acad. Sci., U.S.A., 71, 544.

Meynell, G. G. (1963). Antibacterial mechanisms of the mouse gut. II. The role of Eh and volatile fatty acids in the normal gut. Brit. J. Exp. Pathol., 44, 209.

Meynell, G. G., and Subbaiah, T. V. (1965). Antibacterial mechanisms of the mouse gut. I. Kinetics of infection by Salmonella typhimurium in normal and streptomycin treated mice studied with abortive transductants. Brit. J. Exp. Pathol., 44, 197.

Miller, C. P., and Bonhoff, M. (1962). A study of experimental Salmonella infection in the mouse. J. Infect. Dis., 111, 107.

Miller, H. R. P., and Nawa, Y. (1979a). Nippostrongylus brasiliensis: Intestinal goblet-cell response in adoptively immunized rats. Exp. Parasitol., 47, 81.

Miller, H. R. P., and Nawa, Y. (1979b). Immune regulation of

intestinal goblet cell differentiation. Specific induction of non-specific protection against Helminths? Nouv. Rev. Fr. Hematol., 21, 31.

Moser, I., Hohmann, A., Schmidt, G., and Rowley, D. (1980). Salmonellosis in mice: Studies on oral immunization with live avirulent vaccines. Med. Microbiol. Immunol., 168, 119.

Mossel, D. A. A. (1976). Small infectious doses of Salmonella. Lancet, i, 1130.

Müller-Schoop, J. W., and Good, R. A. (1975). Functional studies of Peyer's patches: Evidence for their participation in intestinal immune response. J. Immunol., 114, 1757.

Mullock, B. M., Hinton, R. H., and Dobrota, M. (1979). Endocytic vesicles in liver carry polymeric IgA from serum to bile. Biochem. Biophys. Act., 587, 381.

Nagura, H., Nakane, P. K., and Brown, W. R. (1979). Translocation of dimeric IgA through neoplastic colon cells in vitro. J. Immunol. 123, 2359.

Nakane, P. K., and Pierce, G. B. (1967). Enzyme-labelled antibodies for the light and electron microscopic localization of tissue antigens. J. Cell. Biol., 33, 307.

Nash, D. R., and Holle, B. (1973). Local and systemic cellular immune responses in guinea pigs given antigen parenterally or directly into the lower respiratory tract. Clin. Exp. Immunol., 13, 575.

Nash, D. R., Vaerman, J. P., Bazin, H., and Heremans, J. F. (1969). Identification of IgA in rat serum and secretion. J. Immunol., 103, 145.

Nelson, D. L., Bundy, B. M., Blaese, R. M., and Strober, W. (1976). Cytotoxic effector cell function in organized gut-associated

lymphoid tissue. Cell. Immunol., 22, 165.

Newcomb, R. W., Normansell, D., and Starworth, D. R. (1968). A structural study of human exocrine IgA globulin. J. Immunol., 101, 905.

Ogra, P. L., and Karzon, D. T. (1971). Formation and function of polio virus antibody in different tissues. Prog. Med. Virol., 13, 156.

Orlans, E., Peppard, J., and Reynolds, J. (1978). Rapid active transport of immunoglobulin A from blood to bile. J. Exp. Med., 147, 588.

Orskov, K., Jensen, K. A., and Kobayashi, K. (1928). Studien über Breslauinfektion der Mause, Speziell mit Rucksicht auf die Bedeutung des Retikuloendothelial gewebes. Z. Immun. Forsch., 55, 34.

Ottaway, C. A., and Parrot, D. M. V. (1979). Regional blood flow and its relationship to lymphocyte and lymphoblast traffic during a primary immune reaction. J. Exp. Med., 150, 218.

Ouchterlony, O., and Nilsson, L. A. (1973). In "Handbook of Immunochemistry" (2nd edition) (ed. Weir, D. M.). Immunodiffusion and immuno-electrophoresis. Blackwell's, Oxford, p 19.1.

Owen, R. L. (1977). Sequential uptake of horseradish peroxidase by lymphoid follicle epithelium of Peyer's patches in the normal unobstructed mouse intestine: an ultrastructural study. Gastroenterology, 72, 440.

Owen, R. L. (1981). Histology and ultrastructure of the gastrointestinal immune system: The mucosal immune system in health and disease. In "Report of the 81st Ross Conference on Pediatric Research" (eds. Ogra and Bienenstock), p 3.

Owen, R. L., and Nemanie, P. (1978). Antigen processing structures of the mammalian intestinal tract: an SEM study of lymphoepithelial organs. In "Scanning Electron Microscope", Vol. 2 (eds. Becker, R. P., Johari, O., and O'Hare, A. M. F.). SEM Inc., pp 367-378.

Ozawa, A., Coto, J., Ito, Y., and Shibata, H. (1973). Histopathological and biochemical responses of germfree and conventional mice with Salmonella infection. In "Germfree Research, Biological Effect of Gnotobiotic Environments" (ed. Heneghan, J. B.). Academic Press, N.Y., p 325.

Parrott, D. M. V., and Ferguson, A. (1974). Selective migration of lymphocytes within the mouse small intestine. Immunol., 26, 571.

Parrott, D. M. V., Tilney, N. L., and Sless, F. (1975). The differen migratory characteristics of lymphocyte populations from a whole spleen transplant. Clin. Exp. Immunol., 19, 459.

Perrotto, J. L., Hang, L. M., Isselbacher, K. J., and Warren, K. S. (1974). Systemic cellular hypersensitivity induced by an intestinal absorbed antigen. J. Exp. Med., 140, 296.

Pesce, A. J., Ford, D. J., Gaizutis, M. A., and Pollack, V. E. (1977). Binding of protein to polystyrene in solid-phase immunoassays. Biochim. Biophys. Acta., 492, 399.

Pesce, A. J., Ford, D. J., and Gaizutis, M. A. (1978). Qualitative and quantitative aspects of immunoassays. Scand. J. Immunol., 8, Suppl. 7, 1.

Pesce, A. J., Mendoza, N., Bareisha, I., Gaizutio, M. A., and Pollack, V. E. (1974). Use of enzyme-linked antibodies to measure serum anti-DNA antibody in systemic lupus erythematosus. Clin. Chem., 20, 353.

Pesce, A. J., Modesto, R. R., Ford, D. J., Sethi, K., Clyne, D. H., and Pollack, V. E. (1976). Preparation and analysis of peroxidase antibody and alkaline phosphatase antibody conjugates. In "Immuno-enzymatic Techniques" (eds. Feldman, G., Druet, P., Bignon, J., and Avraemeas, S.). North Holland, Amsterdam, p 7.

Pierce, N. F., and Gowans, J. L. (1975). Cellular kinetics of the intestinal immune response to cholera toxoid in rats. J. Exp. Med., 142, 1550.

Plant, J., and Glynn, A. A. (1974). Natural resistance to Salmonella infection, delayed hypersensitivity and Ir genes in different strains of mice. Nature, 248, 345.

Plant, J., and Glynn, A. A. (1976). Genetics of resistance to infection with Salmonella typhimurium in mice. J. Infect. Dis., 133, 72.

Poger, M. E., and Lamm, M. E. (1974). Localization of free and bound secretory component in human intestinal epithelial cells. J. Exp. Med., 139, 629.

Polish Typhoid Committee (1965). Evaluation of typhoid vaccine in the laboratory and in a controlled field trial in Poland. Bull. W.H.O., 32, 15.

Rawson, A. J., and Abelson, N. M. (1964). Studies of blood group antibodies. VI. The blood group isoantibody activity of γ1A-globulin. J. Immunol., 93, 192.

Reed, L. J., and Muench, H. (1938). A simple method of estimating fifty per cent. end-points. Am. J. Hyg., 27, 493.

Reitman, M. (1967). Infectivity and antigenicity of streptomycin-dependent Salmonella typhosa. J. Infect. Dis., 117, 101.

Richman, L. K., Chiller, J. M., Brown, W. R., Hanson, D. G., and

Vaz, N. M. (1978). Cellular mechanisms in immunologic tolerance induced by enterically administered soluble protein. Gastroenterology, 74, 1084.

Robson, H. G., and Vas, S. I. (1972). Resistance of inbred mice to Salmonella typhimurium. J. Infect. Dis., 126, 378.

Rossen, C., Hsu, K. C., and Rose, H. M. (1967). Localization of 11S external secretory IgA globulin by immunofluorescence. Clin. Res., 15, 298.

Rossen, R. D., Kasel, J. A., and Couch, R. B. (1971). The secretory immune system: Its relation to respiratory viral infection. Prog. Med. Virol., 13, 194.

Rowley, D., and Jenkin, C. R. (1962). Antigenic cross-reaction between host and parasite as a possible course of pathogenicity. Nature, 193, 151.

Rowley, D., and Whitby, J. L. (1959). The bactericidal activity of mouse macrophages in vitro. Brit. J. Exp. Pathol., 40, 507.

Rubin, W., Fauci, A. S., Sleisenger, M. H., Jeffries, G. H., and Margolis, S. (1965). Immunofluorescent studies in adult celiac disease. J. Clin. Invest., 44, 475.

Rudzik, O., Perey, D. Y. E., and Bienenstock, J. (1975). Differential IgA repopulation after transfer of autologous and allogeneic rabbit Peyer's patches cells. J. Immunol., 114, 40.

Ruitenberg, E. J., and Elgersma, A. (1926). Absence of intestinal mast cell response in congenitally athymic mice during Trichinella spiralis infection. Nature, 264, 258.

Salcedo, M. (1978). Clinica de la fiebre tifoidea en adultos. I. Seminario sobre fiebre tifoidea in Chile. Boletin do Instituto Bacteriologica de Chile, p 24.

Savage, D. (1972). Survival on mucosal epithelia, epithelial penetration and growth in tissues of pathogenic bacteria. Symp. Soc. Gen. Microbiol., 22, 25.

Savage, D. C. (1969). Microbial interference between indigenous yeast and lactobacilli in the rodent stomach. J. Bacteriol., 98, 1278.

Schmidt, G. (1977). Immunization with E. coli hybrids. In "Immunology of the Gut". Ciba Symposium 46. North Holland, Amsterdam, p 355.

Schnaitman, C. A. (1974). Outer membrane proteins of Escherichia coli. III. Evidence that the major protein of Escherichia coli 0111 outer membrane consists of four distinct polypeptide species. J. Bacteriol., 118, 442.

Shklair, I. L., Rovelstad, G. H., and Lamberts, B. L. (1969). A study of some factors influencing phagocytosis of cariogenic streptococci by caries-free and caries-active individuals. J. Dent. Res., 48, 842.

Shuster, J. (1971). Pepsin hydrolysis of IgA-delineation of two populations of molecules. Immunochem., 8, 405.

Socken, D. J., Jeejeebhoy, K. N., and Bazin, H. (1979). Identification of secretory component as an IgA receptor on rat hepatocytes. J. Exp. Med., 50, 1539.

Steele, E. J., Chaicumpa, W., and Rowley, D. (1974). The isolation and biological properties of three classes of rabbit antibody to Vibrio cholerae. J. Infect. Dis., 130, 93.

Strober, W., Hague, N. E., Lum, L. G., and Henkart, P. A. (1978). IgA-Fc receptors on mouse lymphoid cells. J. Immunol., 121, 2440.

Svehg, S. E., and Bloth, B. (1970). Ultrastructure of secretory and high-polymer serum immunoglobulin A of human and rabbit origin.

Science, 169, 847.

Svenborg-Eden, C., and Svennerholm, A-M. (1978). Secretory immunoglobulin A and G antibodies prevent adhesion of E. coli to human urinary tract epithelial cells. Infect. Immun., 22, 790.

Svennerholm, A-M., Lange, S., and Holmgren, J. (1978). Correlation between intestinal synthesis of specific immunoglobulin A and protection against experimental cholera in mice. Infect. Immun., 21, 1.

Takeuchi, A. (1967). Electron microscope studies of experimental Salmonella infection. I. Penetration into intestinal epithelium by Salmonella typhimurium. Am. J. Pathol., 50, 109.

Tannock, G. W., and Savage, D. C. (1974). Influences of dietary and environmental stress on microbial populations in the murine gastrointestinal tract. Infect. Immun., 9, 591.

Tannock, G. W., and Smith, J. M. B. (1972). The effect of food and water deprivation (stress) on Salmonella-carrier mice. J. Med. Microbiol., 5, 283.

Thorne, G. M., and Farrar, W. E. (1973). Genetic properties of R factors associated with epidemic strains of Shigella dysenteriae Type 1 from Central America and Salmonella typhi from Mexico. J. Infect. Dis., 128, 132.

Tomasi, T. B., Jr. (1969). The concept of local immunity and the secretory system. In "Secretory Immunologic System" (eds. Dayton, Small, Chanock, Kaufman, and Tomasi) (Vero Beach Proc.). U.S. Government Printing Office, Washington, p 3.

Tomasi, T. B., Jr., and Bienenstock, J. (1968). Secretory immunoglobulin. Adv. Immunol. (eds Dixon and Kunkel), 9, 1. Academic Press, N.Y.

Tomasi, T. B., Jr., Tan, E. M., Solomon, A., and Prendergast, R. A. (1965). Characteristics of an immune system common to certain external secretions. J. Exp. Med., 121, 101.

Tomasi, T. B., Jr., and Zigelbaum, S. A. (1963). The selective occurrence of γA globulins in certain body fluids. J. Clin. Invest., 42, 1552.

Tourville, D. R., Adler, R. H., Bienenstock, J., and Tomasi, T. B. (1969). The human secretory immunoglobulin system: Immunohistological localization of γA secretory "piece" and lactoferrin in normal human tissues. J. Exp. Med., 129, 411.

Unanue, E. R., Grey, H. M., Rabellino, E., Campbell, P., and Schmitke, J. (1971). Immunoglobulins on the surface of lymphocytes. II. The bone marrow as the main source of lymphocytes with detectable surface-bound immunoglobulins. J. Exp. Med., 133, 1188.

Ushiba, D., Saito, K., Akiyama, T., and Sugiyama, T. (1959). Studies on infection and immunity in experimental typhoid viewed from the host cell-parasite relationship. 11th Symposium, Kanto Branch Meeting of the Japan Microbiologica Association, Tokyo.

Vaerman, J. P., and Heremans, J. F. (1968). Effect of neuraminidase and acidification on complement-fixing properties of human IgA and IgG. Int. Arch. Allergy, 34, 49.

Van Epps, D. E., and Williams, R. C. (1976). Suppression of leucocyte chemotaxis by human IgA myeloma component. J. Exp. Med., 144, 1227.

Van Epps, D. E., Reed, K., and Williams, R. C. (1978). Suppression of human PMN bactericidal activity by human IgA paraproteins. Cell. Immunol., 36, 363.

Van Oss, C. J., and Singer, J. M. (1966). The binding of immune globulins and other proteins by polystyrene latex particles.

J. Reticuloendoth. Soc., 3, 29.

Van Weemen, B. K., and Schuurs, A. H. W. M. (1971). Immunoassay using antigen-enzyme conjugates. FEBS Letters, 15, 233.

Voller, A., Bidwell, D. E., and Bartlett, A. (1976). Microplate enzyme-immunoassays for the immunodiagnosis of virus infections. In "Manual of Clinical Immunology," Ch. 69 (eds. Rose, N., and Friedman, H.). American Society for Microbiology, p 506.

Voller, A., Bidwell, D. F., Huldt, G., and Engvall, E. (1974). A microplate method of ELISA and its application to malaria. Bull. W.H.O., 51, 209.

Von Jeney, N., Günther, E., and Jann, K. (1977). Mitogenic stimulation of murine spleen cells: relation to susceptibility to Salmonella infection. Infect. Immun., 15, 26.

Vos, J. G., Buys, J., Hanstede, J. G., and Hagenaars, A. H. (1979). Comparison of enzyme-linked immunoabsorbent assay and passive haemaglutination method for the quantitation of antibodies to lipopolysaccharide and tetanus toxoid in the rat. Infect. Immun., 24, 798.

Wahdan, M. H., Sérié, C., Cerisier, Y., Sallam, S., and Germanier, R. (1982). A controlled field trial of live Salmonella typhi strain Ty 21a oral vaccine against typhoid: Three-year results. J. Infect. Dis., 145, 292.

Waksman, B. H., and Ozer, H. (1976). Specialized amplification elements in the immune system: The role of nodular lymphoid organs in the mucous membranes. Prog. Allergy, 21, 1.

Walker, W. A., and Isselbacher, K. J. (1972). Intestinal uptake of macromolecules: Effect of oral immunization. Science, 177, 608.

Walker, W. A., Wu, M., and Bloch, K. J. (1977). Stimulation by immune complex of mucous release from goblet cells of the rat small

intestine. Science, 197, 370.

Wells, P. D. (1963). Mucin-secreting cells in rats infected with Nippostrongylus brasiliensis. Exp. Parasitol., 14, 15.

Westphal, O., Luderitz, O., and Bister, F. (1952). Uber die extraktion von bacterien mit phenol/wasser. Z. Naturforsch., 78, 148.

Williams, A. F., and Gowans, J. L. (1975). The presence of IgA on the surface of rat thoracic duct lymphocytes which contain internal IgA. J. Exp. Med., 141, 335.

Williams, R. C., and Gibbons, R. J. (1972). Inhibition of bacterial adherence by secreting immunoglobulin A. Science, 177, 697.

Wilson, I. D., and William, R. C. (1969). Two distinct groups of IgA revealed by peptic digestion. J. Clin. Invest., 48, 2409.

Woodward, T. E. (1970). The unmasking of typhoid fever. S. Afr. Med. J., 44, 99.

Yamamoto, K., Anacker, R. L., and Ribi, E. (1970). Macrophage migration inhibition studies with cells from mice vaccinated with cell walls of Mycobacterium bovis BCG: Relationship between inhibitory activity of lung cells and resistance to airborne challenge with Mycobacterium tuberculosis H37Rv. Infect. Immun., 1, 595.

Yugoslav Typhoid Commission (1964). A controlled field trial of the effectiveness of acetone-dried and inactivated and heat-phenol-inactivated typhoid vaccines in Yugoslavia. Bull. W.H.O., 30, 623.

Zollinger, W. D., Dalrymple, J. M., and Artenstein, M. S. (1976). Analysis of parameters affecting the solid phase radioimmunoassay quantitation of antibody to meningococcal antigens. J. Immunol., 117, 1788.